

## Application Note

# UPLC-MS/MS Analysis of Azole Antifungals in Serum for Clinical Research

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## Abstract

The analysis of Azole antifungals in serum was described in this application note.

Use of UPLC-MS/MS enables separation of itraconazole and voriconazole from their metabolites, and the selectivity provided by mass selective detection provides a reliable means of analysis of antifungal compounds in serum for clinical research purposes.

### Benefits

- Analytical selectivity afforded by mass selective detection
- Wide linear measuring range
- Simple, inexpensive sample preparation using small sample volumes

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## Introduction

Here described is a method for the analysis of azole antifungals in serum. This method may be used for emerging indications, and for understanding pharmacokinetic and pharmacodynamic properties in clinical research.<sup>1,2</sup> Although microbiological test methods are in use to measure azole antifungals, enhanced activity of the itraconazole metabolite – hydroxyitraconazole – can overestimate concentrations.<sup>1,2</sup> Similarly, the use of two or more drugs in combination can impair the utility of microbiological test methods.<sup>1</sup> Furthermore, measurement of hydroxyitraconazole is of unknown utility and remains the subject of research.<sup>3</sup>

The method described utilizes deproteination of serum samples with a deuterated internal standard mixture in methanol. Separation was achieved within three minutes using an ACQUITY UPLC BEH C<sub>18</sub> Column on an ACQUITY UPLC I-Class System followed by detection on a Xevo TQD Mass Spectrometer (Figure 1).



Figure 1. Waters ACQUITY UPLC I-Class System and Xevo TQD Mass Spectrometer.

## Experimental

### Sample preparation

Standards were sourced for fluconazole, itraconazole, posaconazole, and voriconazole (Sigma-Aldrich, Dorset, UK); hydroxyitraconazole and voriconazole-N-oxide (Toronto Research Chemicals). Stable labeled internal standards  $^2\text{H}_4$ -fluconazole,  $^2\text{H}_5$ -hydroxyitraconazole,  $^2\text{H}_5$ -itraconazole,  $^2\text{H}_4$ -posaconazole,  $^2\text{H}_3$ -voriconazole, and  $^2\text{H}_3$ -voriconazole-N-oxide were sourced from Toronto Research Chemicals.

Calibrators were prepared in pooled serum purchased from Golden West Biologicals (California, USA). The calibration range was 0.5–100 µg/mL for fluconazole and 0.05–10 µg/mL for all other compounds. QC materials were also prepared in pooled serum at 1.5, 20, and 80 µg/mL fluconazole and 0.15, 2, and 8 µg/mL for all other compounds.

## Sample extraction

To 50 µL of sample, 950 µL of internal standard in methanol containing 0.1% formic acid (1 µg/mL <sup>2</sup>H<sub>4</sub>-fluconazole, 200 ng/mL for all other internal standards) was added, vortex-mixed, and centrifuged for two minutes at 16,100 g. Supernatant (50 µL) was diluted with 150 µL water to prepare the final extract for analysis.

## LC conditions

System:	ACQUITY UPLC I-Class (FTN)
Needle:	30 µL
Column:	ACQUITY UPLC BEH C <sub>18</sub> , 130Å, 1.7 µm, 2.1 mm x 30 mm
Mobile phase A:	Water + 2 mM ammonium acetate + 0.1% formic acid
Mobile phase B:	Methanol + 2 mM ammonium acetate + 0.1% formic acid
Needle wash solvent:	80% aqueous methanol
Purge solvent:	Mobile phase A
Seal wash:	20% aqueous methanol
Column temp.:	50 °C
Injection volume:	20 µL

Flow rate: 0.80 mL/min

### Gradient:

Time(min)	% Mobile phase A	% Mobile phase B	Curve
Initial	75	25	Initial
2.1	3	97	7
2.5	75	25	11
Run time:	3.0 min (3.7 min injection-to- injection)		

### MS conditions

System:	Xevo TQD
Resolution:	MS1 (0.7 FWHM) MS2 (0.7 FWHM)
Acquisition mode:	Multiple Reaction Monitoring (MRM) (see Table 1 for details)
Polarity:	ESI+ ionization
Capillary:	0.8 kV
Source temp.:	150 °C
Desolvation temp.:	500 °C

Inter-scan delay: 0.02 s

Inter-channel delay: 0.01 s

## Data management

MassLynx v4.1 with TargetLynx Application Manager

Function (acquisition time)	Analyte	Precursor ion ( <i>m/z</i> )	Product ion ( <i>m/z</i> )	Cone voltage (V)	Collision energy (eV)	Dwell time (s)
1. (0.35–0.90 min)	Fluconazole (quan)	307.1	238.0	28	14	0.035
	Fluconazole (quan)	307.1	220.05	28	20	0.035
	Fluconazole- <sup>2</sup> H <sub>4</sub>	311.1	242.0	28	14	0.035
2. (0.95–1.30 min)	Voriconazole-N-Oxide (quan)	366.1	143.05	18	10	0.035
	Voriconazole-N-Oxide (quan)	366.1	224.05	18	12	0.035
	Voriconazole- <sup>2</sup> H <sub>3</sub> -N-Oxide	369.1	146.05	18	10	0.035
3. (1.35–1.75 min)	Voriconazole (quan)	350.1	127.05	26	32	0.035
	Voriconazole (quan)	350.1	281.05	26	16	0.035
	Voriconazole- <sup>2</sup> H <sub>3</sub>	353.1	127.05	26	32	0.035
4. (1.80–2.40 min)	Posaconazole (quan)	701.35	127.05	64	70	0.04
	Posaconazole (quan)	701.35	148.1	64	62	0.02
	Voriconazole- <sup>2</sup> H <sub>3</sub>	705.35	127.05	64	70	0.01
	Itraconazole (quan)	705.25	392.25	60	40	0.04
	Itraconazole (quan)	705.25	119.05	60	72	0.02
	Itraconazole- <sup>2</sup> H <sub>5</sub>	710.25	397.25	60	40	0.01
	Hydroxyitraconazole (quan)	721.35	408.3	62	36	0.04
	Hydroxyitraconazole (quan)	721.35	159.05	62	80	0.02
	Hydroxyitraconazole- <sup>2</sup> H <sub>5</sub>	726.35	413.3	62	36	0.01

Table 1. Guideline MRM parameters for antifungal compounds and their internal standards.

## Results and Discussion

Under these chromatographic conditions, all compounds are separated chromatographically, with the exception of hydroxyitraconazole and posaconazole, which are separated by mass. Figure 2 shows a mid-range calibrator (50 µg/mL fluconazole, 5 µg/mL all other compounds). No carryover was observed for any compounds.

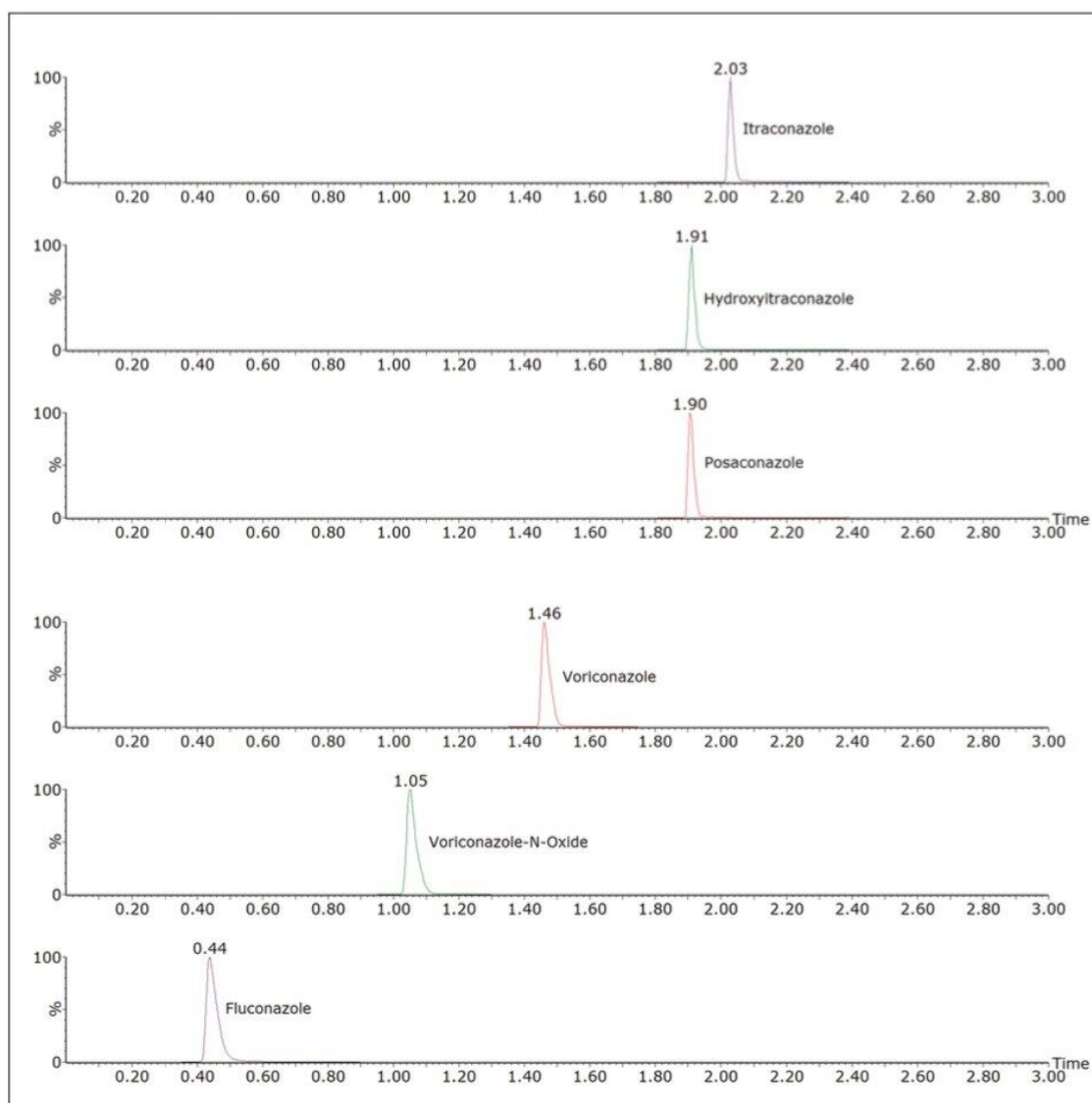


Figure 2. UPLC separation of fluconazole, hydroxyitraconazole, itraconazole, posaconazole, voriconazole, and voriconazole-N-oxide using an ACQUITY UPLC BEH C<sub>18</sub> Column.

Analytical sensitivity investigations demonstrate that the method would allow precise quantification (<20% RSD) at 0.375 µg/mL for fluconazole, 0.05 µg/mL for hydroxyitraconazole and posaconazole, 0.0375 µg/mL for voriconazole-N-oxide, and 0.025 µg/mL for voriconazole.

Total precision was determined by extracting and quantifying five replicates of three concentrations of QC material over five separate days (n=25). Repeatability was assessed by analyzing five replicates at each QC level. Table 2 presents results of these experiments, where total precision and repeatability at the low (1.5 µg/mL fluconazole, 0.15 µg/mL other compounds), medium (20 µg/mL fluconazole, 2 µg/mL other



compounds), and high (80 µg/mL fluconazole, 8 µg/mL other compounds) concentrations were ≤11.5% RSD.

Compound	Total QC precision (RSD)			QC repeatability (RSD)		
	Low	Mid	High	Low	Mid	High
Fluconazole	2.7%	2.6%	2.6%	2.6%	1.8%	2.6%
Hydroxyitraconazole	11.5%	5.4%	5.1%	10.0%	3.0%	4.0%
Itraconazole	8.9%	5.1%	6.4%	8.6%	3.0%	2.6%
Posaconazole	7.7%	3.9%	4.5%	5.2%	2.9%	2.4%
Voriconazole	2.6%	2.4%	2.9%	1.5%	2.1%	1.7%
Voriconazole-N-Oxide	5.4%	1.9%	3.4%	3.5%	1.9%	2.7%

*Table 2. Total precision and repeatability for the analysis of fluconazole, hydroxyitraconazole, itraconazole, posaconazole, voriconazole, and voriconazole-N-oxide.*

The method was shown to be linear over the range of 0.457–117 µg/mL for fluconazole, 0.0457–11.7 µg/mL for hydroxyitraconazole and voriconazole-N oxide, 0.381–11.7 µg/mL for itraconazole and posaconazole, and 0.0381–13.0 µg/mL for voriconazole when different ratios of high and low concentration pools were combined and analyzed.

Matrix effects were evaluated as the peak area of extracted post-spiked serum samples (n=6) taken as a percentage of extraction solvent samples spiked to equivalent concentrations. The internal standard was shown to compensate for significant signal enhancement observed for hydroxyitraconazole, itraconazole, and posaconazole, as shown in Table 3 for the response ratio matrix effect.

Compound	Response ratio		
	Matrix effect	Range	RSD
Fluconazole	0.99	0.99–0.99	0.7%
Hydroxyitraconazole	1.04	1.02–1.07	3.1%
Itraconazole	1.03	1.01–1.04	2.6%
Posaconazole	1.01	1.00–1.02	2.0%
Voriconazole	0.99	0.99–1.00	0.7%
Voriconazole-N-Oxide	0.99	0.99–1.00	0.9%

*Table 3. Matrix effects.*

Potential interference from endogenous compounds (albumin, bilirubin, cholesterol, triglycerides, and uric acid), Intralipid (20% emulsion), and potentially co-administered compounds (cyclosporine, everolimus, mycophenolic acid, sirolimus, and tacrolimus) were assessed by determining the recovery of each compound



from a serum pool of known concentration when spiked with the potential interference (n=3). Recoveries ranged from 85.0 to 107.2% for all compounds.

20 serum samples were purchased from a US national reference laboratory with assigned values for hydroxyitraconazole. Good agreement was demonstrated between the Waters UPLC-MS/MS method and the method used by the reference laboratory.

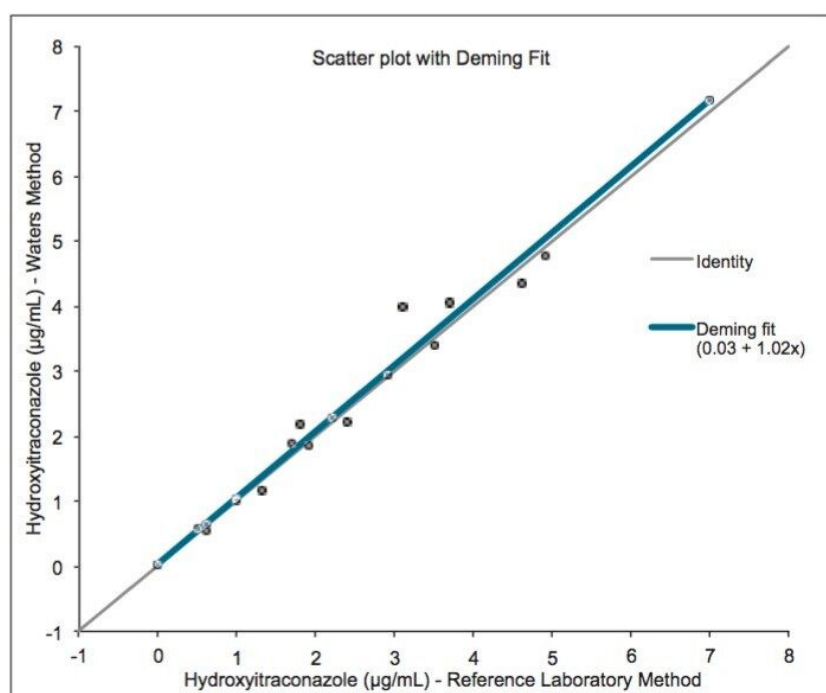


Figure 3. Scatter plot of Deming fit of reference laboratory method versus Waters method for 20 hydroxyitraconazole samples.

## Conclusion

Use of UPLC-MS/MS enables separation of itraconazole and voriconazole from their metabolites, and the selectivity provided by mass selective detection provides a reliable means of analysis of antifungal compounds in serum for clinical research purposes.

This method provides sufficient analytical sensitivity to analyze low levels of fluconazole (0.5 µg/mL), hydroxyitraconazole, itraconazole, posaconazole, voriconazole, and voriconazole N-oxide (all 0.05 µg/mL)

over a large linear range (200-fold) using only 50 µL of sample. Sample preparation is simple, fast, and inexpensive.

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## References

1. Andes D, Pascual A, Marchetti O. Antifungal therapeutic drug monitoring: established and emerging indications. *Antimicrob Agents Chemother*. 2009;53(1):24–34.
2. Dodds Ashley E S, Lewis R, Lewis J S, Martin C, Andres D. Pharmacology of Systemic Antifungal Agents. *Clin Infect Dis*. 2006;43 (Supplement 1):S28 S39.
3. Odds F C, Vanden Bossche H. Antifungal activity of itraconazole compared with hydroxyl-itraconazole in vitro. *Journal of Antimicrobial Chemotherapy*. 2000;45:371–373.

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