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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

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.[1,2] Although microbiological test methods are in use to measure azole antifungals, enhanced activity of the itraconazole metabolite – hydroxyitraconazole – can overestimate concentrations.[1,2] Similarly, the use of two or more drugs in combination can impair the utility of microbiological test methods.1 Furthermore, measurement of hydroxyitraconazole is of unknown utility and remains the subject of research.[3]

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_{18} 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 $^{2}H_{4}$-fluconazole, $^{2}H_{5}$-hydroxyitraconazole, $^{2}H_{5}$-itraconazole, $^{2}H_{4}$-posaconazole, $^{2}H_{3}$-voriconazole, and $^{2}H_{3}$-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 $^{2}H_{4}$-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_{18}, 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 (*m/z*)	Product ion (*m/z*)	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-$^{2}H_{4}$	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-$^{2}H_{3}$-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-$^{2}H_{3}$	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-$^{2}H_{3}$	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-$^{2}H_{5}$	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-$^{2}H_{5}$	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.

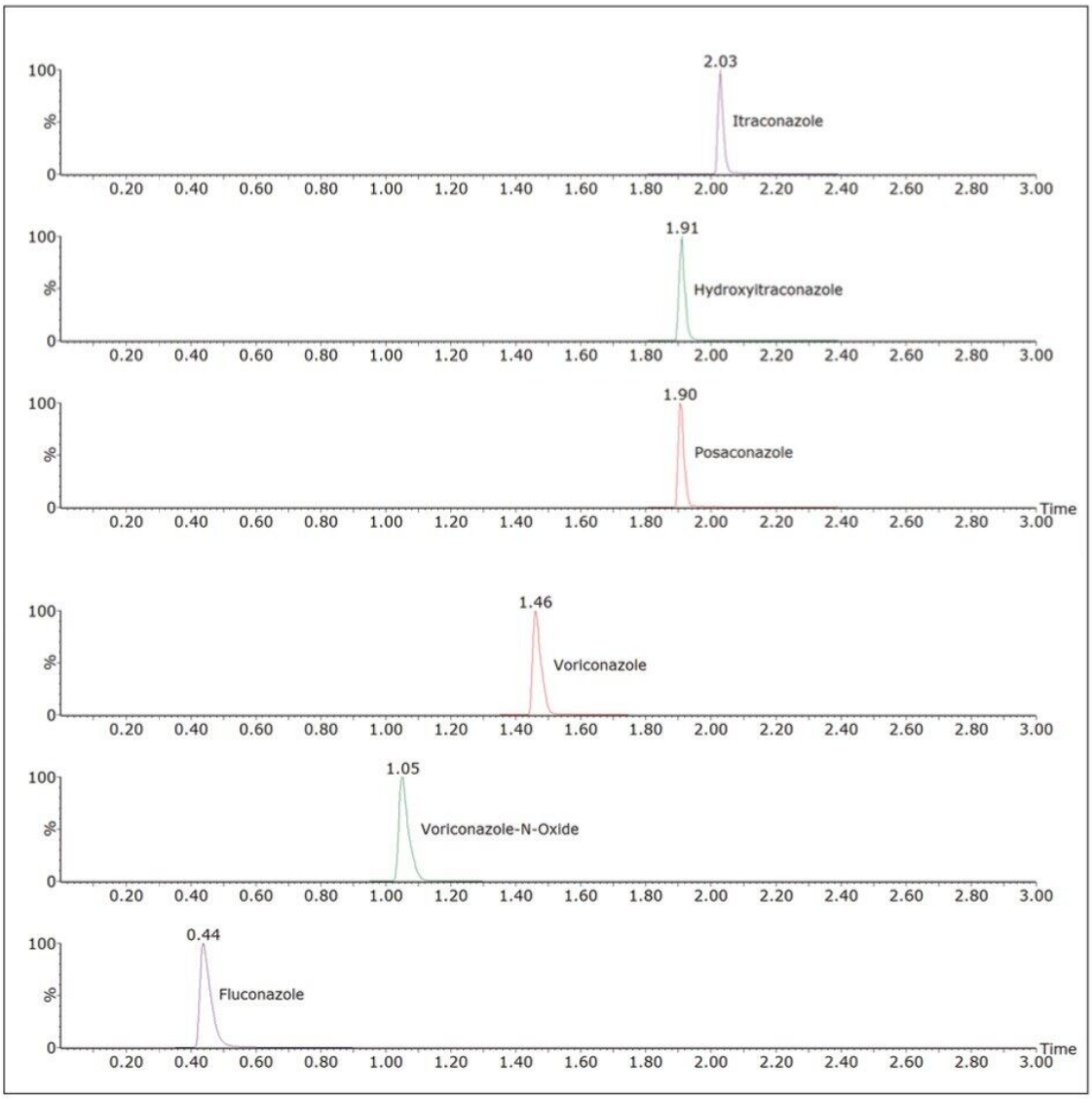

Figure 2. UPLC separation of fluconazole, hydroxyitraconazole, itraconazole, posaconazole, voriconazole, and voriconazole-N-oxide using an ACQUITY UPLC BEH C_{18} Column.

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 85.0 107.2% 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.

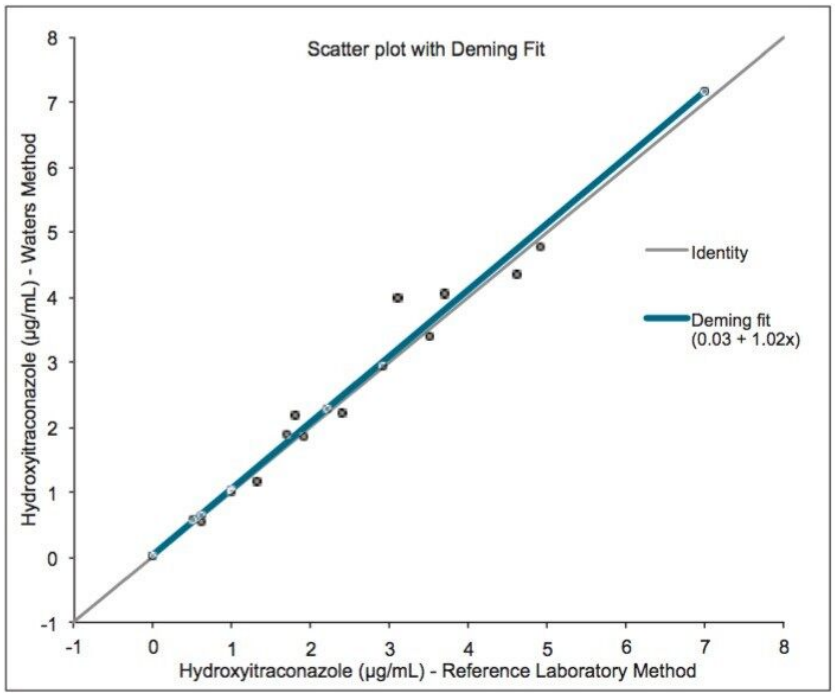

Figure 3. Scatter plot of Deming fit of refereRncees ildaubaolr aptloorty method versus Waters method for 20 hydroxyitraconazole samples.

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.

References

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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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