Application Note

Clinical Research

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Rapid and Sensitive Analysis of Antifungal Agents in Human Serum Using the Agilent 6475 LC/TQ

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Abstract

A rapid, 3.5-minute, sensitive measurement of eight antifungal agents (5-fluorocytosine, fluconazole, hydroxy-itraconazole, isavuconazole, itraconazole, posaconazole, ketoconazole, and voriconazole) in human serum was established using an Agilent 6475 triple quadrupole LC/MS (LC/TQ) and the RECIPE therapeutic drug monitoring (TDM) kit. This method presents the quantitation of antifungal agents for TDM with a hassle-free sample preparation protocol.

Introduction

In addition to minimizing drug-related toxicity, TDM has been hailed as a key step to prevent the emergence of drug resistance. The growing number of invasive fungal infections creates a ripple effect on the rapid advancement of TDM for antifungal agents. Increasing evidence confirms the critical role of TDM in preventing and treating antifungal infections.[1]

The RECIPE ClinMass TDM kit system for antimycotics in serum (Munich, Germany) was used on a 6475 LC/TQ for the analysis of eight antifungal agents. This application note demonstrates the evaluation and validation of the RECIPE TDM kit, covering sensitivity, linearity, and robustness across five consecutive days (n = 5). Quick and simple sample preparation was implemented, providing high-throughput monitoring of antifungal agents in biological samples.

Experimental

Reagents and chemicals

Mobile phases, sample preparation diluents (precipitant and diluting solution D), serum calibration standard set, serum quality control (QC) samples, internal standards (ISTD), prefilter column, and analytical column were purchased from the RECIPE ClinMass TDM kit system (MS9000, MS9600) (Munich, Germany).

Sample preparation

Four levels of human serum calibration standard set, including a blank, and two levels of QC samples were prepared using the protocol outlined in Figure 1.

Instrumentation

Mix 100 µL of ISTD and precipitant with 50 µL of homogenized calibrators/QC samples.

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Mix on a Geno/Grinder (5 minutes at 1,200 rpm) and incubate 5 minutes at room temperature.

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Centrifuge for 5 minutes at 10,000 rpm.

↓

Collect the supernatant, dilute 20 times with diluting solution D, and inject into the Agilent 6475 LC/TQ.

Figure 1. Sample preparation protocol.

Table 1. Instrumentation.

Module	Part Number
Liquid Chromatography System	
Agilent 1290 Infinity II High-Speed Pump	G7120A
Agilent 1290 Infinity II Multisampler	G7167B
Agilent 1290 Infinity II Multicolumn Thermostat	G7116B
Mass Spectrometry System	
Agilent 6475 LC/TQ	G6475AA
Agilent Jet Stream Technology Ion Source (AJS)	

Method

The optimization of multiple reaction monitoring (MRM) transitions for analytes and ISTDs was carried out using Auto MRM Optimizer in the Agilent MassHunter acquisition software to provide optimal sensitivity and selectivity. Data were processed using the Agilent MassHunter Qualitative Analysis software, version B.12, and Agilent MassHunter Quantitative Analysis software, version B.12.

Table 2. Agilent 1290 Infinity II LC parameters.

Parameter	Description
Column	RECIPE prefilter and analytical column (MS9032 and MS9030)
Column Temperature	40 °C
Mobile Phase	RECIPE mobile phase A (MS9007) RECIPE mobile phase B (MS9008)
Flow Rate	0.6 mL/min
Gradient Program	Time (min) %B 0 0 0.05 0 0.10 30 2.10 60 2.20 98 2.40 98 2.41 0 3.50 0
Stop Time	3.5 min
Injection Volume	2 µL

Table 3. Agilent 6475 triple quadrupole LC/MS and source parameters.

Parameter	Description
Drying Gas Temperature	300 °C
Drying Gas Flow	13 L/min
Sheath Gas Temperature	375 °C
Sheath Gas Flow	12 L/min
Nebulizer	45 psi
Capillary Voltage	3,000 V (+)
Nozzle Voltage	500 V (+)
Measurement Mode and Polarity	MRM, positive

Table 4. Optimized MRM transitions.

Compound	Precursor (*m/z*)	Product (*m/z*)	Fragmentor (V)	CE (V)	Polarity
Hydroxy-Itraconazole	721.2	408.2	160	38	Positive
	721.2	392.2	160	38	Positive
d_5-Hydroxy Itraconazole	726.2	413.2	190	38	Positive
Itraconazole	705.2	432.3	180	38	Positive
	705.2	392.3	180	42	Positive
d_5-Itraconazole	710.2	397.2	180	38	Positive
Posaconazole	701.3	614.5	190	42	Positive
	701.3	344.3	190	50	Positive
d_4-Posaconazole	705.3	618.3	190	42	Positive
Ketoconazole	531.1	489.2	200	38	Positive
	531.1	244.0	200	38	Positive
d_8-Ketoconazole	539.1	497.1	200	38	Positive
Isavuconazole	438.1	369.2	115	22	Positive
	438.1	224.0	115	22	Positive
$^{13}C,d_4$-Isavuconazole	443.1	224.0	115	22	Positive
Voriconazole	350.0	281.2	110	14	Positive
	350.0	127.1	110	14	Positive
d_3-Voriconazole	353.0	284.0	110	14	Positive
Fluconazole	306.9	238.1	110	18	Positive
	306.9	220.0	110	18	Positive
d_4-Fluconazole	310.9	242.1	110	18	Positive
5-Fluorocytosine	130.1	113.0	95	22	Positive
	130.1	58.1	95	42	Positive
$^{13}C,^{15}N_2$-5-Fluorocytosine	133.1	115.0	95	42	Positive

Results and discussion

Figure 2 shows the chromatograms of quantifier MRM transitions for the eight antifungal agents at the lowest calibration level. Six out of eight antifungal agents had good chromatographic separation across the run time. The retention times of two antifungals, posaconazole and hydroxy-itraconazole, were slightly overlapped. Despite this overlap, measurement integrity can still be maintained due to MRM selectivity.

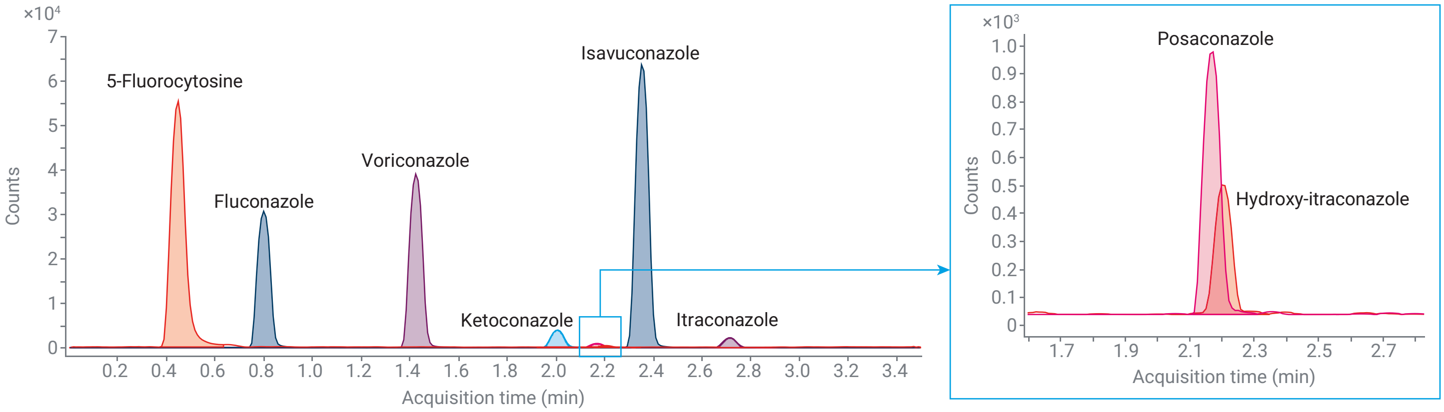

Figure 2. Chromatograms of the quantifier ion for the eight antifungal agents at the lowest calibration level. The inset graph shows the signal of posaconazole and hydroxy-itraconazole.

Validation of the analytical method was carried out by measuring calibration and QC samples six times (n = 6) over five consecutive days (n = 5). Accuracy of QC samples was evaluated based on the ratio of measured over preset concentrations of serum QC samples. Precision was determined as interday relative standard deviation (RSD) of measured QC concentration.

Calibration sets of eight antifungal agents were established with excellent linearity (R^2), 0.995 or more, across five days. The limit of quantitation (LOQ) was set at a signal-to-noise ratio greater than 10, and up to eight-fold higher than the RECIPE recommendation with 2 µL injection volume, as shown in Figure 3.

The LOQ values were:

- 5-Fluorocytosine: 89 µg/L
- Fluconazole: 11.3 µg/L
- Hydroxy-itraconazole: 2.5 µg/L
- Isavuconazole: 7.9 µg/L
- Itraconazole: 2.7 µg/L
- Ketoconazole: 7.0 µg/L
- Posaconazole: 3.5 µg/L
- Voriconazole: 4.5 µg/L

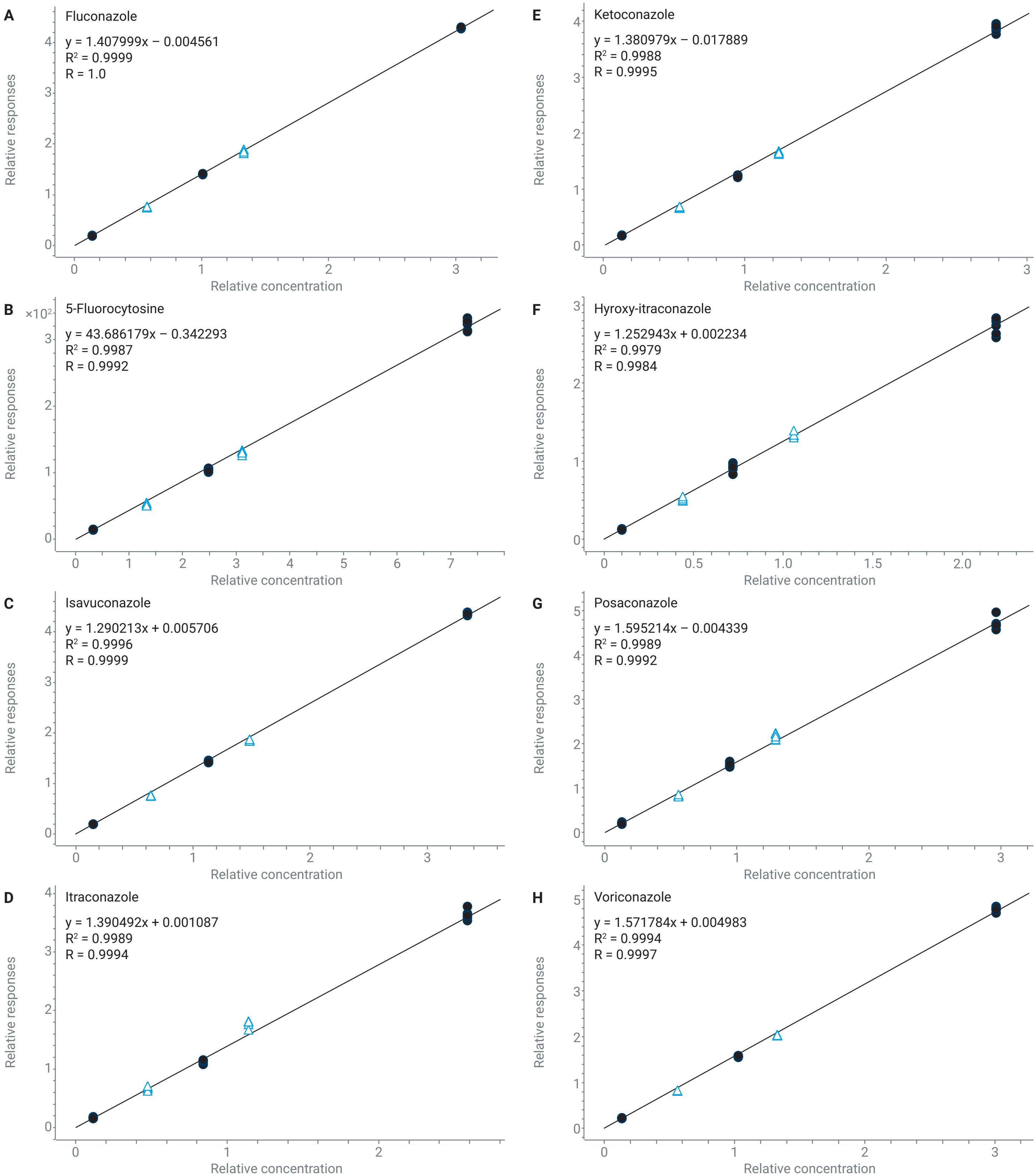

Figure 3. Calibration curves (•) and quality control (∆) sets of eight antifungal agents. (A) Fluconazole, (B) 5-Fluorocytosine, (C) Isovuconazole, (D) Itraconazole, (E) Ketoconazole, (F) Hyroxy-itraconazole, (G) Posaconazole, and (H) Voriconazole.

Interday accuracy of antifungal agents in serum QC samples ranged between 94.1 and 106.2% (Table 5). Outstanding precision was obtained, RSD < 5.6%, over both six intraday and five interday measurements. No significant carryover was observed for the eight antifungal agents in the entire run.

Table 5. Interday accuracy and precision of QC samples.

Compound	Level	Target (µg/L)	Acquired (Mean, µg/L)	Accuracy (%)	RSD (%)
5-Fluorocytosine	I	353.3	335.7	95.0	3.1
	II	830.0	780.9	94.1	3.7
Fluconazole	I	45.8	44.4	96.9	1.7
	II	106.7	103.8	97.3	2.5
Hydroxy-Itraconazole	I	11.0	10.8	98.2	5.1
	II	26.5	25.7	96.9	4.3
Isavuconazole	I	34.2	32.2	94.2	1.6
	II	79.0	74.7	94.5	2.5
Itraconazole	I	9.5	10.0	105.1	3.6
	II	22.8	24.2	106.2	5.5
Ketoconazole	I	28.7	27.3	95.1	1.6
	II	66.2	62.7	94.7	2.6
Posaconazole	I	14.9	14.7	98.6	3.5
	II	34.5	35.6	103.3	2.6
Voriconazole	I	18.7	17.8	95.3	1.6
	II	44.3	41.9	94.6	2.3

Conclusion

A fast and sensitive analysis of eight antifungal agents in serum was validated on an Agilent 6475 triple quadrupole LC/MS. By employing a ready-to-use RECIPE ClinMass TDM kit, this method demonstrates accurate and precise quantitation of antifungal agents in biological samples. Additionally, the streamlined sample preparation method allows high throughput analysis of up to 350 samples per day.

Reference

1. Ashbee, H. R.; Barnes, R. A.; Johnson, E. M.; Richardson, M. D.; Gorton, R.; Hope, W. W. Therapeutic Drug Monitoring (TDM) of Antifungal Agents: Guidelines from The British Society for Medical Mycology. *J. Antimicrob. Chemother.* **2014**, *69(5)*, 1162–1176. DOI: 10.1093/jac/dkt508

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