TECHNICAL NOTE 66059

Quantification of eight antimycotics in human plasma by liquid chromatography-tandem mass spectrometry for clinical research

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Application benefits

- Robust, sensitive hardware enables increased confidence in data
- Simple offline sample preparation by protein precipitation
- Eight antimycotics drugs in a single 3.6-minute quantitative method

Goal

Implementation of an analytical method for the quantification of eight antimycotics in human plasma on a Thermo Scientific™ TSQ Quantis™ triple quadrupole mass spectrometer.

Introduction

Antifungals, also known as antimycotics, typically refer to a class of pharmaceutical fungicide used to treat and prevent mycosis, ranging from athlete's foot to ringworm to serious infections, such as cryptococcal meningitis. Voriconazole, posaconazole, fluconazole, ketoconazole, and other similar antimycotics are used to treat life-threatening fungal infections along with prevention of infections in immunocompromised individuals. The narrow

therapeutic ranges of these antifungal agents, in addition to other complications, could lead to very different drug exposure from even the same dosage regimen and, therefore, very different individual outcomes. Analytical methods to quantify such antimycotics were traditionally performed using high-performance liquid chromatography (HPLC) coupled with UV detectors. However, these methods require complicated extraction procedures and time-consuming chromatography. LC-MS based methods are known for their superior selectivity and often result in significant reduction of the time spent on complicated sample preparation procedures and chromatography.

An analytical method for clinical research to quantify eight antimycotics in human plasma in 3.6 minutes is presented in this report. Samples were prepared by protein precipitation followed by chromatographic separation on a Thermo Scientific™ Vanquish™ Flex Binary UHPLC system. Detection was performed on a TSQ Quantis triple quadrupole mass spectrometer with heated electrospray ionization (HESI) operated in positive ionization mode. Method performance was evaluated using the ClinMass® TDM Platform with the ClinMass Add-On Set for Antimycotics by RECIPE Chemicals + Instruments GmbH (Munich, Germany) in terms of linearity of response, lower limit of quantification (LLOQ), carryover, accuracy, and intra- and inter-assay precision for all analytes.

Experimental

Target analytes

The complete list of analytes with their corresponding internal standards and the concentration ranges covered by the calibrators used are reported in Table 1. They include 5-fluorocytosine, fluconazole, isavuconazole, itraconazole, ketoconazole, OH-itraconazole, posaconazole, and voriconazole.

Sample preparation

Reagents included four calibrators (including blank) and two controls from RECIPE, as well as an internal standard mix for quantitation. Samples of 50 µL of plasma-based calibrators and controls were protein precipitated using 100 µL of acetonitrile containing the internal standards. Precipitated samples were vortex-mixed and centrifuged for 10 minutes. Fifty microliters of the supernatant were transferred to a clean vial and diluted to a volume of 500 µL with water.

Liquid chromatography

The diluted supernatant was injected onto a Vanquish Flex Binary UHPLC system connected to a TSQ Quantis triple quadrupole mass spectrometer. Chromatographic separation was achieved by gradient elution on a Thermo Scientific™ Hypersil GOLD™ 50 × 2.1 mm (1.9 µm) column kept at 40 ^{0}C.

Mobile phases composition was the following:

- Mobile phase A: Water + 0.1 % formic acid
- Mobile phase B: Acetonitrile + 0.1 % formic acid

Injection volume was 2 µL. MeOH/water (50/50) was used as needle wash solvent.

The LC gradient is described in detail in Table 2. Total runtime was 3.6 minutes.

Table 2. LC gradient profile

Time (min)	Flow (mL/min)	%B
0.0	0.5	5
0.5	0.5	5
1.5	0.5	100
2.5	0.5	100
2.6	0.5	5
3.6	0.5	5

Table 1. Analytes, concentrations ranges (MS9613, batch #1369), and internal standards

Compound name	Molecular formula	Concentration range (mg/L)	Internal standard name	Molecular formula
5-Fluorocytosine	$C_4H_4FN_3O$	4.90–108	^{13}C-$^{15}N_2$-5-Fluorocytosine	$^{13}C^{15}N_2C_3H_4FNO$
Fluconazole	$C_{13}H_{12}F_2N_6O$	0.622–13.5	d_4-Fluconazole	$C_{13}H_8D_4F_2N_6O$
Isavuconazole	$C_{22}H_{17}F_2N_5OS$	0.481–10.8	^{13}C-d_4-Isavuconazole	$^{13}CC_{21}H_{13}D_4F_2N_5OS$
Itraconazole	$C_{35}H_{38}Cl_2N_8O_4$	0.146–3.11	d_5-Itraconazole	$C_{35}H_{33}D_5Cl_2N_8O_4$
Ketoconazole	$C_{26}H_{28}Cl_2N_4O_4$	0.430–8.88	d_8-Ketoconazole	$C_{26}H_{20}D_8Cl_2N_4O_4$
OH-Itraconazole	$C_{35}H_{38}Cl_2N_8O_5$	0.171–3.55	d_5-OH-Itraconazole	$C_{35}H_{33}D_5Cl_2N_8O_5$
Posaconazole	$C_{37}H_{42}F_2N_8O_4$	0.233–4.90	d_4-Posaconazole	$C_{37}H_{38}D_4F_2N_8O_4$
Voriconazole	$C_{16}H_{14}F_3N_5O$	0.275–5.96	d_3-Voriconazole	$C_{16}H_{11}D_3F_3N_5O$

Mass spectrometry

Analytes and internal standard were detected by Selected Reaction Monitoring (SRM) on a TSQ Quantis triple quadrupole mass spectrometer using a HESI source operated in positive ionization mode. A summary of the MS conditions is reported in Table 3. One confirming ion for each analyte was included in the acquisition method for confirmation (Table 4).

Table 3. MS parameters

Ion source parameters	
Source type	Heated Electrospray Source Ionization (HESI)
Spray voltage - Positive (V)	3,750
Sheath gas (Arb)	55
Aux gas (Arb)	10
Sweep gas (Arb)	2
Ion transfer tube temp. (°C)	320
Vaporizer temp. (°C)	450
Settings	
Data acquisition mode	SRM
SRM parameters	
Cycle time (s)	0.3
Q1 resolution (FWHM)	0.7
Q3 resolution (FWHM)	1.2
Chromatographic peak width(s)	6

Method evaluation

The performance of the method was evaluated in terms of linearity of response, LLOQ, carryover, accuracy, and intra- and inter-assay precision for all analytes.

A 20-fold serial dilution of the lowest calibrator using blank matrix was performed to evaluate the LLOQ. A full set of calibrators (four levels) and diluted calibrators (two levels) were injected in a single batch and all used for the linear interpolation. The LLOQ was set as the lowest level that could be determined with a CV < 20%.

Carryover was calculated in terms of percentage ratio between peak area of the highest calibrator and a subsequent blank sample injection. Analytical accuracy was evaluated in terms of percentage bias between nominal and average calculated concentrations using quality control samples at two different levels provided by RECIPE (MS9682 batch #1369).

Quality control samples were prepared and analyzed in replicates of five over three different days. Intra-assay precision for each day was evaluated in terms of percentage coefficient of variation (%CV) using the controls at two different levels in replicates of five (n=5). Inter-assay precision was evaluated as the %CV on the full set of samples (control samples at two levels in replicates of five prepared and analyzed on three different days, n=15).

Table 4. Description of the SRM parameters

Analyte / internal standard	Retention (min)	Quantification			Confirmation			RF lens (V)
		Precursor ion (*m/z*)	Product ion (*m/z*)	Collision energy (V)	Precursor ion (*m/z*)	Product ion (*m/z*)	Collision energy (V)	
5-Fluorocytosine	0.3	130.1	58.0	32	130.1	113.0	19	78
Fluconazole	1.4	306.9	238.1	15	306.9	220.0	17	110
Isavuconazole	1.8	438.1	224.0	20	438.1	369.0	18	138
Itraconazole	1.8	705.2	392.2	35	705.2	404.2	33	140
Ketoconazole	1.6	531.1	489.1	30	531.1	244.0	33	120
OH-Itraconazole	1.7	721.2	408.2	35	721.2	392.2	34	140
Posaconazole	1.7	701.3	614.3	34	701.3	344.1	43	200
Voriconazole	1.7	350.0	281.0	15	350.0	127.0	33	111
^{13}C-$^{15}N_2$-5-Fluorocytosine	0.3	133.1	115.0	32	133.1	/	/	78
d_4-Fluconazole	1.4	310.9	242.1	22	310.9	/	/	110
^{13}C-d_4-Isavuconazole	1.8	443.1	224.0	18	443.1	/	/	138
d_5-Itraconazole	1.8	710.2	397.2	35	710.2	/	/	140
d_8-Ketoconazole	1.6	539.1	497.1	33	539.1	/	/	120
d_5-OH-Itraconazole	1.7	726.2	413.2	34	726.2	/	/	140
d_4-Posaconazole	1.7	705.3	618.3	43	705.3	/	/	200
d_3-Voriconazole	1.7	353.0	353.0	33	353.0	/	/	111

Data analysis

Data were acquired and processed using Thermo Scientific™ TraceFinder™ 5.1 software.

Results and discussion

A linear interpolation with 1/x weighting was used for all analytes. The percentage bias between nominal and back-calculated concentration was always within ±6.0% for all the calibrators in all the runs. Representative chromatograms at the LLOQ for 5-fluorocytosine, isavuconazole, ketoconazole, voriconazole, and their corresponding internal standards are reported in Figure 1. Representative calibration curves for the same analytes are reported in Figure 2.

No carryover was observed for any of the analytes, with no signal detected in the blank injected immediately after the highest calibrator.

The data demonstrated good accuracy of the method with the percentage bias between nominal and average back-calculated concentration for control samples ranging between -5.5% and 5.1% (Table 5). The %CV for intra-assay precision was always below 12.4% for all analytes. The maximum %CV for inter-assay precision including all analytes was 10.8%. Results for intra- and inter-assay precision are reported in Table 6.

LLOQs of all compounds are reported in Table 7.

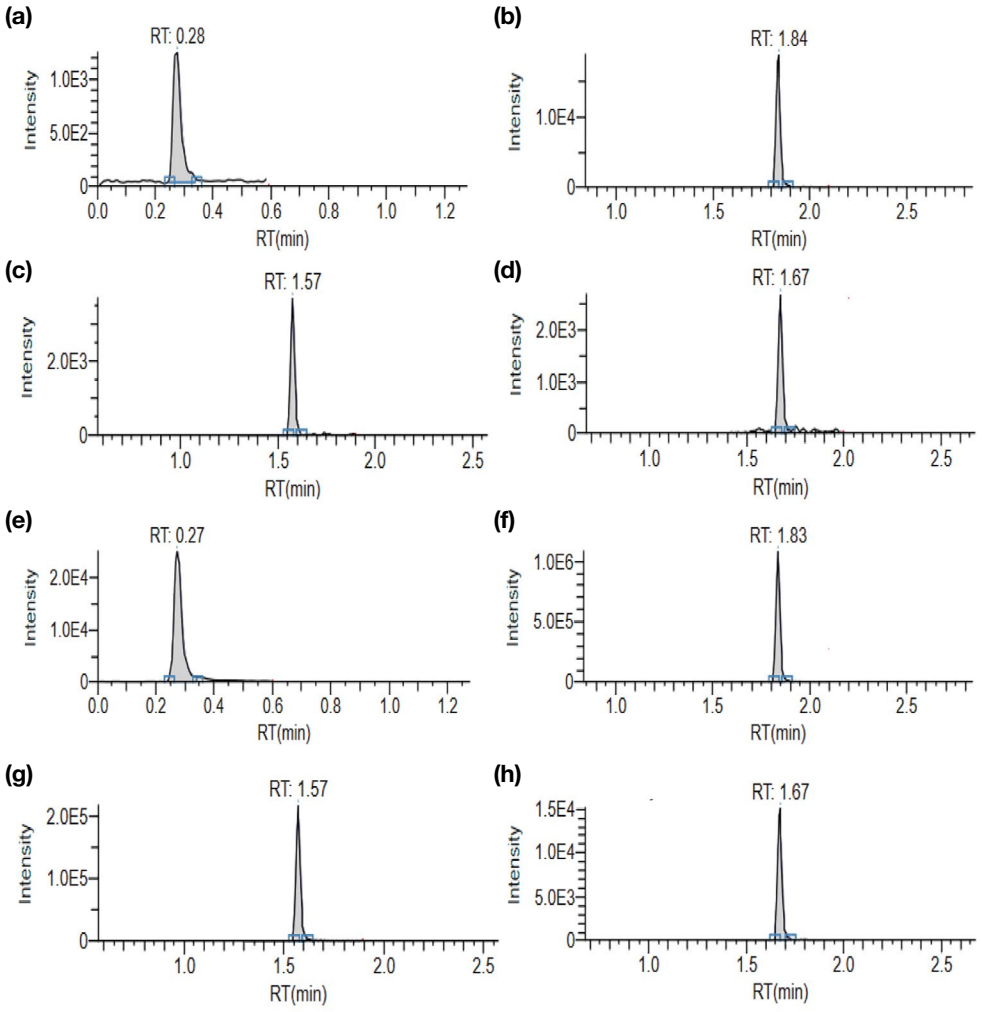

Figure 1. Representative chromatograms of the lower limit of quantification for (a) 5-fluorocytosine, (b) isavuconazole, (c) ketoconazole, (d) voriconazole, (e) ^{13}C-$^{15}N_2$-5-fluorocytosine, (f) ^{13}C- d_4-isavuconazole, (g) d_8-ketoconazole, (h) d_3-voriconazole

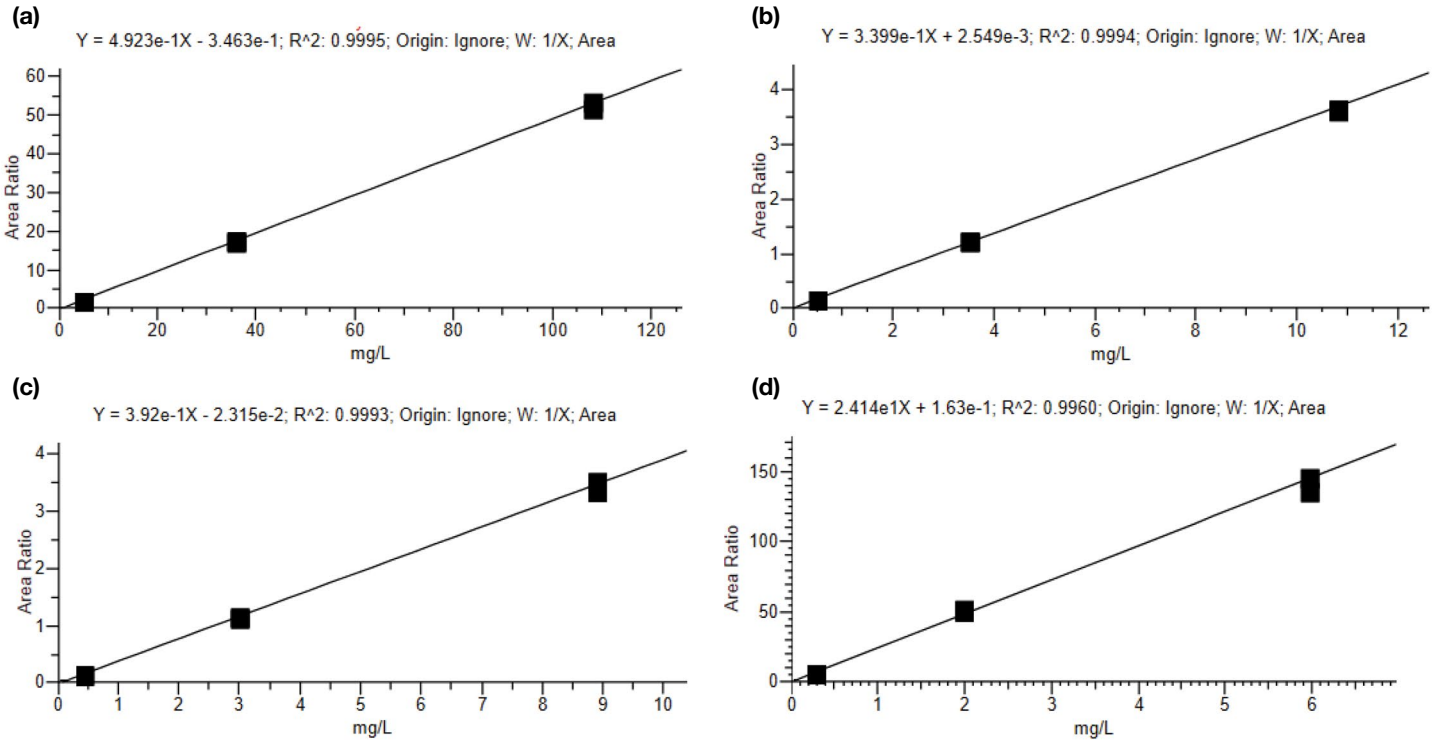

Figure 2. Representative calibration curves for (a) 5-fluorocytosine, (b) isavuconazole, (c) ketoconazole, (d) voriconazole

Table 5. Analytical accuracy results for control MS9682 batch #1369

Analyte	Control	Nominal concentration (mg/L)	Average calculated concentration (mg/L)	Bias (%)
5-Fluorocytosine	Level I	19.9	19.1	-4.0
	Level II	46.7	48.1	3.1
Fluconazole	Level I	2.43	2.42	-0.5
	Level II	5.79	6.00	3.6
Isavuconazole	Level I	1.95	1.98	1.7
	Level II	4.59	4.82	5.1
Itraconazole	Level I	0.590	0.562	-4.8
	Level II	1.31	1.38	5.1
Ketoconazole	Level I	1.71	1.62	-5.5
	Level II	3.90	4.02	3.2
OH-Itraconazole	Level I	0.678	0.665	-2.0
	Level II	1.60	1.57	-1.7
Posaconazole	Level I	0.909	0.883	-2.8
	Level II	2.19	2.17	-1.0
Voriconazole	Level I	1.10	1.14	3.4
	Level II	2.59	2.69	4.0

Table 6. Analytical intra- and inter-assay precision results for control MS9682 batch #1369

Compound name	Control	Intra-assay						Inter-assay	
		Day 1		Day 2		Day 3			
		Average calculated concentration (mg/L)	CV (%)	Average calculated concentration (mg/L)	CV (%)	Average calculated concentration (mg/L)	CV (%)	Average calculated concentration (mg/L)	CV (%)
5-Fluorocytosine	Level I	19.3	1.5	19.0	4.2	19.0	2.7	19.1	1.1
	Level II	48.0	10.6	49.6	4.7	46.8	4.3	48.1	2.9
Fluconazole	Level I	2.39	1.6	2.42	3.10	2.43	2.5	2.42	0.9
	Level II	5.92	11.5	6.28	2.9	5.79	2.9	6.00	4.3
Isavuconazole	Level I	2.06	3.2	1.93	3.8	1.96	3.5	1.98	3.5
	Level II	4.81	12.4	5.00	2.4	4.65	4.1	4.82	3.7
Itraconazole	Level I	0.603	2.7	0.545	2.8	0.538	3.2	0.562	6.3
	Level II	1.41	11.9	1.46	3.4	1.26	6.0	1.38	7.3
Ketoconazole	Level I	1.67	7.7	1.57	3.5	1.61	1.5	1.62	2.9
	Level II	4.09	11.3	4.16	3.5	3.82	4.5	4.02	4.5
OH-Itraconazole	Level I	0.716	5.3	0.657	6.2	0.622	4.9	0.665	7.2
	Level II	1.58	7.9	1.66	6.8	1.48	5.4	1.57	5.6
Posaconazole	Level I	0.97	10.0	0.844	10.8	0.840	7.0	0.883	8.2
	Level II	2.25	7.8	2.35	11.3	1.90	8.5	2.17	10.8
Voriconazole	Level I	1.10	6.1	1.07	4.7	1.04	6.9	1.07	2.4
	Level II	2.61	10.1	2.64	5.1	2.62	4.1	2.62	0.5

Table 7. LLOQs for all compounds

Analyte	LLOQ (mg/L)
5-Fluorocytosine	0.245
Fluconazole	0.0622
Isavuconazole	0.0481
Itraconazole	0.0292
Ketoconazole	0.0430
OH-Itraconazole	0.0342
Posaconazole	0.233
Voriconazole	0.0138

Conclusions

A robust, reproducible, and sensitive liquid chromatography-tandem mass spectrometry method for clinical research for quantification of eight antimycotics in human plasma was developed and implemented. The analytical method was validated on an Vanquish Flex Binary UHPLC system coupled to a TSQ Quantis triple quadrupole mass spectrometer. The method described here offers quick and simple offline protein precipitation with concomitant internal standard addition using the ClinMass TDM Platform with the ClinMass Add-On Set for Antimycotics from RECIPE. The described method meets research laboratory requirements in terms of sensitivity, linearity of response, accuracy, and precision.

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