TECHNICAL NOTE 66046

Quantification of eight antimycotics in human plasma by LC-HRAM-MS for clinical research

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Keywords: Orbitrap Exploris 120 MS, TraceFinder software, Vanquish Flex UHPLC, Antimycotics, LC-HRMS, offline sample preparation, plasma, high-resolution mass spectrometry

Application benefits

- High-resolution mass spectrometry for improved selectivity
- 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™ Orbitrap Exploris™ 120 mass spectrometer.

Introduction

Antifungals, also known as antimycotics, typically refer to a class of pharmaceutical fungicide used in the relief and prevention of 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 address life-threatening fungal infections along with prevention of infections in immunocompromised individuals. Individual variation along with other complications can 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.

In this report, an analytical method for clinical research for the quantification of eight antimycotics in human plasma in 3.6 minutes is presented. Samples were prepared by protein precipitation followed by chromatographic separation on a Thermo Scientific™ Vanquish™ Flex Binary UHPLC system. Detection was performed on an Orbitrap Exploris 120 mass spectrometer with heated electrospray ionization (HESI) operated in positive ionization mode. Method performance was evaluated using the ClinMass® LC-MS/MS calibrators, controls, and internal standards provided by RECIPE Chemicals + Instruments GmbH (Munich, Germany) in terms of linearity of response, carryover, accuracy, and intra- and inter-assay precision for all analytes.

Experimental

Target analytes

The complete list of analytes and corresponding internal standards is reported in Table 1. The retention times obtained and the concentration ranges covered by the calibrators used are reported in Table 2.

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 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 an Orbitrap Exploris 120 mass spectrometer. Chromatographic separation was achieved by gradient elution on a Thermo Scientific™ Hypersil GOLD™ 50 x 2.1 mm (1.9 µm) column (P/N 25002-059428) kept at 40 °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.

Table 1. List of analytes and internal standards

Compound name	Formula	Expected mass (*m/z*)	Internal standard name	Formula	Expected mass (*m/z*)
5-Fluorocytosine	$C_4H_4FN_3O$	130.0411	^{13}C-$^{15}N_2$-5-Fluorocytosine	$^{13}C^{15}N_2C_3H_4FNO$	133.0385
Fluconazole	$C_{13}H_{12}F_2N_6O$	307.1113	d_4-Fluconazole	$C_{13}H_8D_4F_2N_6O$	311.1365
Isavuconazole	$C_{22}H_{17}F_2N_5OS$	438.5078	^{13}C-d_4-Isavuconazole	$^{13}CC_{21}H_{13}D_4F_2N_5OS$	443.1480
Itraconazole	$C_{35}H_{38}Cl_2N_8O_4$	705.2466	d_5-Itraconazole	$C_{35}H_{33}D_5Cl_2N_8O_4$	710.2780
Ketoconazole	$C_{26}H_{28}Cl_2N_4O_4$	531.1560	d_8-Ketoconazole	$C_{26}H_{20}D_8Cl_2N_4O_4$	539.2063
OH-Itraconazole	$C_{35}H_{38}Cl_2N_8O_5$	721.2415	d_5-OH-Itraconazole	$C_{35}H_{33}D_5Cl_2N_8O_5$	726.2729
Posaconazole	$C_{37}H_{42}F_2N_8O_4$	701.3370	d_4-Posaconazole	$C_{37}H_{38}D_4F_2N_8O_4$	705.3621
Voriconazole	$C_{16}H_{14}F_3N_5O$	350.1223	d_3-Voriconazole	$C_{16}H_{11}D_3F_3N_5O$	353.1412

Table 2. Retention times and ranges of concentrations (MS9613 batch #1369)

Compound name	Retention time (min)	Concentration range (mg/L)
5-Fluorocytosine	0.27	4.90–108
Fluconazole	1.4	0.622–13.5
Isavuconazole	1.8	0.481–10.8
Itraconazole	1.8	0.146–3.11
Ketoconazole	1.6	0.430–8.88
OH-Itraconazole	1.7	0.171–3.55
Posaconazole	1.7	0.233–4.90
Voriconazole	1.7	0.275–5.96

The LC method is described in detail in Table 3. Total runtime was 3.6 minutes.

Table 3. 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

Mass spectrometry

Analytes and internal standards were detected by Full Scan – data-dependent MS^2 on an Orbitrap Exploris 120 mass spectrometer using a HESI source operated in positive ionization mode. A summary of the MS conditions is reported in Table 4. Two fragments for each analyte were used for confirmation based on ion ratio.

Method evaluation

The method performance was evaluated in terms of linearity of response within the calibration ranges, carryover, accuracy, intra- and inter-assay precision for all analytes. 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. 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	
Mild trapping	No
Internal mass calibration	RunStart EASY-IC™
Data acquisition mode	Full scan – ddMS
Full Scan parameters	
Resolution (at *m/z* 200)	60,000
Scan range (*m/z*)	100–750
RF lens (%)	70
Expected peak width (s)	6
Maximum injection time mode	Auto
AGC target	Standard (1e6)
Polarity	Positive
Data-Dependent MS^2 scan properties	
Isolation window (*m/z*)	2
Collision energy type	Normalized
HCD collision energy (%)	30
Resolution (at *m/z* 200)	15,000
Scan range mode	Auto

Data analysis

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

Results and discussion

A linear regression was used for all analytes. Among these interpolations, three were applied with 1/x weighting (fluconazole, isavuconazole, and hydroxy-itraconazole). The other interpolations were applied with an equal weighting. The percentage bias between nominal and back-calculated concentration was always within ±15% for all the calibrators in all the runs. Chromatograms for the lowest calibrator for representative analytes and their internal standards are reported in Figure 1. Representative calibration curves are reported in Figure 2.

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

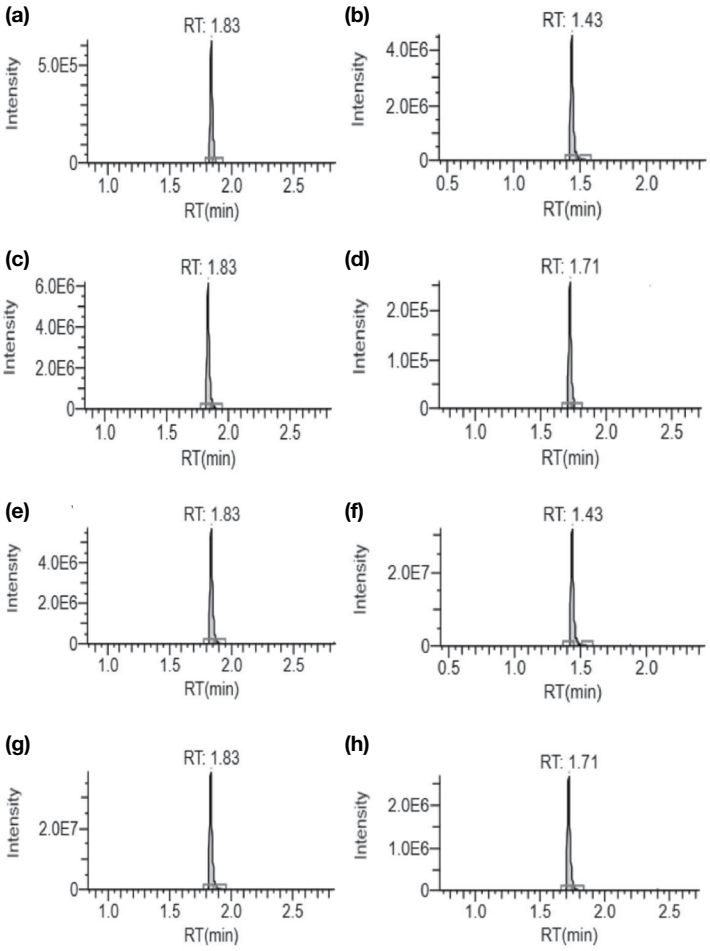

Figure 1. Representative chromatograms of the lowest calibrator for (a) itraconazole, (b) fluconazole, (c) isavuconazole, (d) OH-itraconazole, (e) d_5-itraconazole, (f) d_4-fluconazole, (g) ^{13}C-d_4-isavuconazole, (h) d_5-oh-itraconazole

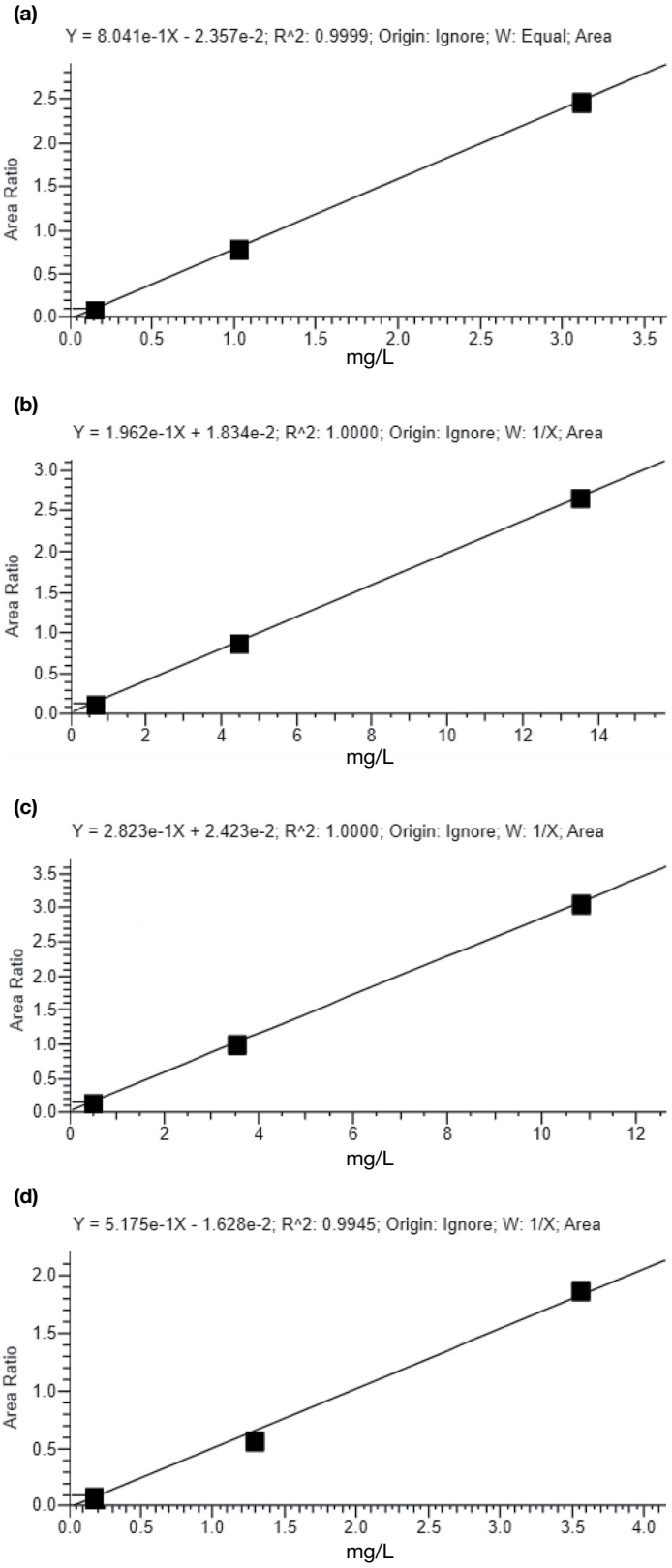

Figure 2. Representative calibration curves for (a) itraconazole, (b) fluconazole, (c) isavuconazole, (d) OH-itraconazole

The data demonstrated good accuracy of the method with the percentage bias between nominal and average back-calculated concentration for the used control samples ranging between -7.6% and 7.2% (Table 5). The %CV for intra-assay precision was always below 8.4% for all the analytes. The maximum %CV for inter-assay precision including all the analytes was 5.8%. Results for intra- and inter-assay precision are reported in Table 6.

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	21.9	20.2	-7.6
	Level II	51.4	49.0	-4.7
Fluconazole	Level I	2.43	2.50	2.7
	Level II	5.79	6.08	5.0
Isavuconazole	Level I	1.95	1.98	1.6
	Level II	4.59	4.85	5.7
Itraconazole	Level I	0.590	0.571	-3.1
	Level II	1.31	1.37	4.3
Ketoconazole	Level I	1.71	1.71	0.3
	Level II	3.90	4.18	7.2
OH-Itraconazole	Level I	0.678	0.656	-3.3
	Level II	1.60	1.58	-1.0
Posaconazole	Level I	0.988	0.942	-4.6
	Level II	2.38	2.25	-5.5
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	21.2	3.7	19.7	1.2	19.7	2.8	20.2	4.3
	Level II	49.3	4.3	49.9	3.3	47.6	5.8	49.0	2.5
Fluconazole	Level I	2.57	4.3	2.49	0.9	2.43	2.1	2.50	2.9
	Level II	6.02	4.6	6.33	2.4	5.88	5.6	6.08	3.9
Isavuconazole	Level I	2.03	4.7	1.98	1.2	1.93	2.2	1.98	2.7
	Level II	4.72	5.5	5.13	3.5	4.71	5.5	4.85	4.9
Itraconazole	Level I	0.574	3.9	0.560	4.5	0.580	2.3	0.571	1.7
	Level II	1.28	4.6	1.42	3.6	1.40	6.2	1.37	5.5
Ketoconazole	Level I	1.75	2.8	1.72	1.4	1.68	2.2	1.71	2.2
	Level II	4.05	5.0	4.32	3.9	4.17	4.9	4.18	3.2
OH-Itraconazole	Level I	0.659	3.8	0.658	7.2	0.650	4.4	0.656	0.8
	Level II	1.51	4.5	1.68	2.7	1.56	8.4	1.58	5.4
Posaconazole	Level I	0.965	4.7	0.937	1.6	0.926	2.3	0.943	2.1
	Level II	2.16	3.8	2.39	2.9	2.19	5.1	2.25	5.3
Voriconazole	Level I	1.17	4.3	1.13	1.4	1.12	2.3	1.14	2.3
	Level II	2.68	4.2	2.86	3.1	2.55	5.6	2.69	5.8

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Conclusions

A robust, reproducible, and sensitive liquid chromatography-HRAM mass spectrometry method for clinical research for quantification of eight antimycotics in human plasma was developed and implemented. Sample preparation consisted of a simple offline protein precipitation with concomitant internal standard addition. The method was evaluated on an Vanquish Flex Binary UHPLC system coupled to an Orbitrap Exploris 120 mass spectrometer. Method performance was evaluated using the ClinMass LC-MS/MS calibrators, controls, and internal standards. The described method meets research laboratory requirements in terms of sensitivity, linearity of response, accuracy, and precision.

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TN66046-EN 0621S

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