TECHNICAL NOTE 73904

Quantification of nine antimycotic drugs in human plasma or serum by LC-HRAM(MS) for clinical research

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Application benefits

- Increased method accuracy by implementation of a comprehensive ClinMass™ kit for sample preparation
- High-resolution mass spectrometry for improved selectivity
- Simple offline sample preparation by protein precipitation
- Nine antimycotic drugs in a single quantitative method

Goal

Implementation of an analytical method for the quantification of nine antimycotic drugs in human plasma or serum on a Thermo Scientific™ Q Exactive™ Plus hybrid quadrupole-Orbitrap™ mass spectrometer.

Introduction

Antifungals, also known as antimycotics, typically refer to a class of pharmaceutical fungicides 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 antimycotics are used to treat life-threatening fungal infections and prevent infections in immunocompromised patients. 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.

A robust, reliable, and accurate LC-MS based analytical method for clinical research for the quantification of nine antimycotic drugs in human plasma or serum is reported in this study. Plasma or serum samples were processed by offline internal standard addition and protein precipitation. Extracted samples were injected onto a Thermo Scientific™ Vanquish™ Duo UHPLC system connected to a Q Exactive Plus hybrid quadrupole-Orbitrap mass spectrometer with a heated electrospray ionization (HESI-II) source. Detection was performed by full scan coupled to data-dependent fragmentation (fullMS-ddMS^2). The full scan experiment was used for quantification, and fragmentation was used for confirmation. Method performance was evaluated using the ClinMass TDM Platform with the ClinMass Add-On Set for Antimycotics from RECIPE Chemicals + Instruments GmbH (Munich, Germany) in terms of linearity of response within the calibration ranges, lower limit of quantification (LLOQ), carryover, accuracy, trueness of measurements, and intra- and inter-assay precision for each analyte.

Most reported LC-MS analyses of antimycotics focus on targeted, sensitive quantitation utilizing triple quadrupole mass spectrometers. The results in this study demonstrate the capabilities of high resolution accurate mass (HRAM) mass spectrometry powered by Orbitrap technology.

Experimental

Target analytes

A comprehensive list of analytes, corresponding internal standards, and concentration ranges covered by the calibrators (MS9613 batch #1367) are reported in Table 1.

Table 1. Analytes, internal standards, and concentration ranges

Analyte	Internal standard	Concentration range (ng/mL)
5-Fluorocytosine	$^{13}C,^{15}N_2$-5-fluorocytosine	5.12–117
Anidulafungin	d_4-posaconazole	0.461–8.98
Fluconazole	d_4-fluconazole	0.564–12.6
Isavuconazole	$^{13}C,d_4$-isavuconazole	0.482–10.6
Itraconazole	d_5-itraconazole	0.133–2.94
Ketoconazole	d_8-ketoconazole	0.406–8.34
OH-Itraconazole	d_5-OH-itraconazole	0.164–3.60
Posaconazole	d_4-posaconazole	0.232–5.01
Voriconazole	d_3-voriconazole	0.265–5.90

Sample preparation

Reagents included four calibrators (including blank) and two controls from RECIPE (MS9682 batch #1367), as well as eight stable isotope-labeled internal standard for the quantification. Samples of 50 µL of plasma or serum were protein-precipitated using 100 µL of precipitating solution containing the internal standards. Precipitated samples were vortex-mixed and centrifuged. 100 µL of supernatant were transferred to a clean plate or vial prior to injection.

Liquid chromatography

A Vanquish Duo UHPLC system, a dual-channel instrument configured for both LC-only and online SPE applications (Figure 1), was used for chromatographic separation. The direct injection flow path was used in this case, utilizing mobile phases and an analytical column provided by RECIPE. Details of the analytical method are reported in Table 2. The injection volume was 10 µL. The total run time was 3.5 minutes.

Mass spectrometry

Analytes and internal standards were detected by FullMS-ddMS^2 mode on a Q Exactive Plus hybrid quadrupole-Orbitrap mass spectrometer with a heated electrospray ionization (HESI-II) source operated in positive ion mode. A summary of the MS conditions is reported in Table 3.

Method evaluation

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

To determine the LLOQ, the lowest calibrator was diluted down to 20-fold with blank matrix; a full set of calibrators (three levels), diluted calibrators (four levels), and controls (two levels) were extracted in replicates of five (n=5), 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 percentage coefficient of variation (%CV) below 20% across the entire batch of samples.

Carryover was calculated in terms of percentage ratio between peak area of the highest calibrator and a blank sample injected immediately after it.

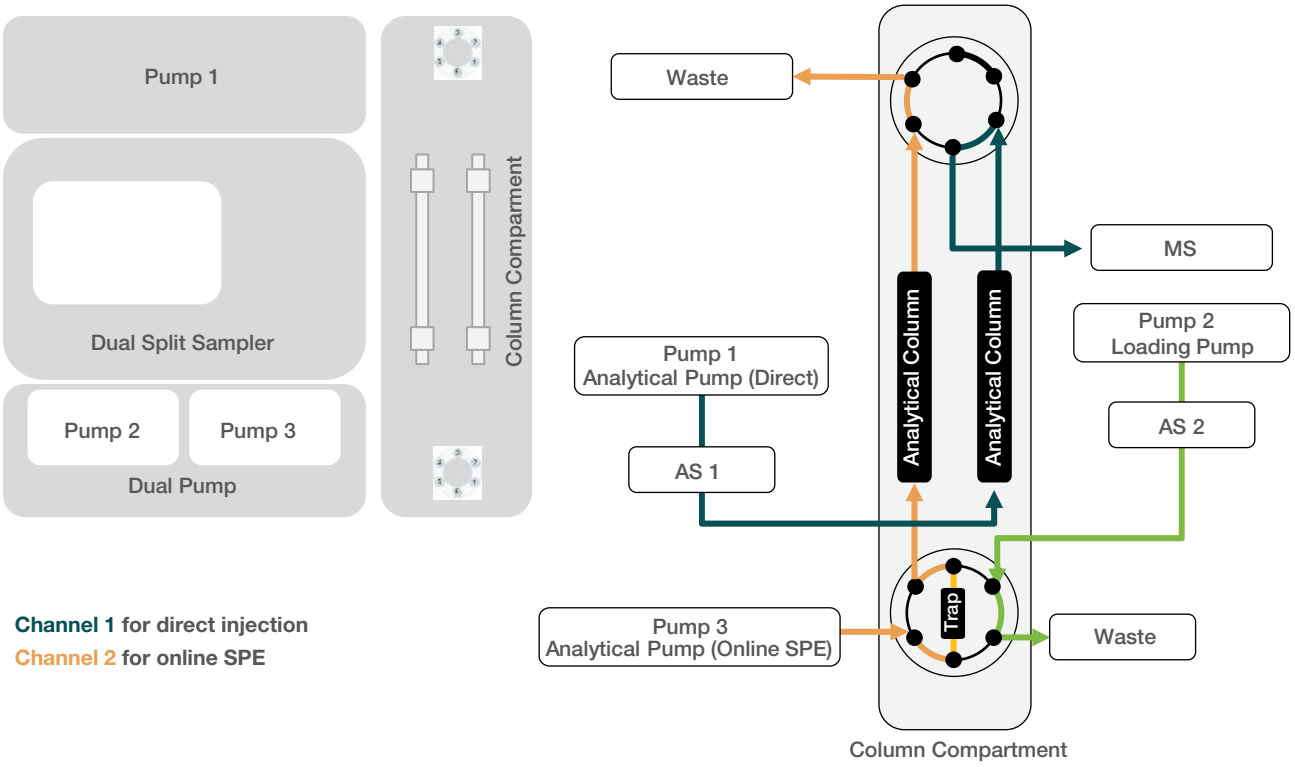

Figure 1. Schematic representation of the Vanquish Duo UHPLC system setup; the direct injection flow path was used.

Table 2. Liquid chromatography method description

Gradient profile			
Time (min)	**Flow rate (mL/min)**	**A (%)**	**B (%)**
0.00	0.6	100	0
0.01	0.6	100	0
0.10	0.6	70	30
2.10	0.6	40	60
2.20	0.6	2	98
2.40	0.6	2	98
2.41	0.6	100	0
3.50	0.6	100	0
Other parameters			
Injection volume		10 µL	
Column temperature		40 °C	

Table 3. MS settings

Parameter	Value
Source type	Heated electrospray ionization (H-ESI II)
Vaporizer temperature	450 °C
Capillary temperature	275 °C
Spray voltage (positive mode)	1500 V
Sheath gas	50 AU
Sweep gas	0 AU
Auxiliary gas	15 AU
S–Lens RF level	60
Data acquisition mode	FullMS–ddMS^2
FullMS resolution @ *m/z* 200	70,000
FullMS scan range	120–1150 *m/z*
ddMS^2 resolution @ *m/z* 200	17,500
ddMS^2 isolation window	2.0 *m/z*
Stepped normalized collision energy (NCE)	15, 25, 35

Analytical accuracy was evaluated in terms of percentage bias between nominal and average back-calculated concentrations at two different levels using the quality control samples provided by RECIPE and prepared and analyzed in replicates of five on three different days.

Trueness of measurement was also evaluated for itraconazole and hydroxyitraconazole as percentage bias using certified external quality controls (Instand RV 602 2019 Probe 21 and 22, Dusseldorf, Germany) prepared and analyzed in replicates of five on a single day.

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).

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

Results and discussion

A linear response (quadratic for ketoconazole) with 1/× weighting was obtained for all the analytes not only in the calibration range covered by the calibrators, but also down to a LLOQ reported in Table 4. The percentage bias between nominal and back-calculated concentration was always within ±10% for all the calibrators (±15% for the lowest calibrator) in all the runs. Representative chromatograms for the LLOQ for fluconazole, voriconazole, and the corresponding internal standards are depicted in Figure 2. Representative calibration curves for the same analytes in the concentration range covered by the kit (three calibrators) are shown in Figure 3.

Table 4. Analytes and corresponding LLOQ

Analyte	LLOQ (ng/mL)
5-Fluorocytosine	0.256
Anidulafungin	0.461
Fluconazole	0.028
Isavuconazole	0.024
Itraconazole	0.067
Ketoconazole	0.041
OH-Itraconazole	0.016
Posaconazole	0.023
Voriconazole	0.013

No carryover was registered, with no peak detected in the blank sample injected immediately after the highest calibrator.

The data presented in this report demonstrate the outstanding accuracy of the method with the percentage bias between nominal and average back-calculated concentration for the control samples ranging between -2.0% and 4.2% (Table 5).

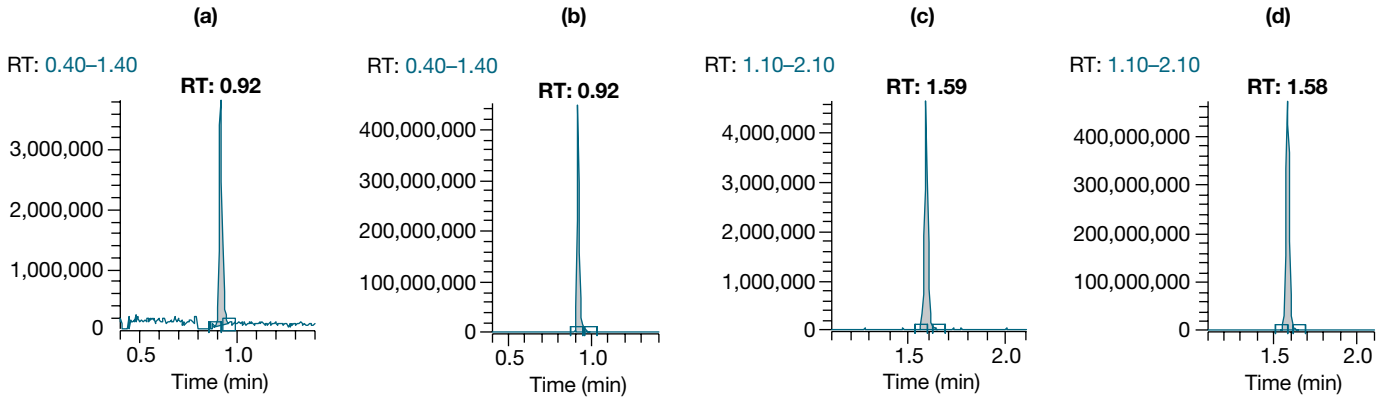

Figure 2. Representative chromatograms of the LLOQ for (a) fluconazole, (b) fluconazole-D_4, (c) voriconazole, and (d) voriconazole-D_3

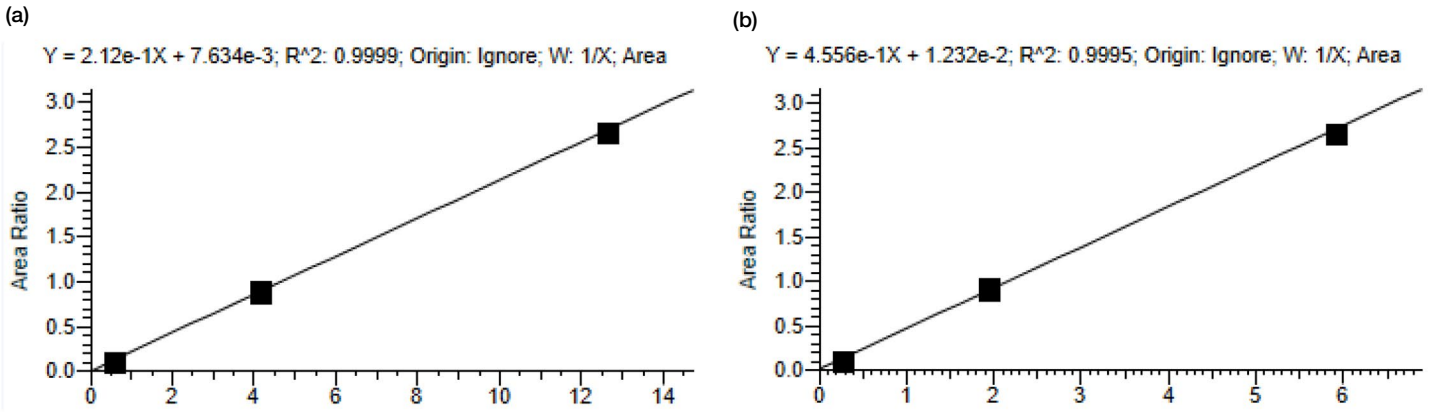

Figure 3. Representative calibration curves for (a) fluconazole and (b) voriconazole

Table 5. Analytical accuracy results for controls MS9682 batch #1367

Analyte	Control 1			Control 2		
	Nominal concentration (ng/mL)	Average calculated concentration (ng/mL)	Bias (%)	Nominal concentration (ng/mL)	Average calculated concentration (ng/mL)	Bias (%)
5-Fluorocytosine	21.7	22.5	3.5	50.9	52.1	2.3
Anidulafungin	1.82	1.85	1.7	4.11	4.10	-0.1
Fluconazole	2.29	2.32	1.5	5.40	5.41	0.1
Isavuconazole	1.92	1.98	3.0	4.55	4.57	0.3
Itraconazole	0.528	0.550	4.2	1.26	1.25	-0.5
Ketoconazole	1.63	1.63	0.0	3.68	3.61	-1.9
OH-Itraconazole	0.654	0.658	0.6	1.56	1.53	-2.0
Posaconazole	0.910	0.934	2.6	2.18	2.19	0.6
Voriconazole	1.07	1.11	3.6	2.53	2.61	3.3

Excellent results were obtained also from the evaluation of trueness of measurement, with a percentage bias between -5.4% and 1.4% (Table 6).

The %CV for intra-assay precision was always below 8.2%. The maximum %CV for inter-assay precision was 6.4%. Results for intra- and inter-assay precision are reported in Table 7 and Table 8, respectively.

Table 6. Analytical accuracy results for controls Instand RV 602 2019

Analyte	Probe 21			Probe 22		
	Nominal concentration (ng/mL)	Average calculated concentration (ng/mL)	Bias (%)	Nominal concentration (ng/mL)	Average calculated concentration (ng/mL)	Bias (%)
Itraconazole	1.45	1.47	1.4	0.287	0.272	-5.4
OH-Itraconazole	1.39	1.38	-0.3	0.537	0.537	0.1

Table 7. Intra–assay precision results for controls MS9682 batch #1367

Analyte	Control 1						Control 2					
	Day 1		Day 2		Day 3		Day 1		Day 2		Day 3	
	Average calculated concentration (ng/mL)	CV (%)	Average calculated concentration (ng/mL)	CV (%)	Average calculated concentration (ng/mL)	CV (%)	Average calculated concentration (ng/mL)	CV (%)	Average calculated concentration (ng/mL)	CV (%)	Average calculated concentration (ng/mL)	CV (%)
5-Fluorocytosine	22.3	4.7	22.4	3.2	22.7	2.8	52.5	4.7	50.9	2.3	52.9	2.1
Anidulafungin	1.94	1.4	1.84	4.9	1.77	8.2	4.36	3.6	4.02	3.3	3.94	4.5
Fluconazole	2.26	1.8	2.35	0.4	2.36	1.1	5.31	1.4	5.43	1.3	5.48	0.8
Isavuconazole	1.95	2.0	1.98	1.4	2.01	1.9	4.53	2.2	4.56	1.8	4.60	2.1
Itraconazole	0.532	5.3	0.547	1.4	0.571	3.7	1.20	3.6	1.26	4.1	1.30	2.6
Ketoconazole	1.55	2.5	1.69	1.9	1.66	2.0	3.45	3.3	3.71	0.4	3.67	3.1
OH-Itraconazole	0.640	2.5	0.668	1.3	0.666	1.6	1.50	3.1	1.54	2.4	1.55	1.1
Posaconazole	0.916	1.8	0.942	1.4	0.943	1.3	2.13	1.2	2.25	2.7	2.20	1.3
Voriconazole	1.09	0.9	1.11	1.6	1.12	1.6	2.55	1.1	2.60	1.1	2.69	1.2

Table 8. Inter–assay precision results for controls MS9682 batch #1367

Analyte	Control 1		Control 2	
	Average calculated concentration (ng/mL)	CV (%)	Average calculated concentration (ng/mL)	CV (%)
5-Fluorocytosine	22.5	3.4	52.1	3.5
Anidulafungin	1.85	6.4	4.10	5.8
Fluconazole	2.32	2.3	5.41	1.8
Isavuconazole	1.98	2.1	4.57	2.0
Itraconazole	0.550	4.6	1.25	4.7
Ketoconazole	1.63	4.4	3.61	4.1
OH-Itraconazole	0.658	2.6	1.53	2.7
Posaconazole	0.934	1.9	2.19	3.0
Voriconazole	1.11	1.6	2.61	2.5

Conclusions

An LC-HRAM(MS)-based method (a Vanquish Duo UHPLC system connected to a Q Exactive Plus hybrid quadrupole-Orbitrap MS) for robust, reliable, and accurate analysis of nine antimycotic drugs is reported here. The power of Orbitrap technology in performing accurate and efficient qualitative analyses and quantitation in an applied environment is demonstrated. The ClinMass TDM Platform with the ClinMass Add-On Set for Antimycotics from RECIPE was used. The method incorporates a quick and simple offline protein precipitation step with concomitant internal standard addition. The described method meets research laboratory requirements in terms of sensitivity, linearity of response, accuracy, and precision.

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