TECHNICAL NOTE No. 73187

Quantification of eleven antimycotics in human serum by TurboFlow chromatography coupled to high-resolution accurate-mass Orbitrap mass spectrometry for clinical research

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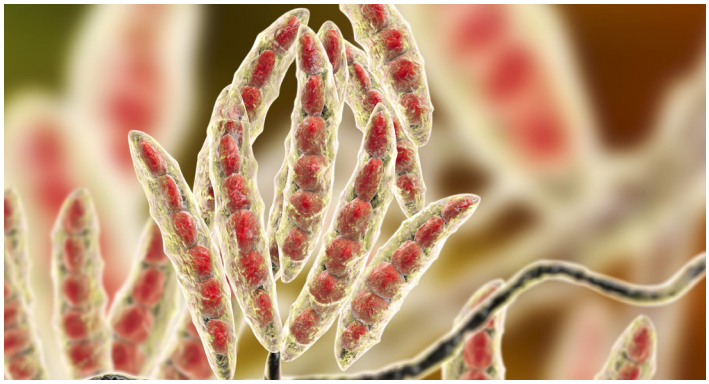
Keywords: Antimycotics, TurboFlow, online sample preparation, serum, Orbitrap, high-resolution accurate-mass mass spectrometry



Application benefits

- Simple protein precipitation followed by online sample cleanup using Thermo Scientific™ TurboFlow™ technology
- 11 antimycotics in a single quantitative method

Goal

Implementation of an analytical method for the quantification of 11 antimycotics in human serum using a Thermo Scientific™ Transcend™ II TLX-1 system coupled to a Thermo Scientific™ Q Exactive™ Focus hybrid quadrupole-Orbitrap™ mass spectrometer

Introduction

Antimycotics or antifungals are pharmaceutical fungicides used for the treatment of and prevention of mycosis (e.g., athlete's foot, ringworm, etc.). The antimycotics typically demonstrate a narrow therapeutic range in addition to other complications (such as, drug–drug interactions,

variations between individuals from absorption to metabolism and clearance). All these factors can possibly lead to different drug exposure from even the same dosage regimen, resulting in different clinical impact. Adjustment of dose based on rapid and accurate drug monitoringresults helps to achieve control of infection in clinical research.

Analytical methods to quantify antimycotics were initially developed on high-performance liquid chromatography (HPLC) coupled with UV detectors in the 1990s. However, these methods require complicated extraction procedures (i.e. liquid–liquid extraction, solid-phase extraction) and time-consuming chromatography. LC coupled to mass spectrometry (MS) exhibits superior selectivity and specificity, while achieving necessary sensitivity, and can be effectively used to measure several antifungal agents in a single analytical run in short time.

In this study, we report an analytical method for clinical research for quantification of eleven antimycotics in human serum. The panel includes 5-flucytosine, amphotericin B, anidulafungin, fluconazole, isavuconazole, itraconazole, ketoconazole, micafungin, OH-itraconazole, posaconazole, and voriconazole. Analytes and internal standards were detected using HRAM mass spectrometry on a Q Exactive Focus mass spectrometer with heated electrospray ionization in positive mode. Detection was performed in FullMS mode using a resolution of 70,000 (FWHM) at *m/z* 200. Method performance was evaluated in terms of linearity of response within the calibration ranges, limits of quantification, intra- and inter-assay precision, carryover, dilution integrity, matrix effect, selectivity, and stability for each analyte. The demonstrated method is simple, fast, accurate, and allows for routine therapeutic drug monitoring and dose adjustments in short intervals while addressing critical cost/sample challenges.

Experimental

Target analytes

The analytes, internal standards, and corresponding concentration ranges are reported in Table 1. Three-level calibrators and two-level controls were provided by RECIPE® Chemicals + Instruments GmbH (Munich, Germany) together with isotope-labeled internal standards $^{13}C^{15}N_2$-5-fluyctosine, d_4-fluconazole, $^{13}Cd_4$-isavuconazole, d_5-itraconazole, d_8-ketoconazole, d_5-OH-itraconazole, d_4-posaconazole, d_3-voriconazole, as well as the precipitation reagent (precipitant P), as part of the LC–MS/MS ClinMass® TDM kit system. Missing isotope-labeled internal standards $^{13}C_6$-micafungin and $^{13}C_6$-anidulafungin were purchased from Alsachim (Illkirch-Graffenstaden, France). The isotope-labeled internal standard d_3-amphotericin B was from Toronto Research Chemicals (North York, Canada). Drug-free serum was purchased from the blood donation service of the Bavarian Red Cross (Wiesentheid, Germany).

Table 1. Analytes, internal standards and concentration ranges

Analyte	Internal standard	Concentration range (ng/mL)
5-flucytosine	13C15N2-5-fluycotsine	3.41–117
Amphotericin B	d3-amphotericin B	0.056–5.23
Anidulafungin	13C6-anidulafungin	0.307–8.98
Fluconazole	d4-fluconazole	0.376–12.6
Isavuconazole	13Cd4-isavuconazole	0.321–10.6
Itraconazole	d5-itraconazole	0.089–2.94
Ketoconazole	d8-ketoconazole	0.271–8.34
Micafungin	13C6-micafungin	1.41–46.3
OH-itraconazole	d5-OH-itraconazole	0.109–3.60
Posaconazole	d4-posaconazole	0.155–5.01
Voriconazole	d3-voriconazole	0.177–5.90

To obtain six different calibrator levels the high and low level calibrator levels were diluted 1.5-fold and the medium calibrator level was diluted 2.5-fold with drug-free serum. The lower QC level from the kit was diluted with drug-free serum (1:3 v/v) to obtain concentrations within 3-fold of the concentration of the lowest limit of quantification (LLOQ). Individual concentrations are displayed in Table 2.

Sample preparation

Fifty microliters of calibrator, control, or serum sample were subjected to protein precipitated using 100 µL of precipitation mix containing all internal standards. Precipitated samples were vortex-mixed and centrifuged, and the supernatants were transferred to a clean plate or vial.

Table 2. Individual concentrations (ng/mL) for the calibrators and the controls

Analyte	Calibrator 1	Calibrator 2	Calibrator 3	Calibrator 4	Calibrator 5	Calibrator 6	Control 1	Control 2	Control 3
5-flucytosine	3.41	5.12	15.9	39.7	78	117	7.23	21.8	50.9
Amphotericin B	0.056	0.084	0.688	1.72	3.49	5.23	0.288	0.864	2.59
Anidulafungin	0.307	0.461	1.27	3.17	5.99	8.98	0.607	1.82	4.11
Fluconazole	0.376	0.564	1.66	4.14	8.40	12.6	0.763	2.29	5.40
Isavuconazole	0.321	0.482	1.40	3.49	7.07	10.6	0.64	1.92	4.55
Itraconazole	0.089	0.133	0.382	0.955	1.96	2.94	0.176	0.528	1.26
Ketoconazole	0.271	0.406	1.16	2.89	5.56	8.34	0.543	1.63	3.68
Micafungin	1.41	2.12	5.96	14.9	30.9	46.3	2.75	8.25	19.1
OH-itraconazole	0.109	0.164	0.476	1.19	2.40	3.60	0.218	0.654	1.56
Posaconazole	0.155	0.232	0.672	1.68	3.34	5.01	0.303	0.91	2.18
Voriconazole	0.177	0.265	0.772	1.93	3.93	5.9	0.357	1.07	2.53

Liquid chromatography

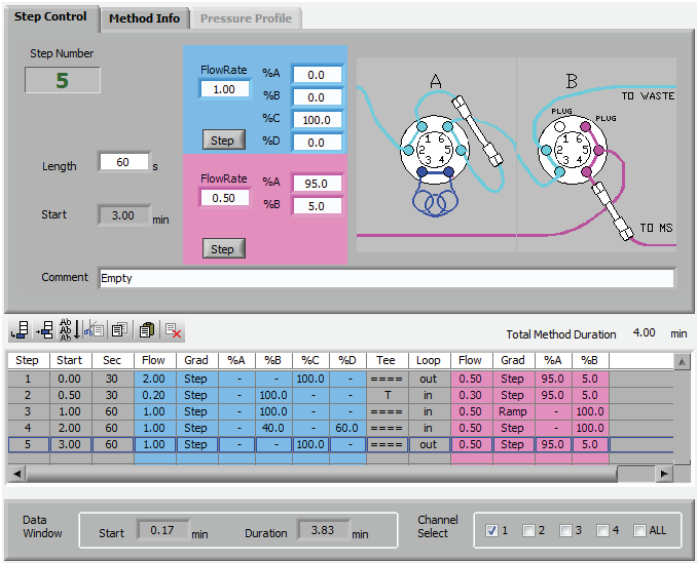
Online sample cleanup was achieved on a 0.5 x 50 mm Thermo Scientific™ TurboFlow™ Cyclone™ MCX-2 column. The LC separation was performed using a 50 x 2.1 mm (1.9 µm) Thermo Scientific™ Hypersil GOLD™ analytical column kept at 30 °C. Details of the analytical method are reported in Figure 1. Total runtime was 4.0 minutes.

Mass spectrometry

Analytes and internal standards were detected using a resolution of 70,000 (FWHM) at *m/z* 200 on a Q Exactive Focus MS system with heated electrospray ionization operated in positive mode. Data were acquired in FullMS mode covering a mass range between *m/z* 110 and 1300. The MS conditions are summarized in Table 3.

Table 3. MS settings

Source type	Heated electrospray ionization (HESI)
Vaporizer temperature	400 °C
Capillary temperature	350 °C
Spray voltage (positive mode)	3500 V
Sheath gas	50 AU
Sweep gas	0 AU
Auxiliary gas	15 AU
Data acquisition mode	FullMS at R=70,000 (FWHM) @ *m/z* 200
Mass range	*m/z* 110–1300 amu



Mobile phases

B	Water Acetonitrile 500 mM ammonium acetate 32% ammonia solution 5:93:1:1 (v/v/v/v)
C	Water Formic acid 99.8:0.2 (v/v)
D	Water 500 mM ammonium acetate 32% ammonia solution 98.9:1:0.1 (v/v/v)
A	Water 500 mM ammonium acetate Formic acid 98.8:1:0.2 (v/v/v)
B	Water Acetonitrile 500 mM ammonium acetate Formic acid 5:93.8:1:0.2 (v/v/v/v)

Figure 1. LC method description

Method evaluation

The method performance was evaluated in terms of calibration curve, inaccuracy, imprecision, limits of quantification, carryover, matrix effect, dilution integrity, and stability. Linearity of response was evaluated on the six calibrators using a linear interpolation with 1/x weighting. A maximum percentage bias between nominal and back-calculated concentration of ±15% was used as an acceptance criterion for all the calibrators (±20% for the lowest). The lower limit of quantification (LLOQ) was defined as the lowest calibrator with a signal-to-noise ratio ≥5 and imprecision/inaccuracy ≤20%. The LLOQ was obtained by 1.5-fold dilutions of the lowest calibrator. The upper limit of quantification (ULOQ) was defined as the highest assay calibrator. Inaccuracy and imprecision were tested using calibrator 1 (LLOQ) and QC samples 1–3. Within-run inaccuracy and imprecision were determined by replicate analysis of five individually prepared QCs. Between-run inaccuracy and imprecision were evaluated by analyzing five individually prepared QC samples per day, on three different days. Mean back-calculated concentrations of all QCs should be within ±15% of the nominal value (±20% for the LLOQ). Carryover was investigated by injecting blank serum samples from different donors (n = 8) after the highest calibrator (ULOQ). The peak area in the drug-free serum sample should not exceed 20% of the LLOQ peak area and 5% of the ISTD peak area.

Matrix effect was tested by adding the analytes at three different concentration levels to drug-free serum samples from different donors (n = 6) and to absolute methanol. After sample extraction the matrix factor (MF) was calculated for each analyte and ISTD as the ratio of the peak area in presence and in absence of the matrix. The ISTD normalized matrix factor was calculated as the ratio of the analyte MF and the MF of the corresponding ISTD. The coefficient of variation (CV) of the ISTD normalized MF should not exceed 15%.

To test dilution integrity, drug-free serum samples (n = 5) were spiked with an analyte concentration approximately 50% above the ULOQ and then diluted 5-fold with drug-free serum. The measured concentration was back calculated and compared to the nominal concentration. Inaccuracy and imprecision should not exceed ±15%. Stability was tested using QC samples 1–3 that were stored up to six hours at room temperature and at 4 °C, up to five weeks at –20 °C and –80 °C. Autosampler stability was tested up to 24 h. Freeze-thaw stability was determined in three cycles at –20 °C and –80 °C (freeze time >12 h, thawing at room temperature). Stored QC samples were analyzed using freshly prepared calibration samples. Mean calculated concentration should be within ± 15% of the nominal concentration.

Data analysis

Data were acquired and processed using Thermo Scientific™ TraceFinder™ 4.1 software. An extraction window of 5 ppm was used to extract the individual chromatograms.

Results and discussion

The method proved to be linear for each analyte in the calibration ranges covered by the calibrators, with a correlation factor (R^2) always above 0.995. Representative calibration curves for anidulafungin and voriconazole are reported in Figure 2. Representative chromatograms of the lowest calibrator for the same analytes are reported in Figure 3.

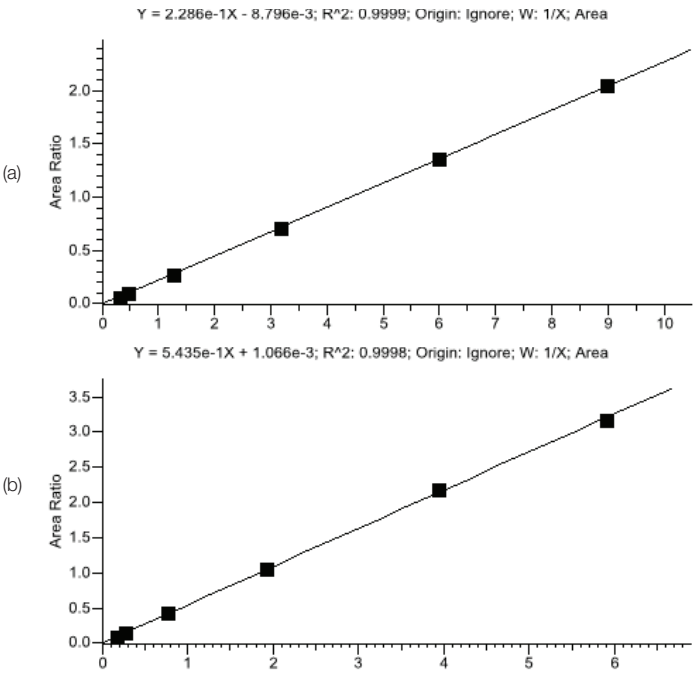


Figure 2. Representative calibration curves for (a) anidulafungin and (b) voriconazole

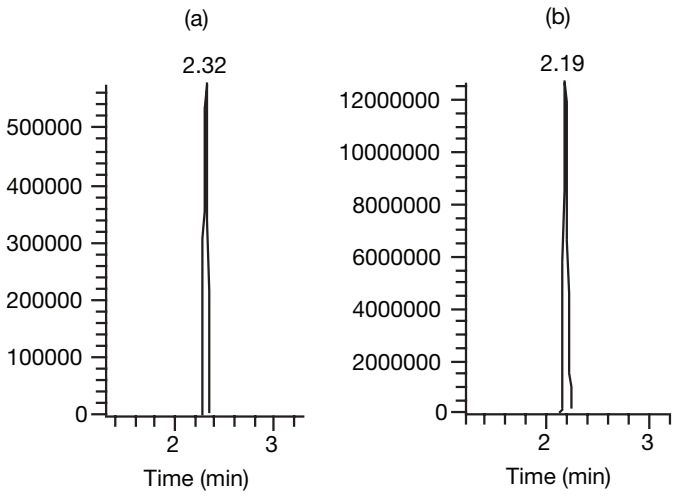


Figure 3. Representative chromatograms of the lowest calibrator for (a) anidulafungin and (b) voriconazole

The LLOQ signal-to-noise ratio of all analytes was at least seven times greater when compared to the blank samples at respective antimycotic retention times. Back-calculated LLOQ concentrations both between- and within-run inaccuracy and imprecision were ≤4.6% and ≤16.6%, respectively (Table 4).

For all analytes within-run inaccuracy was ≤12.0%, within-run imprecision was ≤8.1%, between-run inaccuracy was ≤9.0% and between-run imprecision was ≤7.1% in QC samples 1–3. Results are summarized in Table 4.

Table 4. Mean results for inaccuracy and imprecision of the LLOQ (Cal 1) and QCs

	5-FC	AM-B	ANF	FCZ	IVZ	ITZ	KTZ	MCF	OH-ITZ	PSZ	VRZ
Within-run inaccuracy (run 1) (n = 5) [%]											
LLOQ	–0.18	4.64	2.28	–1.12	–0.75	0.45	1.33	4.18	0.55	0.26	0.34
QC1	3.06	–6.81	9.79	4.95	5.69	4.89	1.73	3.25	1.74	4.69	2.97
QC2	–2.31	–8.38	–11.99	0.06	–0.53	–3.98	–6.87	–8.54	–3.33	–1.49	–1.44
QC3	–1.57	–1.37	–0.59	0.92	–0.80	–2.89	–1.68	–4.38	–1.83	–0.68	–0.23
Between-run inaccuracy (n = 15) [%]											
LLOQ	–0.31	3.33	2.52	–0.76	–0.75	–0.15	0.71	3.95	–0.43	0.56	0.08
QC1	2.43	–7.99	3.76	3.23	4.32	2.46	0.16	–2.23	2.54	1.28	2.00
QC2	–2.63	–7.45	–8.96	–1.11	–0.60	–2.65	–4.99	–7.39	–1.92	–0.65	–1.60
QC3	2.62	–2.17	–3.92	–0.85	–1.33	–3.29	–1.82	–2.97	–2.12	–1.72	–1.76
Within-run imprecision (run 1) (n = 5) [%]											
LLOQ	3.05	11.86	12.1	1.39	1.76	1.87	1.2	16.57	2.29	2.35	1.62
QC1	2.26	3.07	5.99	2.49	2.01	1.98	2.42	8.09	1.73	2.39	2.18
QC2	1.58	5.65	2.28	1.66	0.81	1.40	1.07	7.66	1.42	0.61	1.33
QC3	0.37	3.33	3.41	1.52	0.75	0.81	0.34	4.16	1.43	0.61	0.59
Between-run imprecision (n = 15) [%]											
LLOQ	2.78	11.4	8.42	1.98	1.66	3.58	1.79	12.57	3.3	2.95	2.25
QC1	2.96	6.58	6.87	2.17	2.06	4.27	4.04	6.85	3.07	5.02	2.3
QC2	3.27	4.79	4.79	3.03	3.19	2.07	2.04	5.98	4.44	1.91	3.59
QC3	1.97	7.09	6.06	3.16	1.39	2.94	1.97	7.12	2.38	3.21	2.35

LLOQ: lower limit of quantification. QC: Quality control. 5-FC: 5-flucytosine. AM-B: amphotericin B. ANF: anidulafungin. FCZ: fluconazole. IVZ: isavucaonazole. ITZ: itraconazole. KTZ: ketoconazole. MCF: micafungin. OH-ITZ: OH-itraconazole. PSZ: posaconazole. VRZ: voriconazole.

As far as carryover is concerned, peak areas in eight different drug-free serum samples injected after a highest calibrator sample were consistently below 20% of the LLOQ for all analytes and below 5% for the respective ISTD. The highest peak area of all analytes was 12.7% of the LLOQ and 1.1% of the ISTD.

Evaluation of matrix effect showed constant compensation of potential matrix effects by the ISTD for all analytes at the three tested concentration levels. ISTD normalized matrix factors were as follows: 102.3% 5-FC, 103.7% AM-B, 98.6% ANF, 101.8% FCZ, 100.5% IVZ, 99.0% ITZ, 101.6% KTZ, 93.5%MCF, 99.1% OH-ITZ, 98.2% PSZ, and 100.4% VRZ. The variation coefficient of the ISTD normalized MF for all analytes was ≤9.2%.

Dilution integrity was tested for all analytes. Inaccuracy ranged from 9.6% to 13.1% and imprecision ranged from 1.1% to 4.1%. However, amphotericin B did not meet the suggested requirements of inaccuracy and imprecision ranging ±15% as inaccuracy was -20.5% and imprecision 16.5%.

All analytes were stable for at least five weeks at –20 °C and –80 °C. Freeze-thaw stability was acceptable for all analytes, except for amphotericin B where QC1 slightly exceeded the criteria with a deviation of -16.3% for the third freeze-thaw cycle. Benchtop stability (up to six hours at room temperature) was within acceptance criteria for all analytes except for anidulafungin where QC2 and QC3 showed a deviation of -16.6% and -18.2% after 4 h and -25.2% and -21.4% after 6 h, respectively. Additionally, micafungin did not meet the requirements with a deviation of -18.5% in QC2 after six hours at room temperature. All analytes were stable for at least 6 hours at 4 °C. Autosampler stability was proven for all analytes within 24 hours at 10 °C.

Conclusions

A liquid chromatography – HRAM mass spectrometry method for clinical research for the quantification of 11 different antimycotics in human serum was implemented. The use of online sample cleanup using TurboFlow technology offers improved robustness and sensitivity, while the FullMS acquisition in high resolution gives the flexibility to expand the panel of analytes without modifying the method. The reported method meets research laboratory requirements in terms of sensitivity, linearity of response, accuracy, and precision.

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