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A High Sensitivity LC/MS/MS Method for Quantitative Analysis of Eight Antifungal Drugs in Human Serum

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Introduction

Triazoles and echinocandins are commonly used for the treatment of invasive fungal infections via systemic antifungal chemotherapy. However, these drugs exhibit substantial pharmacokinetic variability in patients such as bioavailability and drug-drug interactions [1,2]. Clinicians often find it challenging to select proper drug doses and evaluate the potential toxicity effects. Therapeutic drug monitoring (TDM) of antifungals is essential to maximise the efficacy and minimise drug overdose risk in patients, hence individualising the treatment [3]. In this study, we aim at developing a fast and reliable LC/MS/MS method with high sensitivity and simple sample pre-treatment. The method is established for simultaneous determination of two classes of antimycotic compounds, five triazoles and three echinocandins in human serum. The method performance is evaluated with spiked serum samples thoroughly before further implementation and validation with clinical samples.

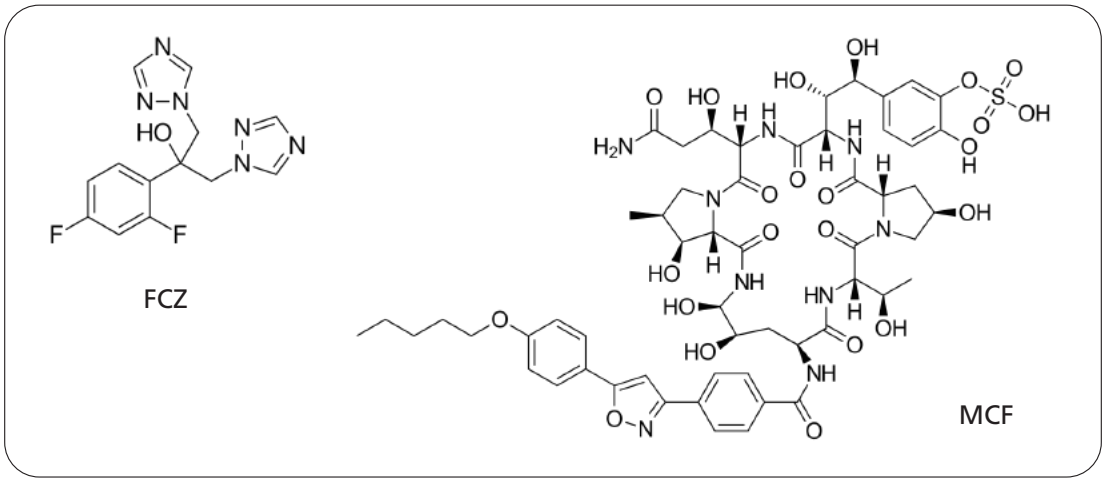

Figure 1 Structure of fluconazole (FCZ) with triazoles and micafungin (MCF) with a cyclic hexapeptoid

Methods and Materials

Human serum was treated with methanol/acetonitrile mixture (1:1, v/v) in the ratio of 1:3 to precipitate the proteins, followed by vortex and centrifugation. A Shimadzu LCMS-8060 triple quadrupole with a heated ESI interface coupled with a Nexera UHPLC was used to develop the method for high sensitivity quantitative analysis of eight antifungal drugs: fluconazole (FCZ), posaconazole (PCZ), voriconazole (VCZ), hydroxyitraconazole (h-ICZ), itraconazole (ICZ), anidulafungin (ANF), caspofungin (CSF) and micafungin (MCF) .

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Table 1 Analytical conditions and MS/MS parameters on LCMS-8060

Column	: Kinetex C18 100Å (100 x 2.1 mm, 1.7 µm)
Flow rate	: 0.4 mL/min
Mobile phase	: A: Water with 0.1 % formic acid
	B: Acetonitrile with 0.1 % formic acid
Elution mode	: 0.00 min (5% B) → 4.00 – 5.50 mins (90% B) → 5.51 – 9.00 min (5% B)
Oven temp.	: 40 °C
Inj. Vol.	: 10 µL
Interface	: ESI, 300 °C
MS mode	: Positive, MRM
Block temp.	: 400 °C
DL temp.	: 250 °C
CID gas	: Ar (270 kPa)
Nebulizing gas flow	: N_2, 2 L/min
Drying gas flow	: N_2, 10 L/min
Heating gas flow	: 0 air, 10 L/min

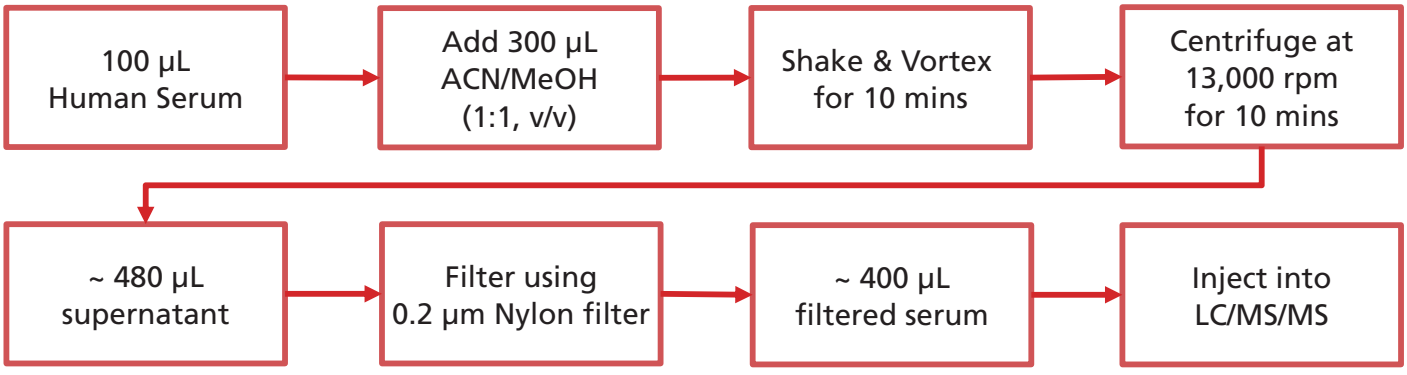

Figure 2 Pre-treatment and Protein Crash procedure for Human Serum

Results and Discussion

Method development for antifungals

Table 1 shows the summary of LC/MS/MS analytical conditions and parameters while Table 2 describes the optimised MRM transitions and parameters for the eight antifungal drugs. With the automated MRM optimisation program, three transitions were chosen for each compound, with one quantifier and two confirmatory transitions. Predominantly, the antifungals form $[M+H]^+$ ions except $[M-H]^-$ ion for ANF.

Table 2 MRM transition and parameters for eight antifungals on LCMS-8060

Antifungals		Precursor ion	Product ion	Q1 Pre-bias (V)	CE (V)	Q3 Pre-bias (V)
Triazole	Fluconazole (FCZ)	307.20	220.10	-27	-20	-22
			238.15	-29	-15	-11
			169.15	-28	-24	-11
	Posaconazole (PCZ)	701.40	683.30	-32	-35	-26
			126.95	-20	-55	-24
			614.35	-20	-38	-22
	Voriconazole (VCZ)	350.20	281.15	-30	-19	-20
			127.10	-30	-37	-13
			224.15	-30	-20	-11
	Hydroxy-itraconazole (h-ICZ)	721.30	408.15	-22	-42	-29
			392.20	-20	-36	-20
			430.30	-36	-37	-13
	Itraconazole (ICZ)	705.30	392.20	-34	-39	-27
			432.20	-36	-36	-30
			256.05	-36	-42	-18
Echinocandin	Anidulafungin (ANF)	1140.40	1122.30	-34	-19	-44
			1104.55	-34	-26	-32
			343.15	-34	-45	-24
	Caspofungin (CSF)	547.50	131.10	-22	-23	-26
			137.05	-20	-26	-14
			86.10	-22	-51	-16
	Micafungin (MCF)	(-)1268.45	(-)247.05	36	55	15
			(-)469.15	36	55	21
			(-)390.00	36	55	12

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Quantitative performance with spiked human serum

Figure 3 shows the total MRM chromatograms for the eight antifungal drugs with a total LC/MS/MS runtime of nine minutes through gradient elution. Calibration curves with external standard method were plotted on standard-spiked serum matrix, obtaining linearity of R^2 > 0.996. The linear ranges 5~5000 ppt for FCZ and VCZ, while 20~5000 ppt for PCZ, ICZ, h-ICZ and CSF. Due to lower sensitivity, the dynamic ranges of the method for ANF and MCF are 200~5000 ppt.

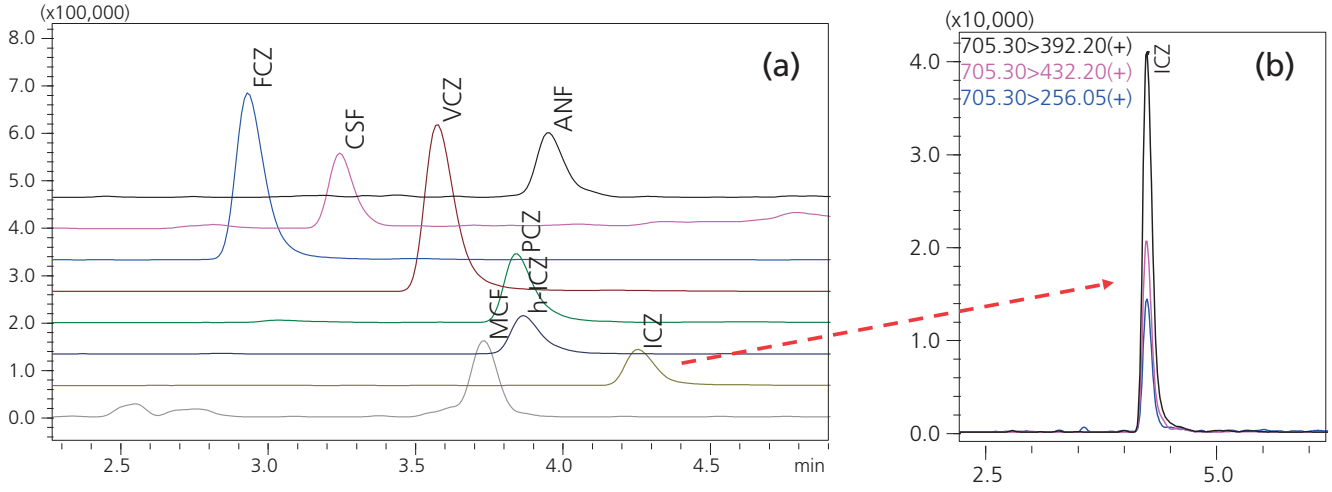

Figure 3 (a) Total MRM chromatograms of antifungal drugs spiked in serum (5ppb each); (b) Individual MRMs of ICZ

Table 3 Calibration results for eight antifungal drugs in spiked human serum

Antifungal	RT (min)	Range (ppt)	R^2	LOD (ppt)	LOQ (ppt)	% RSD (n=3)	
						200 ppt	1000 ppt
FCZ	3.912	5 ~ 5000	0.9995	1.6	4.9	13.5	11.3
VCZ	3.192	5 ~ 5000	0.9998	0.4	1.3	10.8	9.1
PCZ	3.681	20 ~ 5000	0.9994	3.0	9.5	9.6	10.6
h-ICZ	2.860	20 ~ 5000	0.9987	6.0	18.3	13.3	11.3
ICZ	3.503	20 ~ 5000	0.9987	6.2	18.9	18.7	13.2
ANF	3.797	200 ~ 5000	0.9988	41.6	126.2	23.6	14.9
CSF	3.835	20 ~ 5000	0.9968	3.1	9.3	17.3	14.1
MCF	4.230	200 ~ 5000	0.9989	43.2	130.8	32.8	13.2

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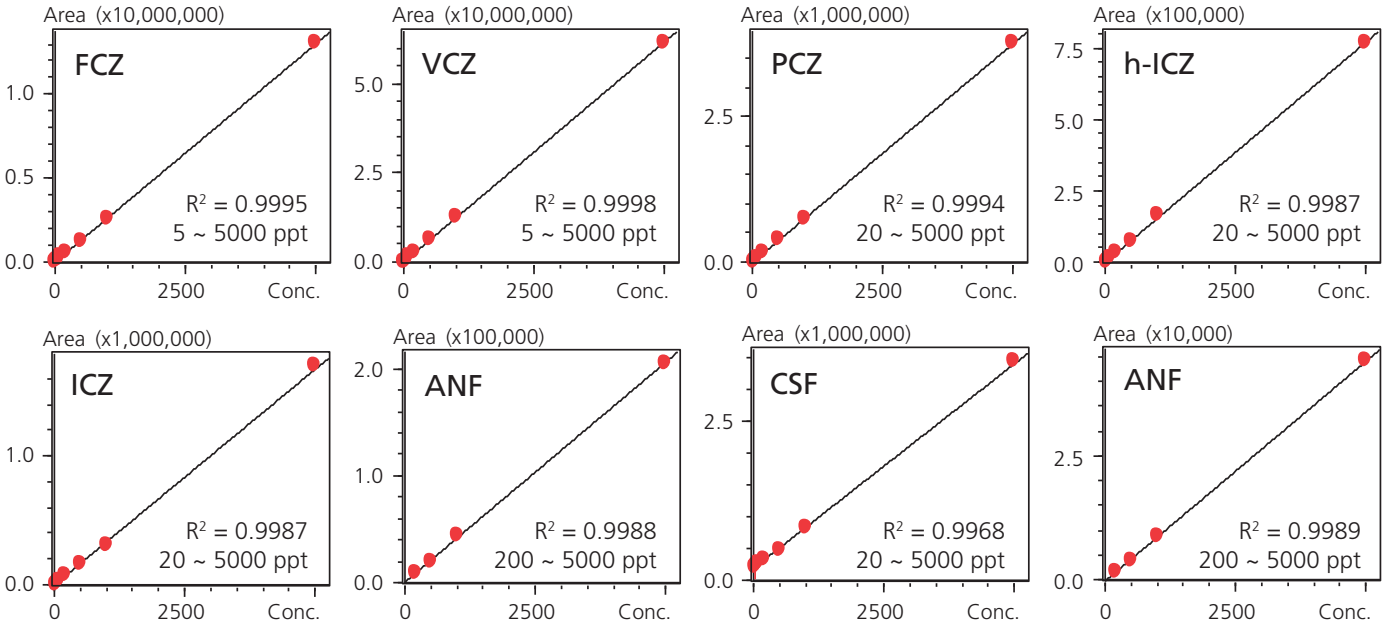

Figure 4 Calibration curve and respective linearity of eight antifungal drugs

The quantitative performance for the eight antifungal drugs are shown in Table 3. It shows good linearity (R^2) of 0.9968 – 0.9998, and their respective limit of detection (LOD) and limit of quantitation (LOQ) are also described. Repeatability study were carried out at two concentrations, 200 ppt (mid level) and 1000 ppt (high level) with n=3. ANF and MCF have larger %RSD values as compared to other antifungals as the low concentration level done was the LOQ.

Table 4 Recovery and matrix effect of antifungal drugs in spiked human serum

Antifungal	Recovery (%)		Matrix effect (%)	
	200 ppt	1000 ppt	200 ppt	1000 ppt
FCZ	124.1	110.3	82.8	80.7
VCZ	117.0	117.9	53.3	50.8
PCZ	108.3	128.9	61.0	55.5
h-ICZ	96.6	125.7	51.4	61.5
ICZ	103.0	119.0	38.9	41.9
ANF	135.0	123.6	86.2	65.0
CSF	101.4	95.4	119.2	103.7
MCF	69.7	88.2	94.2	63.2

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Recovery (RE%) of the MRM method was determined by comparing the ratio between the peak area the pre-spiked standards and post-spiked in serum, while matrix effect (ME%) was determined with the ratio between post-spiked standards in serum to those in the diluent. The study was performed in triplicates and the results are shown in Table 4. The recovery for all the antifungals ranges from 69% to 135% while the matrix effect calculated is in between 39% and 119%. The recovery shows that the protein precipitation would cause some variations on the extraction efficiency whereas human serum poses effects on most antifungal analytes.

Conclusions

A fast MRM-based method for high sensitivity quantitative analysis of eight antifungal drugs, fluconazole (FCZ), posaconazole (PCZ), voriconazole (VCZ), hydroxyitraconazole (h-ICZ), itraconazole (ICZ), anidulafungin (ANF), caspofungin (CSF) and micafungin (MCF), has been developed on LCMS-8060. With remarkable sensitivity of the LOQ ranging at 1.3~131 ppt, the LC/MS/MS method could be applied for sensitive quantitation of the antifungal drugs in human serum for therapeutic drug monitoring (TDM) or other clinical research study.

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