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ORIGINAL ARTICLE

Rapid and Simple Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC) Method for Simultaneous Quantifications of Triazole Antifungals in Human Serum

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Abstract

Purpose To develop and validate a one-step, rapid and simple reversed-phase high-performance liquid chromatography (HPLC)-based protocol for the simultaneous measurement of voriconazole (VCZ), posaconazole (POSA), itraconazole (ITC) in serum/plasma.

Methods Calibration standards (CS) and quality control samples were prepared in drug-free serum by spiking with the triazoles at different concentrations. HPLC was performed with C_{18} column, isocratic mobile phase after extraction with cold acetonitrile. The standardized method was tested in 2693 patients' serum/plasma samples.

Results Linearity of CS for ITC, VCZ and POSA was proportional to the nominal concentration (correlation coefficient > 0.999). Limit of detection (mg/L) for ITC, VCZ and POSA was 0.25, 0.25 and 0.125, respectively. The lower limit of quantification (mg/L) for ITC, VCZ and POSA was 0.5, 0.5 and 0.25, respectively. Precision and accuracy were in acceptable range with 100% average percentage recovery. No interferences from endogenous substances and other antimicrobial compounds were noted. In clinical samples, the therapeutic range achieved for VCZ was 39.9%. Whereas, 61.1% and 44% of samples with ITC and POSA, respectively, were in the sub-therapeutic range.

Conclusion We developed a rapid and simple HPLC method to quantify common triazoles in a single chromatographic run allowing simultaneous measurement of different antifungals in a small volume of serum/plasma. Thus, therapeutic drug monitoring requests can be processed in one run without changing the protocol parameters, column or column conditioning thereby improving turnaround time.

Keywords HPLC · Chromatography · Protocol · Antifungals · Triazole · Therapeutic drug monitoring

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Introduction

Fungi are affecting over a billion people with a mortality of more than 1.5 million [1]. Invasive fungal infection (IFI) is a matter of serious concern in modern clinical practice where increasing use of immunosuppressive therapies is generating large numbers of susceptible hosts. Limitations of antifungal drugs and lack of monitoring antifungal therapy may adversely affect patient management [1, 2].

Voriconazole (VCZ), itraconazole (ITC) and posaconazole (POSA) are broad-spectrum triazole antifungal drugs used primarily for the management of IFI [3–5]. Hydroxy-itraconazole (OHITC), a major metabolite of itraconazole, is bioactive and also has antifungal potency comparable to the parent drug [6, 7]. Azole antifungal drugs are characterized by marked pharmacokinetic variability and drug-interaction, which affect outcome and toxicity [8]. Poor bioavailability, marked intraindividual and interindividual variability and drug-drug interactions associated with azoles necessitates therapeutic drug monitoring (TDM) to optimize therapy and reduce drug toxicity [9–11]

Various methods have been developed over time to measure the level of these antifungals in patient serum [12–21]. Liquid chromatography-mass spectrometry (LC–MS) or LC- tandem mass spectrometry (LC–MS/MS) is a powerful technique for the accurate quantitative determination of drugs and metabolites in biological fluids with high selectivity [14]. However, LC–MS is very expensive and not available in the majority of diagnostic clinical laboratories. On the other hand, high-performance liquid chromatography (HPLC) is largely available for TDM in diagnostic laboratories. The HPLC protocols practiced for triazole quantification vary for different triazoles, and the method is tedious and complex in terms of sample extraction, gradient elution, pH maintenance, etc.

Therefore, the present study aimed to develop and validate a 'one-run protocol' for the rapid and simple reversed-phase HPLC (RP-HPLC) for simultaneous measurement of voriconazole, posaconazole, itraconazole and hydroxyl-itraconazole in human serum/plasma.

Table 1 Chromatographic and instrumental conditions

Instrumental parameters	Conditions
Elution mode	Isocratic
Flow rate	1.0 ml/min
Injection volume	20 µl
Wavelength	262, 255 nm
Mobile Phase	ACN:Water; 70:30
Column	ZORBAX Eclipse XDB-C_{18} (5 µm 4.6 × 250 mm)
Temperature of column	25 °C
Temperature of autosampler	25 °C
Analyte retention time	Itraconazole 8.846 ± 0.1 min
	Hydroxy-itraconazole 4.401 ± 0.1 min
	Posaconazole 4.287 ± 0.1 min
	Voriconazole 3.297 ± 0.1 min
Run time	12 min

Materials and Methods

Antimicrobial Drugs and Reagents

Pure powder of antifungal compounds, itraconazole (ITC), hydroxy-itraconazole (OHITC), voriconazole (VCZ), posaconazole (POSA), amphotericin B (AMB), fluconazole (FLU), caspofungin (CAS), micafungin (MIC) and anidulafungin (ANI) and antibiotics minocycline (MIN), ceftazidime (CFZ), cefoxitin (CFX), colistin sulfate (COS), metronidazole (MTZ) (Sigma-Aldrich, Bengaluru, India) was procured and used to prepare the stock solution in suitable solvents. HPLC grade acetonitrile (ACN), dimethyl sulfoxide (DMSO), methanol and ultrapure water were used as the solvent. Pooled serum from healthy volunteers who did not receive the above-mentioned drugs in the last 4 weeks was used to prepare working calibration standards (CS) and quality control (QC) samples.

Instruments and Chromatographic Conditions

HPLC analysis was performed on an Agilent 1290 infinity system (Agilent technologies Inc., USA) equipped with a quaternary pump and a variable wavelength detector set at 255 nm and 262 nm. The Agilent OpenLAB software enabled the system control, acquisition and processing of data. The mobile phase consisted of a filtered and degassed mix of ACN: H_20 (70:30, v/v). The chromatographic

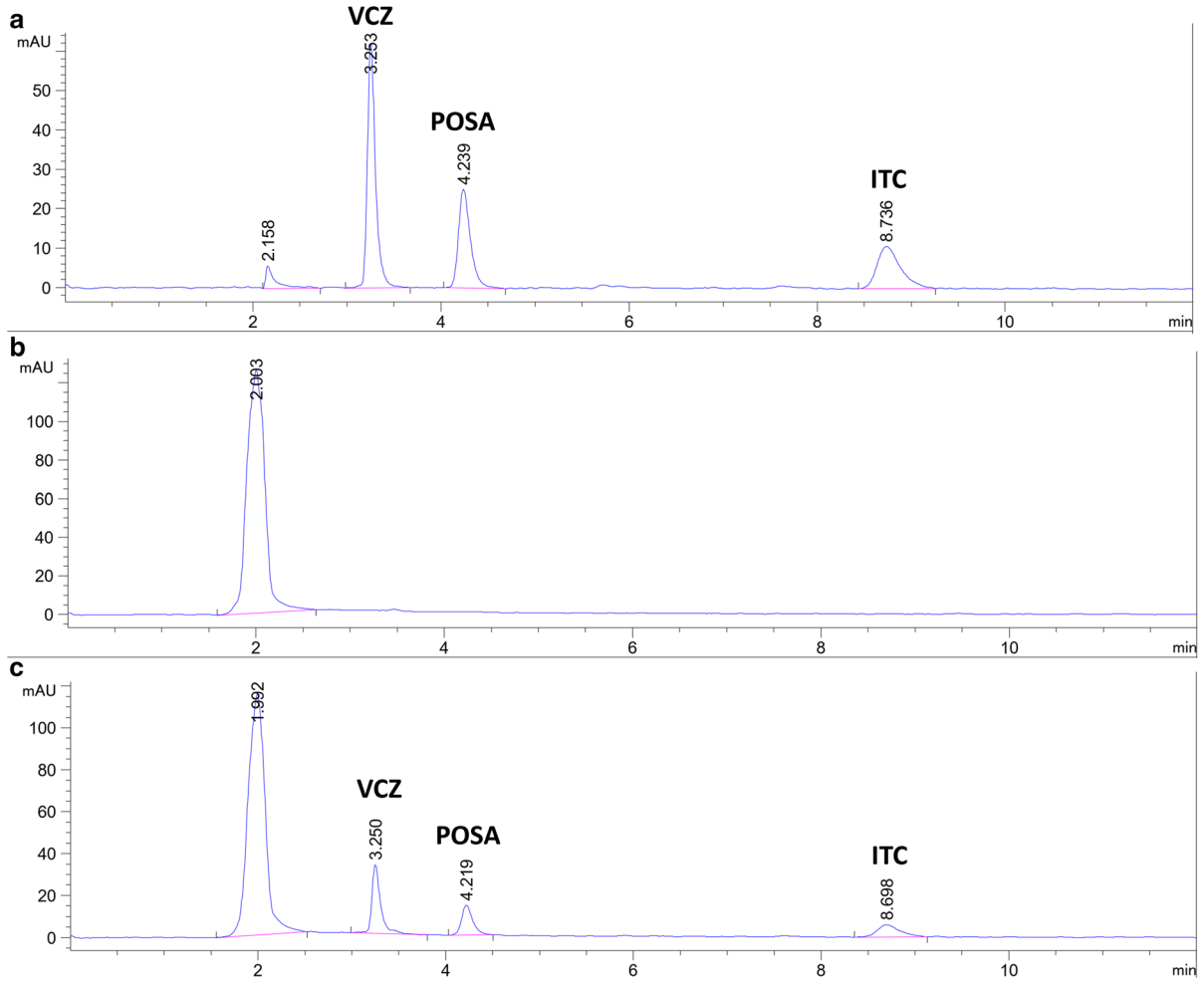

Fig. 1 Representative chromatograms of itraconazole, posaconazole and voriconazole in spiked human serum. a Mobile phase spiked with all three triazole drugs; b Blank pooled human serum; c Pooled human serum spiked with all three triazole drugs. *ITC* Itraconazole; *POSA* Posaconazole; *VCZ* Voriconazole; *RT* Retention time (RT: ITC = 8.846 ± 0.1 min, POSA = 4.287 ± 0.1 min and VCZ = 3.297 ± 0.1 min)

separation was achieved on an Agilent ZORBAX XDB C_{18} column (5 µm, 4.6 × 250 mm) at 25 °C at a flow rate of 1.0 ml/minute (Table 1). ITC eluted at 8.846 ± 0.1 min, OHITC at 4.401 ± 0.1 min, POSA at 4.287 ± 0.1 min and VCZ eluted at 3.297 ± 0.1 min (Fig. 1).

Preparation of Stock Solutions, Working Solutions and Quality Control Samples

Two batches of stock solutions of ITC, OHITC, VCZ and POSA were prepared by dissolving 3.2 mg of ITC, OHITC, VCZ and POSA in 2 ml of dimethyl sulfoxide (DMSO) yielding stock concentrations of 1600 mg/L each. Working CS were prepared by spiking adequate amounts of each drug solution from the first batch into pooled human serum with the concentration of drugs ranging from 0.25–16 mg/L for POSA and 0.5–16 mg/L for ITC and VCZ. For simultaneous detection of all triazole in one protocol, QC samples for ITC, OHITC, VCZ and POSA were prepared from the second batch of stock solution to obtain serum samples containing all triazole in one sample at

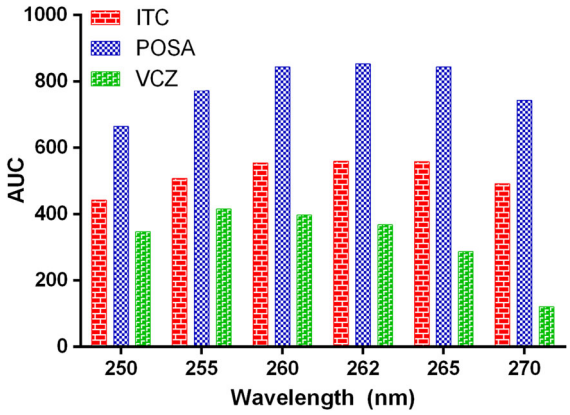

Fig. 2 Maximum absorption spectra of triazole drugs at different wavelengths. Itraconazole and posaconazole shows maximum absorption at 262 nm while voriconazole shows maximum absorption at 255 nm. *ITC* Itraconazole; *POSA* Posaconazole; *VCZ* Voriconazole; *AUC* Area under curve

various concentrations (0.612, 1.25, 2.5, 5 and 10 mg/L, each).

Sample Preparation and Wavelength Screening

An equal volume of CS and QC were mixed with an equal volume of ice-cold ACN. The mixture was vigorously vortexed for 30 s, followed by high-speed centrifugation at 14,800 rpm at 25 °C for 15 min. The supernatant was transferred into the chromatographic vials and placed in the autosampler of HPLC for injection. Wavelengths ranging 250 to 270 nm were screened for selection of maximum absorption spectra of analyte drug. Detection was achieved by monitoring the maximum absorbance at 262 nm for ITC, OHITC and POSA and 255 nm for VCZ (Fig. 2).

Linearity

Linearity was evaluated by analyzing CS using a linear regression equation. Experiments were performed in triplicate on nonconsecutive days. The best fit was determined by plotting both the area under the curve (AUC) and peak height, separately against the drug concentrations measured by HPLC for each drug. (Fig. 3).

Accuracy and Precision

The accuracy and precision of the method were evaluated based on the CS and QC response. QC samples were consecutively measured at three different time points on a given day (intra-day) on three nonconsecutive days (inter-day). Precision was expressed as coefficient of variation, whereas accuracy was expressed as a percentage of the relative error

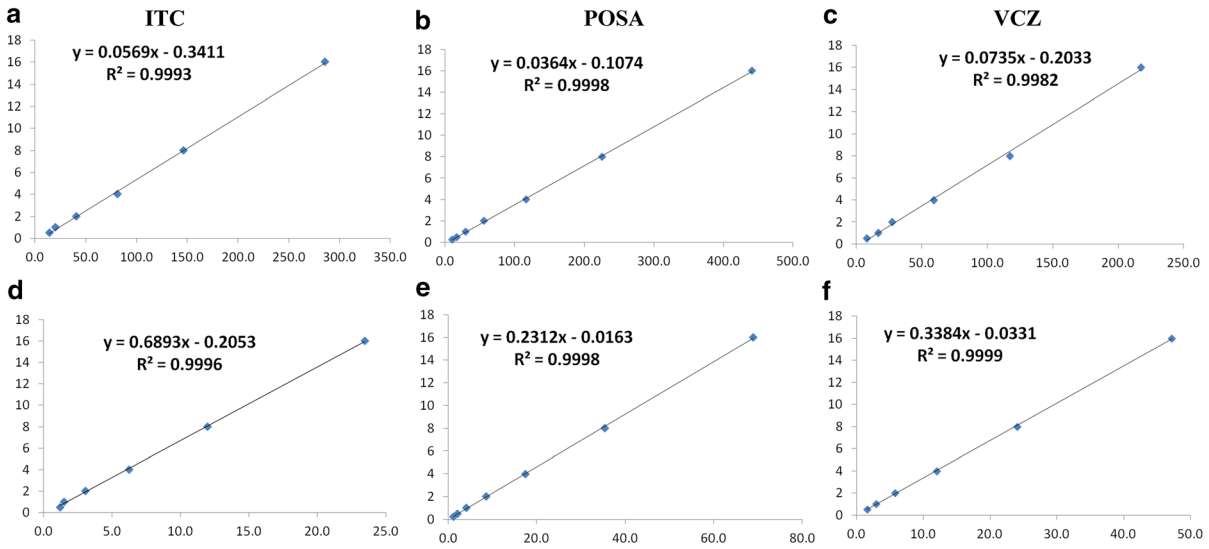

Fig. 3 Best fit curve (linearity response $R^2 > 0.99$) of triazole antifungals using different drug concentrations (ITC,POSA,VCZ) on *y* axis with AUC (**a**–**c**) and peak height (**d**–**f**) on *x* axis

Table 2 ITC, POSA and VCZ calibration curves calculated according to the AUC and peak height of chromatographic peaks

Itraconazole concentration (mg/L)	AUC							Peak height						
	Day 1 Exp-1	Day 1 Exp-2	Day 1 Exp-3	Day 2	Day 3	Day 4	Avg.	Day 1 Exp-1	Day 1 Exp-2	Day 1 Exp-3	Day 2	Day 3	Day 4	Avg.
16	274.6	282.8	311.0	283	274	289.5	285.8	23.2	23.7	23.9	24.1	22.2	23.6	23.5
8	139.0	154.0	147.0	143	148.5	146.7	146.4	12.1	12.3	11.9	12.2	11.3	12.1	12.0
4	82.2	88.7	89.4	74	67.6	86.8	81.4	6.3	6.4	6.8	6.1	5.5	6.5	6.3
2	46.0	43.4	40.5	36.2	35.9	43.3	40.9	3.2	3.0	3.1	2.9	3.1	3.1	3.1
1	29.6	16.8	19.7	18	17.2	21.0	20.4	1.8	1.4	1.4	1.6	1.3	1.5	1.5
0.5	11.3	12.5	18.8	15.2	14	13.3	14.2	1.3	1.1	1.2	1.3	1.2	1.2	1.2
Posaconazole Concentration (mg/L)														
16	466.1	435.4	443.7	442.0	419.0	441.2	441.2	69.2	68.8	69.5	71.3	65.8	68.9	68.9
8	231.4	222.5	220.5	226.0	226.0	225.3	225.3	35.5	35.0	35.3	36.6	34.5	35.4	35.4
4	110.0	122.1	109.6	114.0	126.0	116.3	116.3	17.3	17.7	17.6	18	16.7	17.5	17.5
2	54.6	53.5	64.4	55.0	50.6	55.6	55.6	8.5	8.4	9.3	8.6	8.3	8.6	8.6
1	29.1	36.7	25.7	27.0	28.0	29.3	29.3	4.2	4.2	4.1	4.2	3.9	4.1	4.1
0.5	20.3	14.9	15.1	16.0	14.8	16.2	16.2	2.5	1.8	2.1	2.1	2.1	2.1	2.1
0.25	11.5	10.0	9.0	9.3	9.0	9.8	9.8	1.5	1.1	1.1	1.3	1	1.2	1.2
Voriconazole concentration (mg/L)														
16	220.5	216.3	227.0	219	200	221.3	217.3	47.3	47.3	47.3	49.3	45	47.2	47.2
8	137.5	108.8	106.9	106	127	117.7	117.3	24.5	24.1	23.7	24.5	23.5	24.0	24.1
4	54.9	54.9	69.1	54	62.7	59.6	59.2	12.0	11.9	12.3	12.2	11.8	12.0	12.0
2	23.3	29.1	28.8	29	25.5	27.1	27.1	5.4	5.9	5.8	6.1	5.6	5.9	5.8
1	17.2	22.1	15.8	14	12.3	18.4	16.6	3.2	3.1	3.0	2.9	2.8	2.9	3.0
0.5	9.7	7.0	8.0	6.9	6.5	8.2	7.7	1.7	1.3	1.6	1.6	1.8	1.7	1.6

ITC Itraconazole; *POSA* Posaconazole; *VCZ* Voriconazole; *AUC* Area under curve

(%RE). The European Medical Agency criterion was used to ascertain protocol acceptability [22]. Briefly, accuracy within ± 15% deviation from the nominal values and precision within ± 15% CV were used to define protocol as acceptable, except for the lower limit of quantification (LLOQ), where the %CV limit was extended up to 20%.

Recovery, the Limit of Detection and Lower Limit of Quantification

Experiments were performed to assess the recovery of the drug after the extraction procedure. Briefly, pre-extracted samples were prepared by adding an increasing concentration of ITC, VORI and POSA (16 μg, 8 μg, 4 μg, 2 μg and 1 μg) to the pooled serum from healthy controls and subjected to extraction procedure followed by HPLC. Un-extracted samples in mobile phase matrix were prepared similarly and subjected to HPLC. The analytical results from pre-extracted samples were compared with un-extracted samples, and the % recovery was defined as the peak area (of pre-extracted samples/ un-extracted samples) × 100. Limit of detection (LOD), the concentration at which the analyte signal can be distinguished from the background signal was determined by measuring the peak area of ≥ 2 times the average of the blanks [16]. The lower limit of quantification (LLOQ) was defined as the analyte signal which was ≥ 5 times the average of the blanks [16].

Specificity

To determine the specificity of the standardized extraction protocol chromatogram obtained from the spiked pooled serum samples were evaluated for the

Table 3 Inter day, intraday precision (expressed as CV %) and accuracy as RE % of ITC, POSA and VCZ based on AUC and peak height of calibration standards

C nom (mg/L)	C (μg/ml)-(mean ± SD)			Precision (CV %)			Accuracy (RE %)		
Intraday variability (n = 3)	ITC	POSA	VCZ	ITC	POSA	VCZ	ITC	POSA	VCZ
AUC									
16	16.17 ± 1.11	16.21 ± 0.58	16.11 ± 0.40	6.84	3.57	2.47	1.06	1.34	0.67
8	8.02 ± 0.43	8.08 ± 0.21	8.48 ± 1.26	5.34	2.62	14.91	0.29	0.94	5.95
4	4.62 ± 0.24	4.04 ± 0.26	4.19 ± 0.60	5.2	6.40	14.41	15.54	0.96	4.86
2	2.15 ± 0.15	1.99 ± 0.22	1.79 ± 0.24	6.84	11.00	13.41	7.25	− 0.72	− 10.29
1	0.93 ± 0.37	1.0 ± 0.21	1.15 ± 0.24	39.83	20.44	21.15	− 6.66	0.28	15.29
0.5	0.49 ± 0.24	0.50 ± 0.11	0.41 ± 0.10	48.4	22.16	24.77	− 2.49	0.58	− 18.78
0.25		0.26 ± 0.05			17.44			5.07	
Inter day variability (n = 3)									
16	15.73 ± 0.47	15.69 ± 0.48	15.53 ± 0.86	2.97	3.03	5.54	− 1.66	− 1.92	− 2.95
8	8.01 ± 0.16	8.11 ± 0.02	8.42 ± 0.78	1.99	0.19	9.22	0.11	1.38	5.20
4	4.02 ± 0.56	4.22 ± 0.23	4.13 ± 0.33	13.83	5.49	7.87	0.57	5.40	3.28
2	1.88 ± 0.24	1.85 ± 0.10	1.80 ± 0.13	12.72	5.39	7.17	− 6.18	− 7.56	− 9.84
1	0.77 ± 0.15	0.92 ± 0.04	0.90 ± 0.23	19.14	4.59	25.72	22.87	− 8.46	− 10.34
0.5		0.46 ± 0.03	0.33 ± 0.07		6.01	20.22		− 7.38	− 33.85
0.25		0.23 ± 0.01			5.98			− 6.78	
C nom (mg/L)	C (μg/ml)-(mean ± SD)			Precision (CV %)			Accuracy (RE %)		
Intraday variability (n = 3)	ITC	POSA	VCZ	ITC	POSA	VCZ	ITC	POSA	VCZ
Peak height									
16	16.09 ± 0.25	15.98 ± 0.08	15.97 ± 0.00	1.55	0.51	0.00	0.54	− 0.16	− 0.19
8	8.15 ± 0.14	8.14 ± 0.06	8.12 ± 0.14	1.69	0.72	1.67	1.89	1.72	1.51
4	4.29 ± 0.18	4.04 ± 0.05	4.05 ± 0.07	4.26	1.19	1.74	7.16	0.94	1.24
2	1.94 ± 0.07	2.00 ± 0.11	1.90 ± 0.09	3.56	5.69	4.72	− 3.00	0.14	− 5.21
1	0.86 ± 0.16	0.95 ± 0.01	1.02 ± 0.03	18.56	1.41	3.33	14.11	− 5.30	1.62
0.5	0.63 ± 0.07	0.48 ± 0.08	0.49 ± 0.07	10.97	17.02	14.48	25.76	− 4.61	− 2.75
0.25		0.27 ± 0.05			19.86			7.54	
Inter day variability (n = 3)									
16	15.88 ± 0.68	15.86 ± 0.64	15.92 ± 0.73	4.28	4.02	4.57	− 0.75	− 0.76	− 0.51
8	7.99 ± 0.34	8.19 ± 0.24	8.09 ± 0.17	4.26	2.98	2.09	− 0.13	− 0.02	1.08
4	3.96 ± 0.35	4.00 ± 0.15	4.03 ± 0.07	8.76	3.77	1.68	− 0.89	0.50	0.68
2	1.89 ± 0.08	1.95 ± 0.04	1.95 ± 0.08	4.21	2.12	4.34	− 5.30	0.08	− 2.67
1	0.82 ± 0.11	0.93 ± 0.04	0.93 ± 0.02	13.25	3.88	1.82	17.95	− 3.07	− 6.83
0.5	0.65 ± 0.04	0.47 ± 0.00	0.54 ± 0.03	6.11	0.57	6.23	30.36	− 16.19	8.52
0.25		0.25 ± 0.04			13.94			− 23.32	

C nom Nominal concentration; *ITC* Itraconazole; *POSA* Posaconazole; *VCZ* Voriconazole; *CV* Coefficient of variation [CV % = (standard deviation/mean of measured values) × 100]; *RE* relative error [RE % = (mean measured concentration-nominal concentration)/nominal concentration] × 100); *SD* Standard deviation; *AUC* Area under curve

presence of any endogenous interference at the respective retention time of the triazoles. Additionally, stock solution of antifungals (AMB, FLU, CAS, MIC, ANI) and antibiotics (MIN, CFZ, CFX, COS, MTZ) was spiked at a concentration of 16 mg/L each, in pooled serum containing ITC, POSA and VORI. All

Table 4 Inter day, intraday precision, accuracy of ITC, OHITC, POSA and VCZ based on AUC and peak height results of QC

C nom (mg/L)	C (μg/ml)-(mean ± SD)				Precision (CV %)				Accuracy (RE %)			
Intraday variability	ITC	OHITC	POSA	VCZ	ITC	OHITC	POSA	VCZ	ITC	OHITC	POSA	VCZ
AUC												
10	9.56 ± 0.72	9.91 ± 0.13	9.56 ± 0.72	8.26 ± 0.33	7.56	1.36	2.63	4.04	− 4.42	− 0.92	− 13.59	− 17.35
5	5.12 ± 0.32	4.70 ± 0.06	5.12 ± 0.32	4.89 ± 0.13	6.29	1.24	6.01	2.57	2.36	− 6.00	4.38	− 2.26
2.5	2.51 ± 0.30	2.51 ± 0.11	2.51 ± 0.30	2.26 ± 0.36	11.81	4.19	4.40	14.64	0.37	0.31	− 0.05	− 1.61
1.25	1.16 ± 0.15	1.24 ± 0.06	1.16 ± 0.15	1.27 ± 0.2	12.81	4.57	11.46	16.66	− 6.88	− 1.11	5.66	1.47
0.612	0.59 ± 0.11	0.73 ± 0.02	0.59 ± 0.11	0.80 ± 0.20	18.76	3.21	14.91	25.57	− 2.94	19.11	− 22.19	30.18
Inter day variability												
10	10.51 ± 1.01	10.26 ± 0.16	9.02 ± 0.96	9.79 ± 1.44	9.62	1.52	10.62	14.69	5.06	2.61	− 9.83	− 2.14
5	5.09 ± 0.11	4.80 ± 0.13	5.10 ± 0.10	5.03 ± 0.44	2.24	2.78	2.02	8.79	1.76	− 3.96	2.04	0.64
2.5	2.54 ± 0.05	2.56 ± 0.07	2.49 ± 0.07	2.54 ± 0.27	1.84	2.89	2.73	10.60	1.48	2.43	− 0.48	1.49
1.25	0.88 ± 0.17	1.25 ± 0.01	1.21 ± 0.11	1.11 ± 0.11	19.68	1.16	9.40	10.31	− 29.53	0.38	− 2.85	− 11.40
0.612	0.47 ± 0.06	0.72 ± 0.05	0.46 ± 0.08	0.48 ± 0.05	11.83	6.69	17.16	10.64	− 23.95	16.99	− 24.04	− 20.80
C nom (mg/L)	C (μg/ml)-(mean ± SD)				Precision (CV %)				Accuracy (RE %)			
Intraday variability	ITC	OHITC	POSA	VCZ	ITC	OHITC	POSA	VCZ	ITC	OHITC	POSA	VCZ
Peak height												
10	9.28 ± 0.28	10.29 ± 0.12	8.49 ± 0.65	8.81 ± 0.82	3.01	1.16	7.67	9.29	− 7.22	2.91	− 15.08	− 11.92
5	5.23 ± 0.04	5.14 ± 0.03	5.21 ± 0.12	5.17 ± 0.10	0.76	0.58	2.31	2.00	4.59	2.87	4.18	3.32
2.5	2.52 ± 0.22	2.66 ± 0.06	2.53 ± 0.00	2.66 ± 0.20	8.82	2.32	0.00	7.66	0.60	6.54	1.08	6.50
1.25	1.11 ± 0.12	1.31 ± 0.03	1.22 ± 0.04	1.30 ± 0.10	10.75	2.61	2.88	7.52	− 11.05	4.80	− 2.04	3.85
0.612	0.58 ± 0.08	0.61 ± 0.03	0.57 ± 0.13	0.69 ± 0.17	13.67	4.87	22.36	24.21	− 4.77	− 0.56	− 6.96	12.62
Inter day variability												
10	10.36 ± 0.76	9.73 ± 0.28	10.73 ± 0.76	10.62 ± 0.51	7.33	2.83	7.11	4.78	3.59	− 2.72	7.35	6.24
5	4.84 ± 0.07	4.77 ± 0.21	5.01 ± 0.06	4.96 ± 0.08	1.43	4.35	1.15	1.71	− 3.23	− 4.57	0.25	− 0.85
2.5	2.42 ± 0.07	2.42 ± 0.20	2.48 ± 0.07	2.40 ± 0.03	2.85	8.40	2.80	1.41	− 3.08	− 3.07	− 0.77	− 3.87
1.25	1.01 ± 0.03	1.18 ± 0.09	1.15 ± 0.01	1.12 ± 0.10	3.42	7.66	1.00	9.08	− 19.33	− 5.47	− 7.90	− 10.58
0.612	0.66 ± 0.03	0.60 ± 0.02	0.58 ± 0.05	0.56 ± 0.02	5.20	2.86	7.91	3.02	8.39	− 2.18	− 4.44	− 8.57

C nom Nominal concentration; *ITC* Itraconazole; *OHITC* Hydroxy itraconazole; *POSA* Posaconazole; *VCZ* Voriconazole; *CV* Coefficient of variation [CV % = (standard deviation/mean of measured values) × 100]; *RE* relative error [RE % = (mean measured concentration-nominal concentration)/nominal concentration] × 100); *SD* standard deviation

Table 5 Percentage recovery of triazoles at different concentrations in spiked serum samples

Drug concentration (μg/ml)	Percentage recovery of triazoles (%)		
	Itraconazole	Voriconazole	Posaconazole
16	97.3	104.9	103.2
8	106.2	112.5	104.4
4	92.1	116.3	104.1
2	104.6	104	103.1
1	101.6	126	100.7
Average % recovery	100.4	112.9	100.6

spiked samples were run following the standardized HPLC protocol. The obtained chromatograms were compared with the chromatogram of the serum samples spiked only with ITC, VORI and POSA.

Evaluation of Clinical Specimens

For evaluation of the standardized protocol, clinical serum/plasma samples (trough) received from 2017 through 2019 were used. The samples were obtained both from adults and pediatric patients who were either on prophylaxis, empiric or targeted therapy with ITC or VORI or POSA. A total of 400 μl of the clinical sample were tested as described for CS and QC above.

Ethics Statement

The ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the approval from the Institute ethical committee, PGIMER was sought for the study.

Results

The linearity (response function) of the CS for ITC, VCZ and POSA was proportional to nominal concentration, and the correlation coefficient (linear regression) was higher than 0.999 for all tested compounds (Table 2). The LOD of both ITC and VCZ was 0.25 mg/L, and for POSA it was 0.125 mg/L. The LLOQ for both ITC and VCZ was 0.5 mg/L and for POSA it was 0.25 mg/L. The chromatogram revealed good resolution and different retention times for all the three triazoles (Fig. 1).

Precision (%CV) and accuracy (%RE) of CS are shown in Table 3. For QC, the %CV calculated using AUC for obtaining the best fit was in the range of 1.24 to 25.57% for intra-day and 1.52 to 19.68% for inter-day. When peak height was used to calculate the best fit, the intraday %CVs for QC ranged from 0.00 to 24.21% and the inter-day %CVs ranged 1.0 to 9.08% for all three triazoles (Table 4). The %RE of QC using AUC for obtaining the best fit was in the range of 22.2 to 30.2% for intra-day and 29.5 to 17% for inter-day accuracy. When peak height was used to calculate the best fit, the intraday %REs for QCs ranged from 15.1 to 12.62% and the inter-day %RE ranged from 19.3 to 8.4% (Table 4). The results of recovery experiments are shown in Table 5. The % recovery at different drug concentrations ranged between 92.1–106.2% for ITC, 104–126% for VCZ, and 100.7–104.4% for POS. All drugs have an excellent average % recovery (> 100%). Selectivity and specificity of the method indicated no substantial interferences from endogenous substances as well as other tested antimicrobial compounds (Fig. 4).

A total of 2693 trough serum/plasma samples (ITC, OHITC = 2124, VCZ = 540 and POSA = 29) were used for testing the standardized protocol. No internal standard was used due to excellent mean recovery close to 100% for the extraction method. Out of 540 serum/plasma samples tested for VCZ, only 211 (39.1%) were in the desired therapeutic range of 1–5 mg/L [23, 24]. Of 2124 samples tested for ITC, 1298 (61.1%) serum/plasma samples had level less than 1.0 mg/L; majority (895 samples) had levels less than 0.5 mg/L [4, 11]. OHITC levels were higher compared to the parent drug and in only 45.8% (944 samples) the level was below 1.0 mg/L. We received 29 samples for testing POSA level during this period, and 13 (44.8%) out of 29 were below 0.7 mg/L (Fig. 5) [9, 11, 25].

Discussion

Triazole antifungals (ITC, VCZ and POSA) are commonly used for the effective management of IFI. These drugs are indicated both for the prophylaxis, empiric and targeted therapy of IFI, particularly in candidiasis and aspergillosis. Factors such as

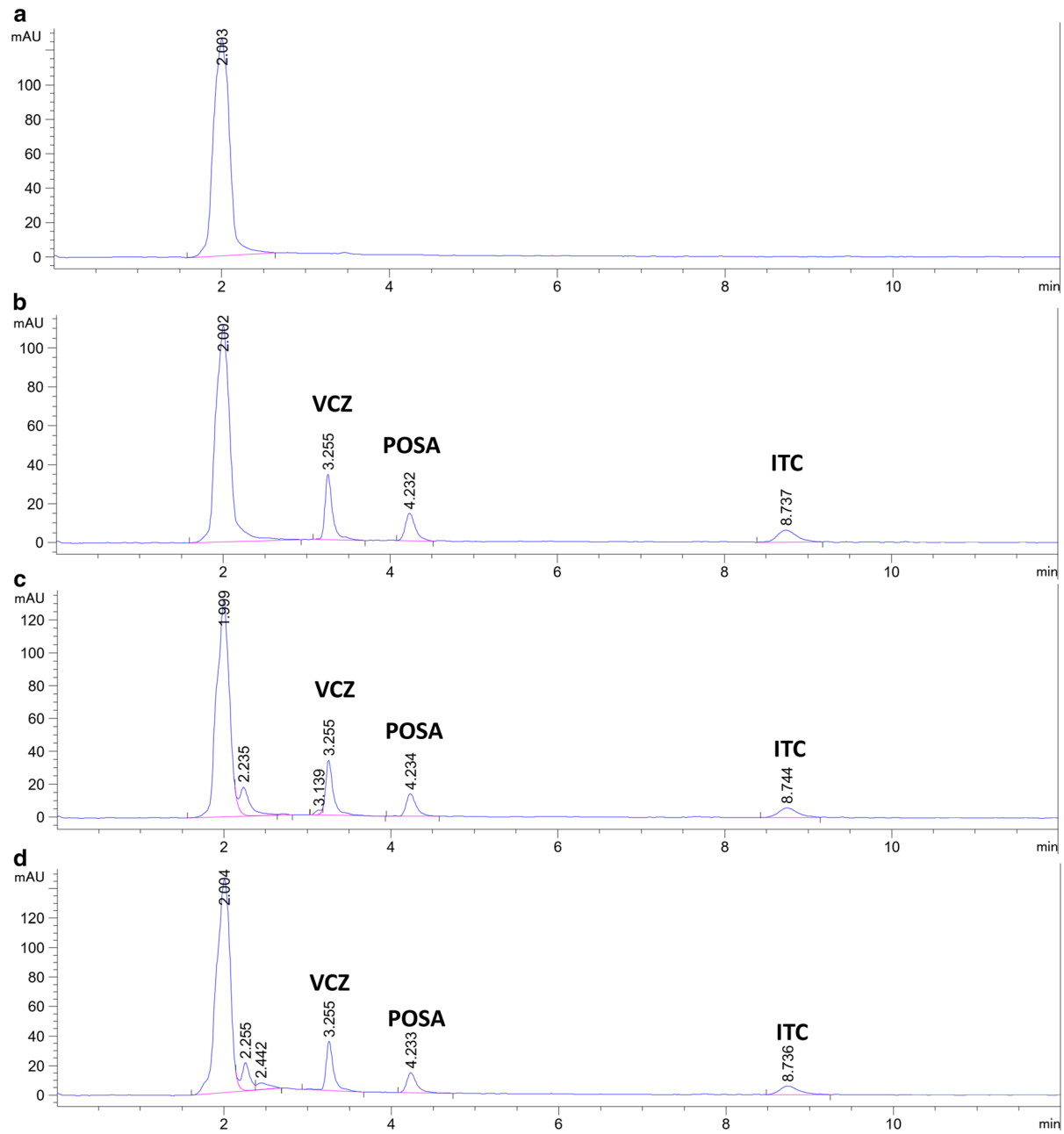

Fig. 4 Representative chromatograms of triazole in presence of common antifungal and antibiotic to check for potential interference. **a** Blanked human serum; **b** Pooled human serum spiked with triazole; **c** Pooled human serum spiked with triazole along with other antifungals (16 mg/L); **d** Pooled serum spiked with triazole and antibiotics (16 mg/L)

interindividual genetic polymorphisms, variable drug formulations, different drug brands, food habits, compliance issues and drug-drug interactions may lead to sub-therapeutic or toxic concentrations of antifungals thereby necessitating TDM ([11, 24–27]).

LC–MS or LC–MS/MS-based measurement of antifungals is the best technique for TDM due to its

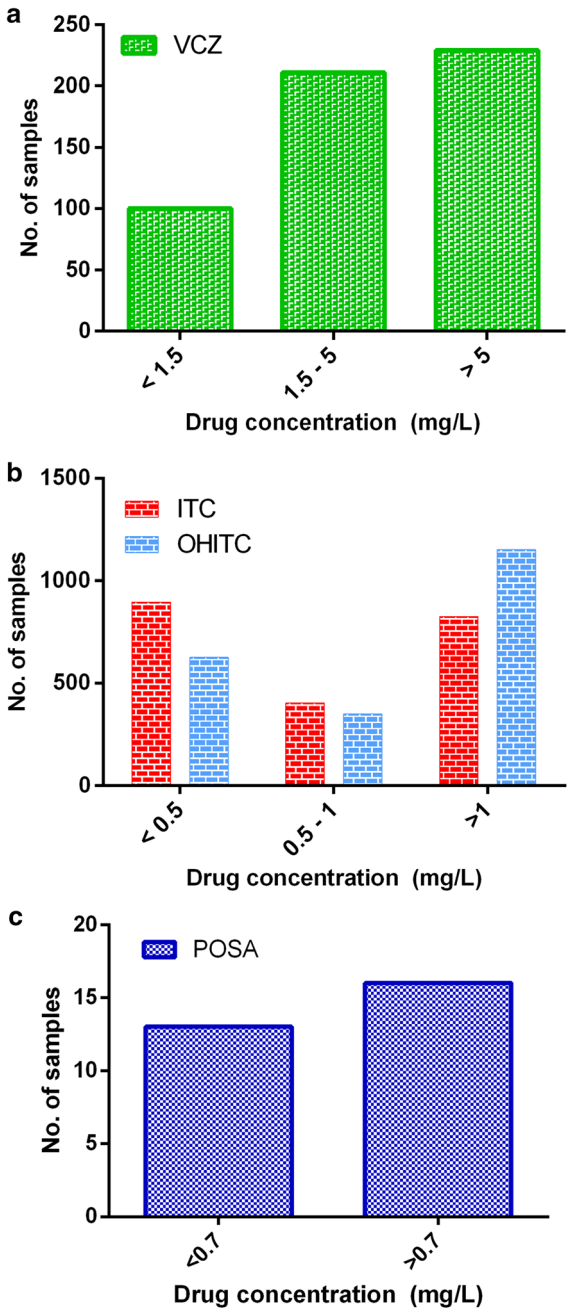

Fig. 5 Concentrations of a voriconazole; b itraconazole and hydroxyl-itraconazole; c posaconazole in clinical samples. *ITC* Itraconazole; *OHITC* Hydroxy-Itraconazole

higher selectivity. The high cost of the instrument and the cumbersome protocol hamper its utility in routine clinical diagnostic laboratories [12–21, 28]. HPLC-based methods provide enough accuracy and quantitation for routine TDM but the available protocols for triazole quantification vary for each drug and the described protocols are lengthy and technically demanding. The steps involved in the available protocol have tedious sample preparation, needs gradient elution, maintenance of buffer rendering their application difficult in clinical laboratories. To circumvent these issues, we developed a method for the quantification of commonly used systemic triazoles (ITC, VCZ and POSA) in a single chromatographic run. Although the protocol described in the present study is a modified version of previously known protocols, it has the advantage of being technically simple and rapid in addition to having a high throughput. The application of this modified protocol holds potential in routine clinical laboratory at centers lacking sophisticated instruments and technical expertise such as those needed with LCMS.

The excellent sample extraction efficacy precluded the need for internal standard and this simple and cost-effective HPLC method provided results with high linearity (0.25–16 µg/ml), precision and accuracy within acceptable limits as per bioanalytical method validation [22]. The results of drug measurement in QC samples revealed that the peak height was superior to AUC for determining the drug concentration for all the tested triazoles. Therefore, we used peak height for all calculations. Precision was less than 10% except for the samples with low concentrations (i.e., 0.612 mg/L) which was $< 15\%$ which were still below the acceptable limit of 20% [22]. Estimation of triazole drug from the spiked serum samples revealed no possible interference by other antifungals and common antibiotics used in clinical practice. The developed protocol is promising even in the cases where combination of antifungal drugs and concomitant administration of antibiotics is instituted. Although the chromatogram revealed clear differences in the retention time for all triazoles, OHITC showed a retention time close to posaconazole (4.401 ± 0.1 min vs. 4.287 ± 0.1 min) which needs to be interpreted with care (data not shown). Further optimization is needed to resolve these two compounds on the chromatogram. However, the use of a combination of posaconazole with itraconazole is not frequent in clinical practice and thus overlapping retention time generally should not affect the interpretation. All the triazoles, both the unprocessed QC serum samples and the processed samples, after

Table 6 Comparison of present and previously published HPLC-based antifungal estimation protocols

Method parameters	Present study	Gómez-López et al. [18]	Verweij et al. [29]	Kahle et al. [20]	Gholam et al. [19]	Pennick et al. [21]
Sample preparation	Extraction with cold ACN, Vortexing and Centrifugation	1:1 Serum:ACN, Vortexing and Centrifugation	Liquid–liquid extraction with hexane–dichloromethane, methanol and NaOH, supernatant layer freezing in alcohol bath and drying of organic layer under stream of nitrogen gas at 37 °C	Liquid–liquid extraction with diethyl ether, drying of organic layer under a gentle stream of nitrogen gas at 37 °C	Extraction with heptane-isoamyl alcohol and drying of organic layer under a gentle stream of nitrogen gas at 50 °C	Solid-phase extraction (SPE)
Column	ZORBAX Eclipse XDB-C_{18} (5 µm 4.6 × 250 mm)	Sunfire C18, 5 µm, 4.6 × 150 mm, Waters Corporation	Acquity UPLC BEH Phenyl column (100 mm × 2.1 mm, 1.7 µm particle size)	ReproSil-Pur Basic C18(5 µm 2 × 150 mm)	Zorbax SB-C18(5 µm 4.6 × 250 mm)	Luna 5 µm C18 column (250 by 4.6 mm)
Mobile phase	ACN:Water; 70:30 (Constant)	ACN:Water (Gradient)	10 mM phosphate buffer pH 2.5 and acetonitrile (Gradient)	0.09 M aqueous ammonium phosphate monobasic and acetonitrile	Phosphate buffer, pH 6.0 (adjusted with 1 M KOH), acetonitrile and methanol	0.01 M Phosphate buffer: acetonitrile
pH, buffer requirement	No	No	Yes	Yes	Yes	Yes
Elution mode	Isocratic	Multiple-step gradient	linear gradient	Isocratic elution	Isocratic	Isocratic
Flow rate	1.0 ml/min	1.0 ml/min	0.4 ml/min	0.2 mL/min	1.7 ml/min	–
Run time	12 min	16 min	6 min	> 15 min	20 min	12 min
TAT	< 30 min	45 min	–	–	> 60 min	> 60 min
Antifungal measurement	ITC, OHITC, VCZ and POSA	Triazole along with metabolites	ITC, OHITC, VCZ, POSA and Isavuconazole	VCZ and POSA	ITC, OHITC and VCZ	VCZ
Advantages and limitations	Simple sample preparation, ready to use mobile phase, isocratic mode allows protocol suitability to different system, single common protocol for different triazole, shorter TAT	Gradient elution limits applicability over different system, costly, longer TAT	Gradient elution, complex liquid–liquid extraction and requirement of pH maintenance, Buffer	Complex extraction procedure, Buffer requirement and cannot measure itraconazole	Complex extraction procedure, pH maintenance, Buffer requirement and longer TAT, cannot measure posaconazole	Solid phase extraction is complex and costly, Longer TAT, can measure only voriconazole

ITC Itraconazole; *OHITC* Hydroxy itraconazole; *POSA* Posaconazole; *VCZ* Voriconazole; *TAT* Turnaround time; *ACN* Acetonitrile

extraction were stable for up to 2 days at 4 °C and 7 days at − 20 °C. (data not shown).

This protocol possibly can be applied for routine practice given the high extraction efficiency, good reproducibility and ability to perform the simultaneous quantification of all the three azoles using a small volume of serum/plasma. This allows us to process different antifungal TDM requests in one run without changing the protocol parameter, column or column conditioning. Thereby, substantially reducing the time and efforts needed to perform TDM in the clinical diagnostic laboratory when a request for many antifungals are received in a given day. This 'one-run protocol' can suffice the detection of > 1 triazole in one sample or even for testing of multiple samples, each with any of the three azoles. In a single run, this protocol can also possibly quantify the levels of any of these triazoles given as combination therapy.

However, this needs to be verified using the samples obtained from patients taking a combination of these tested azoles.

A comparison of the present study protocol with previously published.

HPLC-based antifungal estimation methods are provided in Table 6 [18–21, 29]. The method described in the present study provides results in 30 min including extraction and chromatographic procedures which is faster compared to the other methods (45–60 min). The use of simple protein precipitation with ice-cold ACN resulted in excellent recovery of analyte making the method easier and faster than other previously reported HPLC protocols that generally requires complex solid-phase extraction and prolongs the assay time. Another important advantage of the present technique is the use of simple mobile phase in isocratic elution mode without any requirement for buffers or additives. ACN-water mobile phase without any salt or buffer allows rapid column equilibration obviating the need for steps such as preparation of buffer or flushing solvents to remove salts before shutting down the system. The availability of such an integrated assay allows real-time, instantaneous results with a reduction in turnaround time (TAT) that enables prompt adjustment of dosage thus promoting therapeutic effectiveness with minimal toxicity.

Lower values of antifungals noted in large number of clinical samples of the patients treated with VCZ, ITC and POSA (60.9%, 61.1% and 44.8%, respectively) could be related to age of the patient, poor gastric absorption, antacids use, poor compliance, poor quality of the drug and concomitant medications. Frequent performance of TDM is recommended in view of the high number of patients with triazole levels outside the therapeutic range. TDM will also help to ensure optimal antifungal selection, adjustment of dosage and duration of treatment which is in line with antifungal stewardship efforts [30].

The major limitations of this study are the absence of the complete clinical details of all patients that could have helped in determining inter-patient variability of drug levels. We also did not compare our protocol directly with the standard described protocol to define the sensitivity, precision and accuracy of our protocol. Detailed stability analysis and multi-centric evaluation of this protocol are also warranted for its wider acceptance and general acceptability.

To conclude, this rapid, simple and robust HPLC method is suitable for routine use in diagnostic laboratories. This method is less labor intensive, reduces the TAT, provides simultaneous quantification of three triazoles in a single chromatographic run and can thus, be used to individualize antifungal therapy in the clinical settings.

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Availability of Data and Materials Data will be made available on request.

Compliance with Ethical Standards

Conflict of interest The authors have none to declare.

Ethical approval Ethical approval was taken from Institute ethical committee, PGIMER Chandigarh.

Consent for Publication All authors give consent for publication.

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