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Development and validation of a highly sensitive and robust LC–MS/MS with electrospray ionization method for simultaneous quantitation of itraconazole and hydroxyitraconazole in human plasma: Application to a bioequivalence study

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ABSTRACT

A highly sensitive and specific LC–MS/MS method has been developed for simultaneous estimation of itraconazole (ITZ) and hydroxyitraconazole (OH-ITZ) with 500 μL of human plasma using fluconazole as an internal standard (IS). The API-4000 LC–MS/MS was operated under the multiple reaction-monitoring mode (MRM) using the electrospray ionization technique. Solid phase extraction process was used to extract ITZ, OH-ITZ and IS from human plasma. The total run time was 3.0 min and the elution of ITZ, OH-ITZ and IS occurred at 2.08 min, 1.85 min and 1.29 min, respectively; this was achieved with a mobile phase consisting of 0.2% (v/v) ammonia solution:acetonitrile (20:80, v/v) at a flow rate of 0.50 mL/min on a HyPurity C_{18} (50 mm × 4.6 mm, 5 μm) column. The developed method was validated in human plasma with a lower limit of quantitation of 0.50 ng/mL for both ITZ and OH-ITZ. A linear response function was established for the range of concentrations 0.5–263 ng/mL ($r > 0.998$) for both ITZ and OH-ITZ. The intra- and inter-day precision values for ITZ and OH-ITZ met the acceptance as per FDA guidelines. ITZ and OH-ITZ were stable in the battery of stability studies, viz., bench-top, auto-sampler, dry extract and freeze/thaw cycles. The developed assay method was applied to an oral bioequivalence study in humans.

1. Introduction

Itraconazole (ITZ, CAS no. 84625-61-6, Sporanox) is an orally active triazole antifungal drug with a broad spectrum activity against most human fungal pathogens. ITZ mechanism of action is inhibiting 14-alpha-demethylase, a cytochrome P_{450} enzyme that catalyses the synthesis of ergosterol, a major component of cell membrane of yeast and fungal cells [1,2]. Following oral absorption, it is extensively metabolized by side chain hydroxylation (by CYP3A4) to form hydroxyitraconazole (OH-ITZ). OH-ITZ, which is the major metabolite, is biologically active and its plasma concentration is twofold higher than parent at steady state [3].

Few methods have reported for the quantitation of ITZ [4] or ITZ and OH-ITZ [5] using LC–MS. To date there are only few bioanalytical methods using LC–MS/MS reported for the simultaneous estimation of ITZ and OH-ITZ [6,7]. Vogeser et al. [6] have reported an automated SPE method for samples processing with a lower limit of quantification (LLOQ) of 10 ng/mL for both ITZ and OH-ITZ. Kousoulos et al. [7] have reported a LLOQ of 2 ng/mL and 4 ng/mL for ITZ and OH-ITZ, respectively in human plasma by using automated and sophisticated instrumentation. Herein we are reporting a highly sensitive and rugged LC–MS/MS method, which has overcome the drawbacks of the previously reported methods, viz., usage of automated methods and enabled us to get a reproducible LLOQ of 0.5 ng/mL (fourfold and eightfold less than previously reported lowest LLOQ for ITZ and OH-ITZ, respectively by Kousoulos et al. [7]) for both ITZ and OH-ITZ and application of this method to a bioequivalence study in healthy volunteers following oral administration of 100 mg ITZ capsule.

2. Experimental

2.1. Chemicals and reagents

ITZ was obtained from Neuland Laboratories, Hyderabad, India. OH-ITZ was obtained from Syncom, AT Groningen, The

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Netherlands. Fluconazole (IS) was obtained from Unit-II, Dr. Reddy's Laboratories Ltd., Hyderabad. All the compounds were found to be >98.5% purity determined by chromatographic (HPLC, LC–MS/MS) analysis. HPLC grade of acetonitrile; analytical grade 30% (v/v) ammonia and hydrochloric acid were purchased from Merck Specialties Pvt. Ltd., Mumbai, India. All aqueous solutions including the buffer for the mobile phase were prepared with Milli Q (Millipore, Milford, MA, USA) grade water. The control K2 EDTA human plasma was purchased from Cauvery Diagnostics and Blood Bank, Secunderabad, India.

2.2. Instrumentation and chromatographic conditions

An Agilent (Agilent Technologies, Waldbronn, Germany) 1100 series LC system equipped with degasser (G1322A), isopump (G1310A) along with auto-sampler (G13167B) was used to inject 20-μL aliquots of the processed samples on a HyPurity C_{18} column (4.6 mm × 50 mm, 5 μm, Thermo Electron Corporation, Cheshire, UK), which was kept at room temperature (24 ± 2 °C). The isocratic mobile phase, a mixture of 0.2% (v/v) ammonia and acetonitrile mixture (20:80, v/v) was delivered at 0.50 mL/min into the mass spectrometer's electrospray ionization chamber.

Quantitation was achieved by MS/MS detection in positive ion mode for both ITZ and OH-ITZ and IS, using a MDS Sciex (Foster City, CA, USA) API-4000 mass spectrometer, equipped with a Turboionspray™ interface at 400 °C. The ion spray voltage was set at 5500 V. The common parameters, viz., nebulizer gas, curtain gas, auxillary gas and collision gas were set at 30 psi, 10 psi, 40 psi and 5 psi, respectively. The compound parameters, viz., declustering potential (DP), collision energy (CE), entrance potential (EP) and collision exit potential (CXP) were 124 V, 48 V, 10 V, 14 V for ITZ; 120 V, 49 V, 10 V, 12 V for OH-ITZ and 65 V, 25 V, 10 V, 15 V for IS. Detection of the ions was performed in the multiple reaction-monitoring (MRM) mode, monitoring the transition of the *m*/*z* 705.3 precursor ion to the *m*/*z* 392.3 for ITZ, *m*/*z* 721.4 precursor ion to the *m*/*z* 408.3 for OH-ITZ and *m*/*z* 307.0 precursor ion to the *m*/*z* 220.1 product ion for IS. Quadrupoles Q1 and Q3 were set on unit resolution. The analytical data were processed by Analyst software (Version 1.4.2).

2.3. Standard solutions

Primary stock solutions of ITZ and OH-ITZ for preparation of standard and quality control (QC) samples were prepared by weighing separately. The primary stock solution (1.00 mg/mL) of ITZ and OH-ITZ and IS were prepared in methanol and stored at −20 °C, which were found to be stable for 1 month (data not shown). Appropriate dilutions were made in methanol for ITZ and OH-ITZ to produce working stock solutions of 26.4 μg/mL, 23.2 μg/mL, 19.7 μg/mL, 12.8 μg/mL, 3.84 μg/mL, 1.34 μg/mL, 0.25 μg/mL, 0.10 μg/mL and 0.05 μg/mL on the day of analysis and these stocks were used to prepare calibration curve (CC). Another set of working stock solutions of both ITZ and OH-ITZ were made in a mixture of 0.1N HCl:methanol (30:70, v/v) (from primary stock) at 23.3 μg/mL, 12.8 μg/mL, 1.41 μg/mL and 0.05 μg/mL for preparation of QC samples accordingly. Working stock solutions were stored at approximately 5 °C for a week (data not shown). Individually stock QC and CC two-in-one working solutions of ITZ and OH-ITZ were made before spiking into QC and CC samples accordingly. A working IS solution (300 ng/mL) was also prepared in methanol. Calibration samples were prepared by spiking 490 μL of control human plasma with the appropriate amount of analytes (10 μL) and IS (50 μL) on the day of analysis. Samples for the determination of recovery, precision and accuracy were prepared by spiking control human plasma in bulk at appropriate concentrations and 500 μL volumes were aliquoted into different tubes and depending on the nature of experiment samples were stored at −80 ± 10 °C until analysis.

2.4. Sample preparation

To an aliquot of 500 μL human plasma sample, IS solution (50 μL) was added; diluted with 500 μL of Milli Q water and vortex mixed for 30 s on a cyclomixer (Remi Instruments, Mumbai, India). This sample mixture was loaded on pre-conditioned (1 mL acetonitrile followed by 1 mL water) Oasis HLB cartridges (1 cm^3, 30 mg) and washed with 0.5 mL water followed by 1 mL 10% acetonitrile in water and finally eluted with 1 mL of mobile phase. From the eluate 20 μL was directly injected onto LC–MS/MS system.

2.5. Method validation

The method was validated to meet the acceptance criteria of industrial guidance for the bioanalytical method validation [8].

The specificity of the method was determined by analyzing six different batches of human plasma as is, to demonstrate the lack of chromatographic interference from endogenous plasma components. Sets of spiked standards and QC samples (n = 6 at each concentration) were prepared and analyzed on four different occasions to evaluate linearity, precision and accuracy. Precision and accuracy was also assessed at the lowest concentration of the standards (0.5 ng/mL), representing the LLOQ for the assay.

The recovery of ITZ, OH-ITZ and IS was determined by comparing the responses of the analytes extracted from replicate QC samples (n = 6) with the response of analytes from post-extracted plasma standard sample at equivalent concentrations [9]. Recovery was determined at low, mid and high quality control concentrations, whereas the recovery of the IS was determined at a single concentration of 300 ng/mL. The effect of plasma constituents over the ionization of analytes and IS was determined by comparing the responses of the post-extracted plasma standard QC samples (n = 6) with the response of analytes from neat samples at equivalent concentrations [9,10]. Matrix effect was determined at LLOQ for ITZ and OH-ITZ and for IS at 300 ng/mL.

The stability of analytes and IS in the injection solvent was determined periodically by injecting replicate preparations of processed samples up to 18 h (in auto-sampler) after the initial injection. The peak areas of the analytes and IS obtained at initial cycle were used as the reference to determine the relative stability of the analytes at subsequent points. Stability of analytes in the biomatrix after 10 h exposure in an ice bath (bench-top) was determined at two concentrations in six replicates. Freezer stability of the analytes in biomatrix was assessed by analyzing the QC samples stored at −80 ± 10 °C for at least 15 days. The stability of analytes in biomatrix following repeated three freeze/thaw cycles (stored at −80 ± 10 °C between cycles) was assessed using QC samples spiked with analytes. Samples were processed as described under Section 2.4. Samples were considered to be stable if assay values were within the acceptable limits of accuracy (i.e., ±15% S.D.) and precision (i.e., 15% R.S.D.) [8].

2.6. Pharmacokinetic study

A bioequivalence study was performed in healthy male subjects. The ethics committee approved the protocol and the volunteers provided with informed written consent. Blood samples were

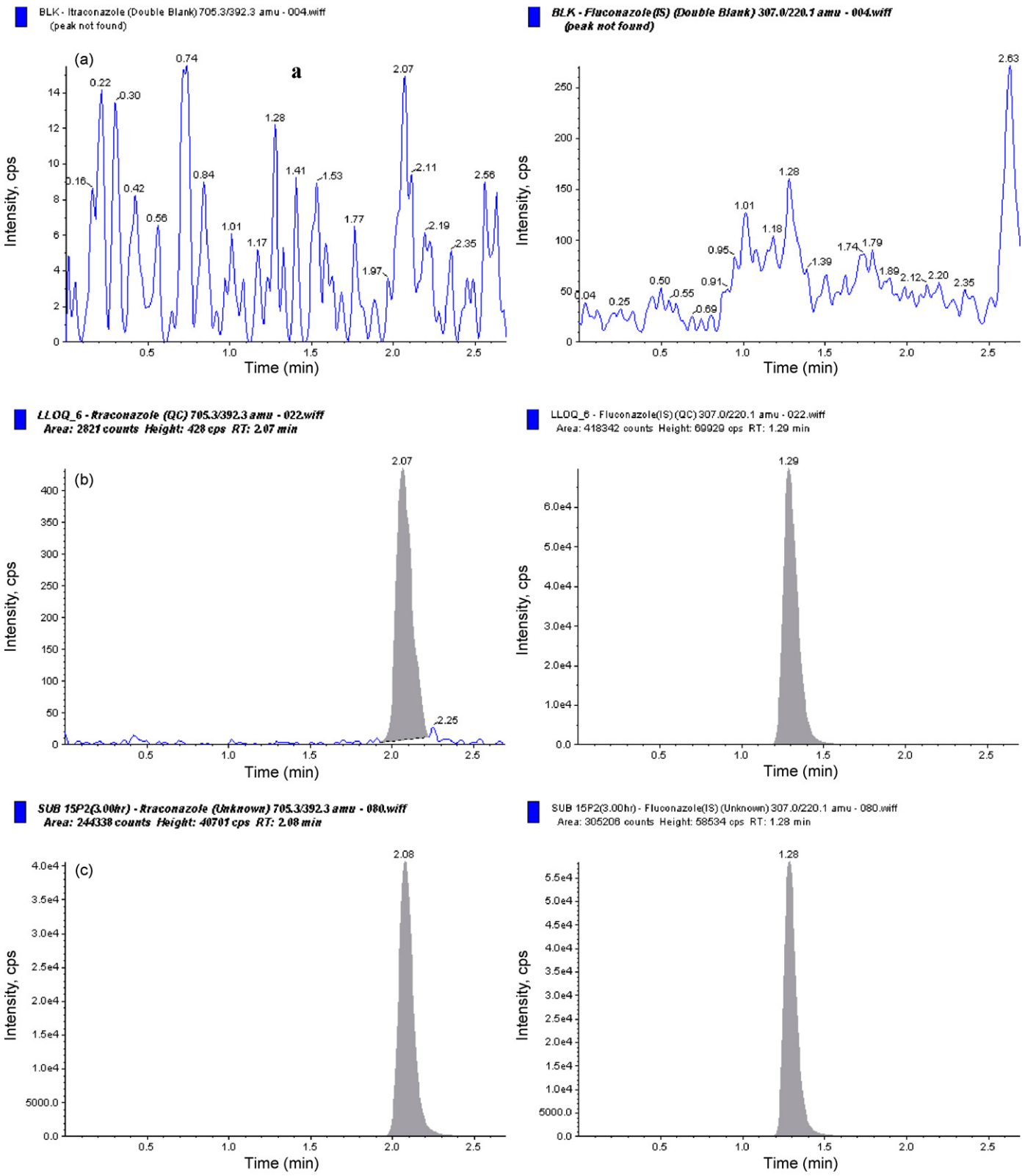

Fig. 1. Typical MRM chromatograms of ITZ (left panel) and IS (right panel) in (a) human blank plasma (b) human plasma spiked with ITZ at LLOQ (0.50 ng/mL) and IS (c) a 3.0-h plasma sample showing ITZ peak (46.9 ng/mL) obtained following oral dose of ITZ capsule to healthy volunteer along with IS.

obtained following oral administration of 100 mg of ITZ capsule into polypropylene tubes containing K2 EDTA solution as an anti-coagulant at pre-dose, 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 7 h, 8 h, 9 h, 10 h, 12 h, 24 h, 36 h, 48 h and 72 h. Plasma was harvested by centrifuging the blood using Biofuge (Hereaus, Germany) at 1760 × *g* for 5 min and stored frozen at −80 ± 10 °C until analysis.

An aliquot of 500 μL of thawed plasma samples were spiked with IS and processed as mentioned in Section 2.4. Along with

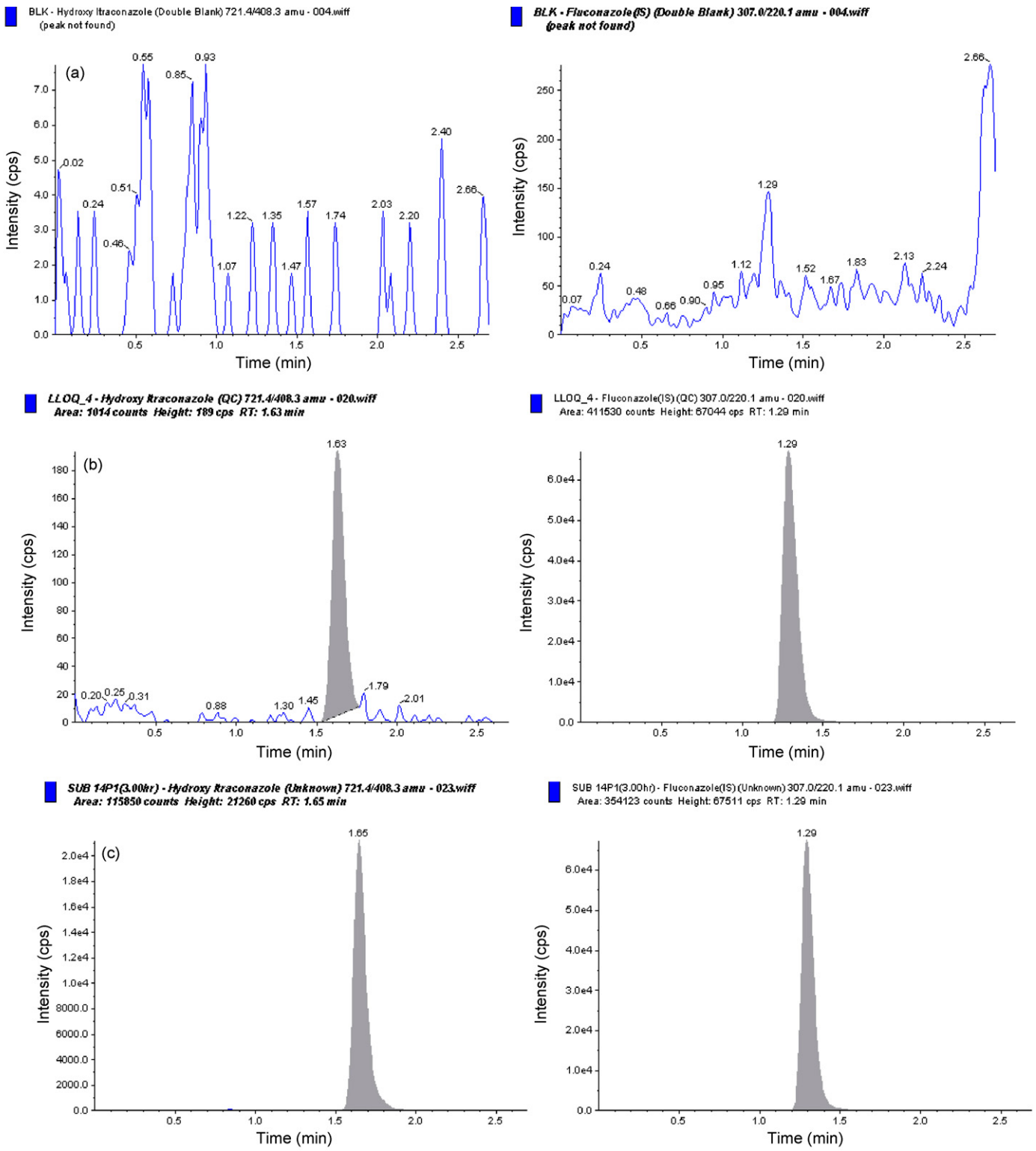

Fig. 2. Typical MRM chromatograms of OH-ITZ (left panel) and IS (right panel) in (a) human blank plasma (b) human plasma spiked with OH-ITZ at LLOQ (0.50 ng/mL) and IS (c) a 3.0-h plasma sample showing OH-ITZ peak (62.8 ng/mL) obtained following oral dose of ITZ capsule to healthy volunteer along with IS.

study samples, QC samples at low, medium and high concentration were assayed in duplicate and were distributed among unknown samples in the analytical run. The criteria for acceptance of the analytical runs encompassed the following: (i) not more than 33% of the QC samples were greater than ±15% of the nominal concentration and (ii) not less than 50% at each QC concentration level must meet the acceptance criteria. Plasma concentration–time data of ITZ and OH-ITZ was analyzed by non-compartmental method using WinNonlin Version 5.1 (Pharsight Corporation, Mountain View, CA, USA).

Table 1
Intra- and inter-day precision determination of ITZ and OH-ITZ quality controls in human plasma

Theoretical concentration (ng/mL)	Run	Measured concentration (ng/mL) (ITZ)				Measured concentration (ng/mL) (OH-ITZ)			
		Mean	S.D.	R.S.D.	Accuracy (%)	Mean	S.D.	R.S.D.	Accuracy (%)
Intra-day variation (six replicates at each concentration)									
ITZ: 0.51, OH-ITZ: 0.49	1	0.53	0.02	4.60	104	0.49	0.07	15.0	99.4
	2	0.50	0.02	3.86	98.4	0.48	0.05	9.37	98.4
	3	0.54	0.04	7.44	105	0.44	0.03	6.97	90.5
	4	0.52	0.02	4.56	102	0.48	0.04	7.52	97.7
Average		0.52	0.03	5.12	102	0.47	0.05	9.72	96.5
ITZ: 1.41, OH-ITZ: 1.37	1	1.31	0.06	4.29	92.8	1.45	0.04	2.90	106
	2	1.32	0.04	2.66	93.8	1.35	0.04	3.01	98.4
	3	1.25	0.04	3.57	88.9	1.28	0.10	7.84	93.4
	4	1.36	0.04	3.02	96.2	1.39	0.08	5.77	101
Average		1.31	0.05	3.39	92.9	1.37	0.07	4.88	99.7
ITZ: 128, OH-ITZ: 125	1	117	3.84	3.28	91.2	113	4.18	3.71	90.4
	2	124	3.84	3.09	97.0	115	5.80	5.06	92.1
	3	120	6.55	5.44	93.9	114	4.80	4.21	91.4
	4	123	1.74	1.41	95.9	118	2.78	2.36	94.6
Average		121	3.99	3.31	94.5	115	4.39	3.84	92.1
ITZ: 233, OH-ITZ: 226	1	219	8.67	3.95	94.0	194	4.64	2.39	86.5
	2	235	11.0	4.67	101	201	5.87	2.92	88.7
	3	233	7.56	3.24	100	211	5.05	2.39	93.4
	4	247	16.6	6.73	106	239	11.0	4.77	101.9
Average		234	11.0	4.65	100	211	6.64	3.12	92.6
Inter-day variation (18 replicates at each concentration)									
ITZ: 0.51, OH-ITZ: 0.49		0.52	0.04	6.80	103	0.48	0.06	11.5	98.7
ITZ: 1.41, OH-ITZ: 1.37		1.27	0.07	5.19	90.2	1.38	0.14	10.3	101
ITZ: 128, OH-ITZ: 125		118	5.36	4.52	92.3	114	4.44	3.90	91.5
ITZ: 233, OH-ITZ: 226		224	8.65	3.86	96.2	213	4.55	2.14	94.0

R.S.D.: relative standard deviation (S.D. × 100/mean).

3. Results

3.1. Method development

3.1.1. Sample pre-treatment

Different methods of sample pre-treatment were investigated. Protein precipitation and liquid–liquid extraction with various organic solvents and their mixtures resulted in non-reproducible recoveries and interferences from the sample matrix with the chromatography of the analytes (data not shown). Subsequently, SPE was investigated as samples pre-treatment technique, which has also been used by other investigators [6,7]. Following optimization of various types of SPEs and several dilution, conditioning, washing and elution reagents, we have finally selected Oasis HLB cartridges (1 cm^3, 30 mg) in this assay. The SPEs pre-conditioned (1 mL acetonitrile followed by 1 mL water) were washed with 0.5 mL water followed by 1 mL 10% acetonitrile in water and finally eluted with 1 mL of mobile phase. From this eluate was directly injected into the LC–MS/MS system.

3.1.2. Liquid chromatography

In pursuit of symmetric peak shape and retention time of ~3.0 min, feasibility of various mixture(s) of solvents such as acetonitrile and methanol using different buffers such as ammonium acetate, ammonium formate and formic acid with variable pH range of 4.0–7.0, along with altered flow rates (in the range of 0.3–1.0 mL/min) were tested for complete chromatographic resolution of ITZ, OH-ITZ and IS (data not shown). The resolution of peaks was achieved with 0.2% (v/v) ammonia and acetonitrile mixture (20:80, v/v) with a flow rate of 0.5 mL/min, on a HyPurity C_{18} column and was found to be suitable for the determination of electrospray response for ITZ, OH-ITZ and IS.

3.1.3. Mass spectrometry

In order to optimize ESI conditions for ITZ, OH-ITZ and IS, quadrupole full scans were carried out in positive ion detection mode. During a direct infusion experiment, the mass spectra for ITZ, OH-ITZ and IS revealed peaks at m/z 705.3, 721.4 and 307.0, respectively as protonated molecular ions $[M+H]^+$. Following detailed optimization of mass spectrometry conditions (provided in Section 2.2) m/z 705.3 precursor ion to the m/z 392.3 and m/z 721.4 precursor ion to the m/z 408.3 were used for quantification for ITZ and OH-ITZ, respectively. Similarly, for IS m/z 307.0 precursor ion to the m/z 220.1 was used for quantification purpose.

3.2. Specificity and selectivity

A typical chromatogram for the control human plasma (free of analyte and IS) and human plasma spiked with ITZ and OH-ITZ at LLOQ along with IS are shown in Figs. 1 and 2, respectively. No interfering peaks from endogenous compounds are observed at the retention times of analytes and IS. The retention time of ITZ, OH-ITZ and IS were 2.08 min, 1.85 min and 1.29 min, respectively. The total chromatographic run time was 3.0 min.

3.3. Recovery

Recovery was found to be 84.37 ± 2.81, 79.53 ± 5.07 and 75.54 ± 2.92 at LQC, MQC and HQC, respectively for ITZ; 93.06 ± 2.10, 88.08 ± 6.93 and 85.56 ± 2.73 at LQC, MQC and HQC,

Table 2
Stability data of ITZ and OH-ITZ quality controls in human plasma

Nominal concentration (ng/mL)	Stability	ITZ			OH-ITZ		
		Mean ± S.D.[a] $n = 6$ (ng/mL)	Accuracy (%)[b]	Precision (% CV)	Mean ± S.D.[a] $n = 6$ (ng/mL)	Accuracy (%)[b]	Precision (% CV)
ITZ: 1.41, OH-ITZ: 1.37	0 h (for all)	1.36 ± 0.04	96.2	3.03	1.39 ± 0.08	101	5.78
	3rd freeze/thaw	1.35 ± 0.05	95.4	3.72	1.38 ± 0.11	100	4.77
	10 h (bench-top)	1.27 ± 0.07	89.9	5.68	1.48 ± 0.09	108	5.91
	18 h (in-injector)	1.47 ± 0.06	104	4.35	1.45 ± 0.08	106	5.63
	15 days at −80 °C	1.27 ± 0.09	90.5	7.27	1.37 ± 0.17	100	12.1
ITZ: 233.22, OH-ITZ: 26.36	0 h (for all)	246 ± 16.6	105	6.73	230 ± 10.9	101	4.77
	3rd freeze/thaw	231 ± 13.3	99.3	5.78	220 ± 10.4	97.4	4.72
	10 h (bench-top)	221 ± 10.4	95.0	4.73	226 ± 4.87	96.9	2.22
	18 h (in-injector)	249 ± 22.5	106	9.03	210 ± 12.3	93.0	5.86
	15 days at −80 °C	227 ± 1.48	97.4	0.65	210 ± 1.99	93.1	0.94

[a] Back-calculated plasma concentrations.
[b] (Mean assayed concentration/mean assayed concentration at 0 h) × 100.

respectively for OH-ITZ. The mean recovery for ITZ and OH-ITZ was found to be 79.81 ± 4.42 and 87.23 ± 6.29, respectively. The recovery of IS was 86.70 ± 2.95%.

3.4. Matrix effect

In this study, the matrix effect was evaluated by analyzing LLOQ sample. Average matrix factor values (matrix factor = response of post-spiked concentrations/response of neat concentrations) obtained for ITZ and OH-ITZ were +1.13 (CV 6.72%, $n = 6$) and +1.07 (CV 3.20%, $n = 6$), respectively at LLOQ level, whereas on IS it was found to be +0.99 (CV 0.78%, $n = 6$)% at tested concentration of 300 ng/mL. Matrix effect was not observed at analytes and IS retention times.

3.5. Calibration curve

The plasma calibration curve was constructed using calibration standards of 0.5–263.63 ng/mL and 0.49–255.88 ng/mL for ITZ and OH-ITZ, respectively. Calibration curve was prepared by determining the best fit of peak area ratios (peak area analyte/peak area IS) versus concentration, and fitted to the $y = mx + c$ using weighing factor ($1/X^2$). The average regression ($n = 4$) for ITZ and OH-ITZ was found to be ≥0.998 and 0.997, respectively. The lowest concentration with the R.S.D. <20% was taken as LLOQ [8] and was found to be 0.50 ng/mL for both ITZ and OH-ITZ. The % accuracy observed for the mean of back-calculated concentration for four linearities was within 94.17–105.02%, and 93.94–103.21% for ITZ and OH-ITZ, respectively. The precision (% CV) values ranged from 0.98% to 4.68% and 1.06% to 8.81% for ITZ and OH-ITZ, respectively.

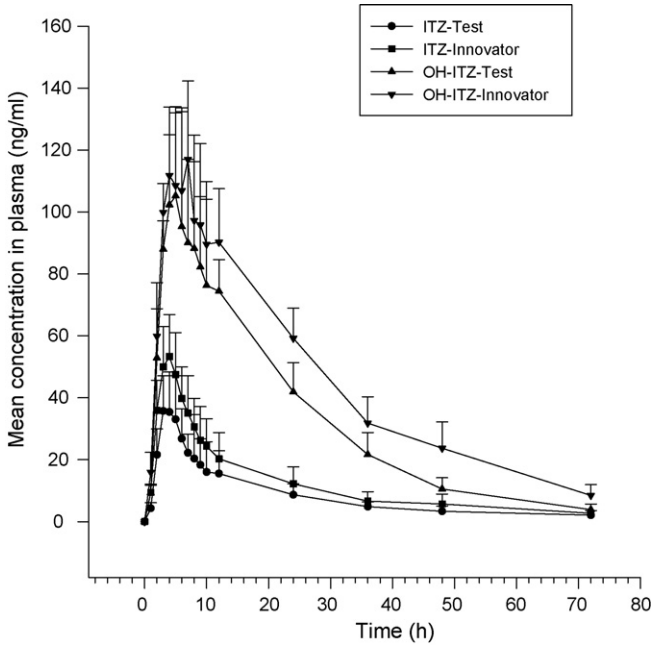

Fig. 3. Mean plasma concentration–time profile of ITZ and OH-ITZ in human plasma following oral dosing of 100 mg ITZ capsule to 24 subjects.

3.6. Precision and accuracy

The accuracy, intra- and inter-assay precision which was determined by analyzing six replicates of QC samples at four concentrations on four different days are shown in Table 1.

3.7. Stability

The predicted concentrations for each analyte at LQC and HQC samples deviated within ±15% of the nominal concentrations in a batter of stability tests, viz., in-injector (18 h), bench-top (10 h), repeated three freeze/thaw cycles and at −80 ± 10 °C for at least for 15 days (Table 2). The results were found to be within the assay variability limits during the entire process.

3.8. Pharmacokinetic study

The present method was applied to the analysis of plasma samples obtained from 24 healthy human volunteers following oral administration of 100 mg of ITZ capsule as a part of bioequivalence study. The sensitivity and specificity of the assay were found to be sufficient for accurately characterizing the plasma pharmacokinetics of ITZ and OH-ITZ in humans. Fig. 3 depicts the mean plasma concentration versus time profile of ITZ and OH-ITZ in these volunteers. Following the oral administration of 100 mg of ITZ capsule (test and reference formulations) to volunteers, the mean maximum plasma concentrations (C_{max}) for ITZ (45.75 ng/mL and 49.94 ng/mL for test and reference formulations, respectively) were attained at ∼4 h (T_{max}) for both test and reference formulations. Whereas the C_{max} values for OH-ITZ were 105 ng/mL and 117 ng/mL for test and reference formulations, respectively and the T_{max} was found to be ∼5 h and ∼6 h for test and reference formulation, respectively. The half-life ($t_{1/2}$) of ITZ and OH-ITZ was found to be ∼29 h and 14 h, respectively in both reference and test

formulations. For ITZ the $AUC_{(0-\infty)}$ values for test and reference formulations were found to be 699 ng h/mL and 798 ng h/mL, whereas for OH-ITZ the values were 2347 ng h/mL and 2801 ng h/mL for test and reference formulations, respectively.

4. Discussion

So far there were only two LC–MS/MS methods [6,7] published for the simultaneous determination of ITZ and OH-ITZ and the reported lowest LLOQ for ITZ and OH-ITZ was 2 ng/mL and 4 ng/mL, respectively. Both reported methods have utilized either fully or semi-automated sample preparation and handling processes using homolog derivative of ITZ as an IS, which is not commercially available extensively. To the best of our knowledge, we have developed an LC–MS/MS method for the determination of ITZ and OH-ITZ simultaneously, which offers the highest sensitivity (0.5 ng/mL) compared to other methods described in the literature using a simple SPE extraction procedure and commercially available IS. The LLOQ reported by us is fourfold and eightfold lower than the earlier reported LLOQ for ITZ and OH-ITZ, respectively.

5. Conclusions

In summary, we have developed and validated a highly sensitive, specific and reproducible LC–MS/MS assay to quantify ITZ and OH-ITZ simultaneously in human plasma. From the results of all the validation parameters, we can conclude that the present method can be useful for bioequivalence studies with desired precision and accuracy.

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