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Simultaneous determination of itraconazole and hydroxyitraconazole in human plasma by high-performance liquid chromatography

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Abstract

The development and validation of a high-performance liquid chromatography (HPLC) method for the simultaneous determination of itraconazole and its metabolite, hydroxyitraconazole, in human plasma is described. The method involved liquid-phase extraction of itraconazole and hydroxyitraconazole using a hexane-dichloromethane (70:30) mixture, after addition of loratidine as an internal standard (IS). Separation was achieved with a reversed-phase C_{18} column (250 mm × 4.6 mm) employing fluorescence detection (excitation: 264 nm, emission: 380 nm). The mobile phase consisted of [0.01% triethylamine solution adjusted to pH 2.8 with orthophosphoric acid–acetonitrile (46:54)]–isopropanol (90:10, v/v) at a flow rate of 1.0 ml/min. For both the drug and metabolite, the standard curve was linear from 5.0 to 500 ng/ml with goodness of fit (r^2) greater than 0.98 observed with four precision and accuracy batches during validation. An observed recovery was more than 70% for drug, metabolite and internal standard. The applicability of this method to pharmacokinetic studies was established after successful application during 35 subjects bioavailibity study. The method was found to be precise, accurate and specific during the study.
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Keywords: Validation; Itraconazole; Hydroxyitraconazole; Loratidine

1. Introduction

Itraconazole is a triazole anti-fungal agent (Fig. 1). It inhibits the biosynthesis of ergosterol, a major component of the cell membrane of yeast and fungal cells. It is used in the treatment of a variety of fungal infections. Itraconazole is extensively metabolized in the liver. A very large number of different metabolites is formed. One metabolite, hydroxyitraconazole has similar antifungal activity in vitro to the parent drug.

Few methods are available for measuring both itraconazole and hydroxyitraconazole. Methods with a sensitivity of 10 ng/ml having sample preparation using solid-phase extraction [1], a sensitivity of 20 ng/ml having sample preparation using three-step liquid–liquid extraction [2] and a sensitivity of 25 ng/ml having sample preparation using protein precipitation are available [3]. Few more methods are also available for the determination of itraconazole and hydroxyitraconazole with lower sensitivity using LC–MS–MS as detection technique [4,5]. None of the above mentioned high-performance liquid chromatography (HPLC) methods achieved a limit of quantification (LOQ) as low as 5 ng/ml. Using LC–MS–MS methods, very low LOQs (< 5 ng/ml) could be achieved, however, this technique is not accessible at many laboratories.

This article describes a sensitive and specific method for the simultaneous determination of itraconazole and hydroxyitraconazole (metabolite) in human plasma. The preparation of the samples was based on simple one-step liquid–liquid extraction instead of multi-step liquid–liquid extraction or solid-phase extraction. This assay allowed determination of both itraconazole and its active metabolite (hydroxyitraconazole) using 1.0 ml plasma sample in a single run using HPLC equipped with fluorescence detector. Now with this validated method it is possible to reach a LOQ as low as 5 ng/ml for both itraconazole and its active metabolite (hydroxyitraconazole) using HPLC.

The calibration curve standards and quality control samples for both itraconazole and hydroxyitraconazole were prepared in bulk and stored in freezer below −60 °C. The above mentioned samples were used during method

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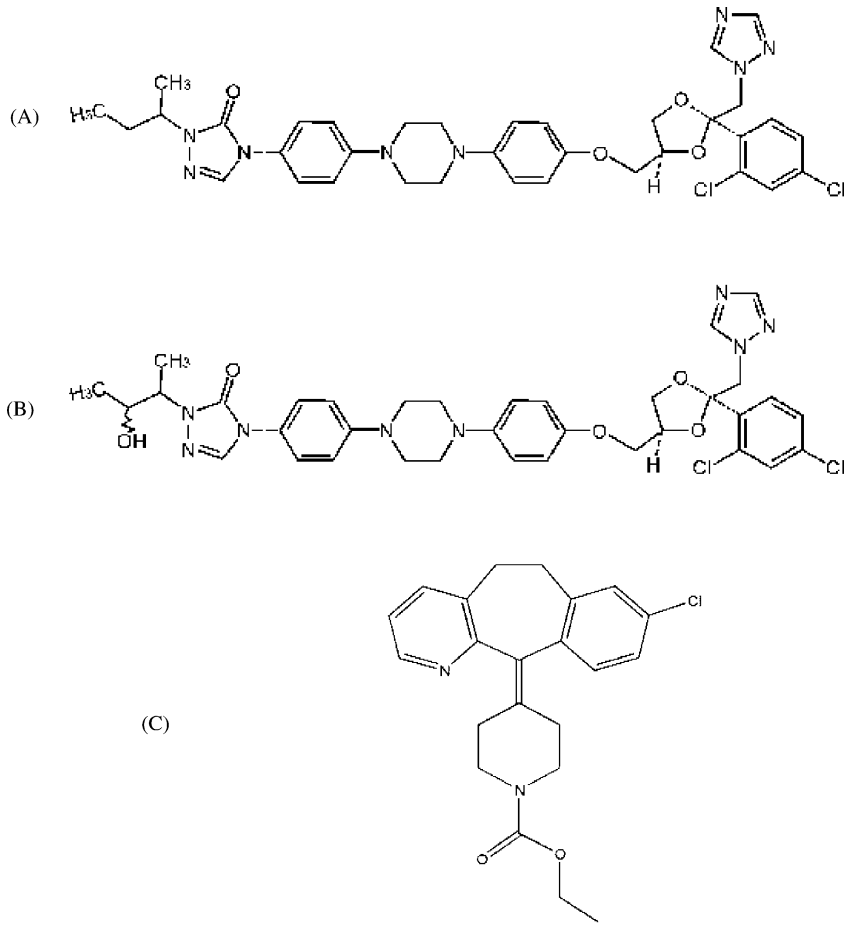

Fig. 1. Structures of (A) itraconazole, (B) hydroxyitraconazole, and (C) loratidine (IS).

validation to establish precision and accuracy as well as stability.

2. Experimental

2.1. Chemicals and reagents

Itraconazole, hydroxyitraconazole, and loratidine working standards were procured from Astron Research, India, Pdi-Research Labs., Canada and Dr. Reddy's Labs. India, respectively. Acetonitrile (HPLC grade) and methanol (HPLC grade) were purchased from Spectrochem India and hexane (HPLC grade), dichloromethane (HPLC grade), 2-propanol (HPLC grade), triethylamine (LR grade), orthophosphoric acid 88% (GR grade) and dipotassium hydrogenphosphate (GR grade) were purchased from Merck, India. Laboratory-prepared water (Milli-Q) was used. Plasma was procured from the Prathama Blood Centre, India.

2.2. Equipment

For HPLC, a Shimadzu 10 A *VP* Series System equipped with RF-10A XL fluorescence detector was used, supplied by Shimadzu, Japan. The Milli-Q water system was supplied by (catalog no.QGARD00R1) Millipore India Ltd., India, the Cyclo Mixer was supplied by Remi equipments, India, a plasma extractor (rotating type) of custom design was used and the centrifuge (Megafuge 2.0 R) was supplied by Heraeus Instruments, Germany.

2.3. Preparation of buffer

A 1.0 M dipotassium hydrogenphosphate buffer was prepared by dissolving 17.419 g of dipotassium hydrogenphosphate, pH adjusted to 8.5 with orthophosphoric acid (88%) and diluted to 100 ml with Milli-Q water.

2.4. Preparation of calibration curve standards

Stock solutions of itraconazole and hydroxyitraconazole of 250 μg/ml were prepared using methanol as solvent (stable for 14 days at 2–8 °C). Calibration standards were prepared by mixing appropriate volumes of the serial dilutions and control plasma to achieve eight different concentrations from 5.0 to 500 ng/ml of each itraconazole and hydroxyitraconazole. The standards were then stored in freezer below −60 °C.

Table 1
Time programme

Time (min)	Flow rate (ml/min)	Mobile phase
0.1	1.0	A
20.0	1.0	A
20.5	1.5	B
25.5	1.5	B
26.5	1.5	A
30.0	1.5	A

Mobile Phase A: [acetonitrile-buffer (pH 2.8) (54:46)]–2-propanol (90:10).
Mobile Phase B: [acetonitrile-buffer (pH 2.8) (54:46)]–2-propanol (50:50).
The above time programme was used to flush the column before next injection.

2.5. Preparation of quality control (QC) samples

Quality control samples were prepared by mixing appropriate volumes of the serial dilutions prepared from the stock solution prepared for calibration curve standards and control plasma to achieve four different concentrations at LOQ, low, medium and high levels each for itraconazole and hydroxyitraconazole. The QC samples were then stored in freezer below −60 °C.

2.6. Sample preparation for analysis

The frozen samples were thawed in water at room temperature. The thawed samples were vortexed to ensure complete mixing of contents. 1.0 ml of plasma was transferred into pre-labeled tubes and 25 μl of internal standard (IS) dilution (about 500 μg/ml of loratidine) was added to each tube and this solution was vortexed. After adding 100 μl of 1 M dipotassium hydrogenphosphate buffer (pH 8.5) it was vortexed to ensure uniform mixing. 4 ml of hexane-dichloromethane (70:30) mixture was added and rotated for 15 min on the plasma extractor followed by centrifugation at 1891 × *g* rcf for 10 min at 10 °C. The aqueous layer was frozen in alcohol freezing bath and the organic layer was transferred to pre-labeled tubes. The organic layer was evaporated at about 40 °C under nitrogen gas stream and reconstituted the residue with 150 μl of reconstitution solution (acetonitrile—Milli-Q water, 65:35). The contents were finally transferred into HPLC autosampler vials for analysis. All the procedures from thawing to placement in the autosampler were performed at room temperature.

2.7. Chromatographic conditions

The sample was chromatographed on a Kromasil C_{18} 5 μm (250 mm × 4.6 mm) column supplied by Flexit, India at 30 °C. The mobile phase consisted of acetonitrile, 2-propanol and 0.01% triethylamine buffer, pH 2.8 (Table 1). The autosampler was set to 4 °C and the injection volume was 40 μl. The pump flow was 1 ml/min with time programme (Table 1) and the fluorescence detector was set at excitation wavelength 264 nm and emission wavelength 380 nm.

2.8. Linearity, precision, and accuracy

Linearity, precision, and accuracy was established for itraconazole and hydroxyitraconazole during the course of the validation by analysing four precision and accuracy batches consisting eight-point calibration curve and eight sets of each LOQ, low, medium and high quality control samples. Eight different calibration curve standards (range from 5.0 to 500 ng/ml) of itraconazole and hydroxyitraconazole along with 8 sets of QC samples (each of LOQ, low, medium and high) were retrieved from the deep freezer and processed as per the procedure described in Section 2.6.

2.9. Stability

2.9.1. Freeze thaw

Eight high and low quality control samples each, which were prepared and stored as described in Section 2.5 were retrieved from the deep freezer after they had been frozen completely after storage (about 24 h) and thawed at room temperature. After complete thawing of the samples the same were put back into the freezer. The above step was

Table 2
Curve parameter summary and back-calculated calibration curve concentrations for itraconazole and hydroxyitraconazole in human plasma

	Concentration (ng/ml)							
	5	10	25	75	250	375	450	500
Itraconazole								
Mean	4.83	11.15	25.76	78.31	264.45	362.80	441.83	475.07
S.D. ±	0.113	0.190	1.806	2.853	2.991	19.037	17.848	17.881
R.S.D. (%)	2.3	1.7	7.0	3.6	1.1	5.2	4.0	3.8
Accuracy (%)	−3.4	11.5	3.0	4.4	5.8	−3.3	−1.8	−5.0
Hydroxyitraconazole								
Mean	5.13	10.05	25.59	74.37	247.12	374.32	451.97	541.68
S.D. ±	0.204	0.564	2.136	3.174	4.366	36.541	33.236	9.077
R.S.D. (%)	4.0	5.6	8.3	4.3	1.8	9.8	7.4	1.7
Accuracy (%)	2.6	0.5	2.4	−0.8	−1.2	−0.2	0.4	8.3

Table 3
Within-batch precision and accuracy of itraconazole and hydroxyitraconazole

	Concentration (ng/ml)			
	LOQ QC (5)	LQC (15)	MQC (240)	HQC (400)
Itraconazole				
Mean	4.76	15.58	259.73	429.28
S.D. ±	0.470	1.172	13.489	48.805
R.S.D. (%)	9.9	7.5	5.2	11.4
Accuracy (%)	−4.8	3.9	8.2	7.3
Hydroxyitraconazole				
Mean	4.83	14.31	251.55	375.96
S.D. ±	0.671	1.757	8.957	29.711
R.S.D. (%)	13.9	12.3	3.6	7.9
Accuracy (%)	−3.4	−4.6	4.8	−6.0

Table 4
Between-batch precision and accuracy of itraconazole and hydroxyitraconazole

	Concentration (ng/ml)			
	LOQ QC (5)	LQC (15)	MQC (240)	HQC (400)
Itraconazole				
Mean	4.74	15.72	265.76	438.22
S.D. ±	0.576	1.784	16.212	25.352
RSD (%)	12.1	11.3	6.1	5.8
Accuracy (%)	−5.2	4.8	10.7	9.6
Hydroxyitraconazole				
Mean	5.23	15.12	264.30	419.36
S.D. ±	0.770	1.750	27.850	39.307
R.S.D. (%)	14.7	11.6	10.5	9.4
Accuracy (%)	4.6	0.8	10.1	4.8

repeated twice after freezing the samples for 12–24 h. Thus, all the samples went through three freeze thaw cycles.

All the stability samples (after three freeze thaw cycles) were processed as per Section 2.6 with freshly thawed QCs (eight each high and low). The means of responses were compared of all the QC samples (that had gone through three freeze thaw cycles) with those of freshly thawed QC samples for stability.

2.9.2. Bench top

Eight samples each of high and low QCs were taken out from deep freezer, thawed and kept at room temperature for 3 h. After 3 h another set of eight high and eight low QC samples was taken out from deep freezer and thawed. Both the sets of low and high QC samples were processed as per Section 2.6 and the means of responses of stability samples with those freshly thawed QC samples were compared for stability.

2.9.3. Auto sampler

After completion of the analysis of precision and accuracy batch, all the QC samples (low, medium and high) were stored in autosampler at 4 °C and re-injected after 50 h as stability samples along with another set of freshly processed QCs (eight each of high, medium, and low). The means of responses of stability samples with those of freshly processed QC samples were compared for stability.

2.9.4. Long term

The QC samples (low and high) prepared and stored below −20 °C as described in Section 2.5 were stored for 237 days. Few days prior to the analysis fresh QC samples were prepared (as per Section 2.5) from freshly prepared drug stock and used as comparison samples. All the stability samples (eight high and eight low) and comparison samples (eight high and eight low) were processed as per Section 2.6. The mean of responses was compared of all the QC sample (stored below −20 °C for 237 days) with those of freshly prepared QC samples for stability.

2.10. Collection and storage of plasma samples

The blood samples were collected in vacutainers containing EDTA as the anticoagulant. The samples were then

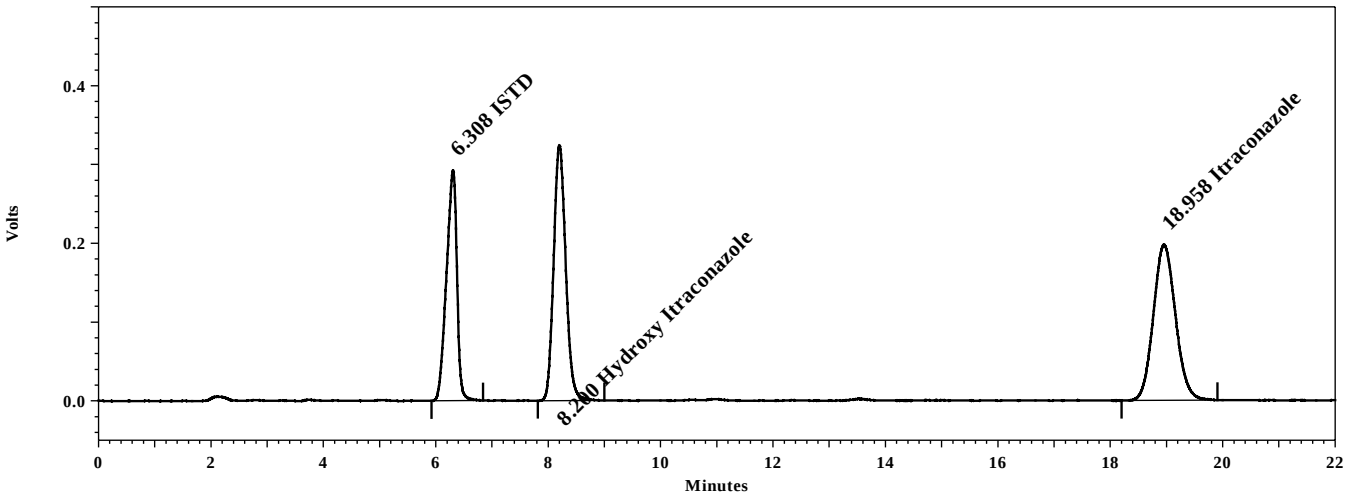

Fig. 2. Representative chromatogram of aqueous standard. ISTD = internal standard.

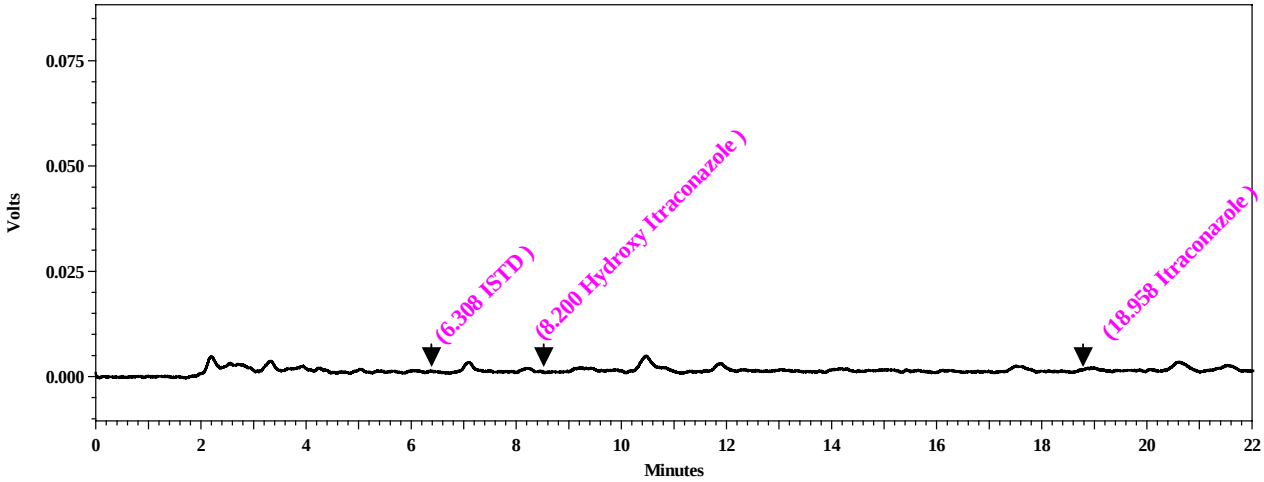

Fig. 3. Representative chromatogram of plasma blank.

centrifuged (1891 × *g*, 10 °C, 15 min) and separated plasma samples were stored in polypropylene tubes below –20°C.

3. Results and discussion

3.1. Time programme

In this method time programme was used to flush the column and remove late eluting interferences using mobile phase B (Table 1). The gradient was not used between 0 and 20 min. to get good resolution between analyte peaks and interference peaks.

3.2. Linearity

A linear equation was judged to produce the best fit for the concentration/response relationship. The regression type was 1/amount^2 and peak area ratio for an eight-point calibration curve was found to be linear from 5.047 to 504.400 ng/ml for itraconazole and 5.054 to 505.150 ng/ml for hydroxyitraconazole. The goodness of fit for both itraconazole and hydroxyitraconazole were consistently greater than 0.98 during the course of validation (Table 2).

3.3. Precision and accuracy

The precision of the assay was measured by the percent coefficient of variation over the concentration range of LOQ, low (*L*), medium (*M*) and high (*H*) quality control samples, respectively of itraconazole and hydroxyitraconazole during the course of the validation by analysing for precision and accuracy batches. The accuracy of the assay was defined as the absolute value of the ratio of the back calculated mean values of the quality control samples to their respective nominal values, expressed as percentage (Tables 3 and 4).

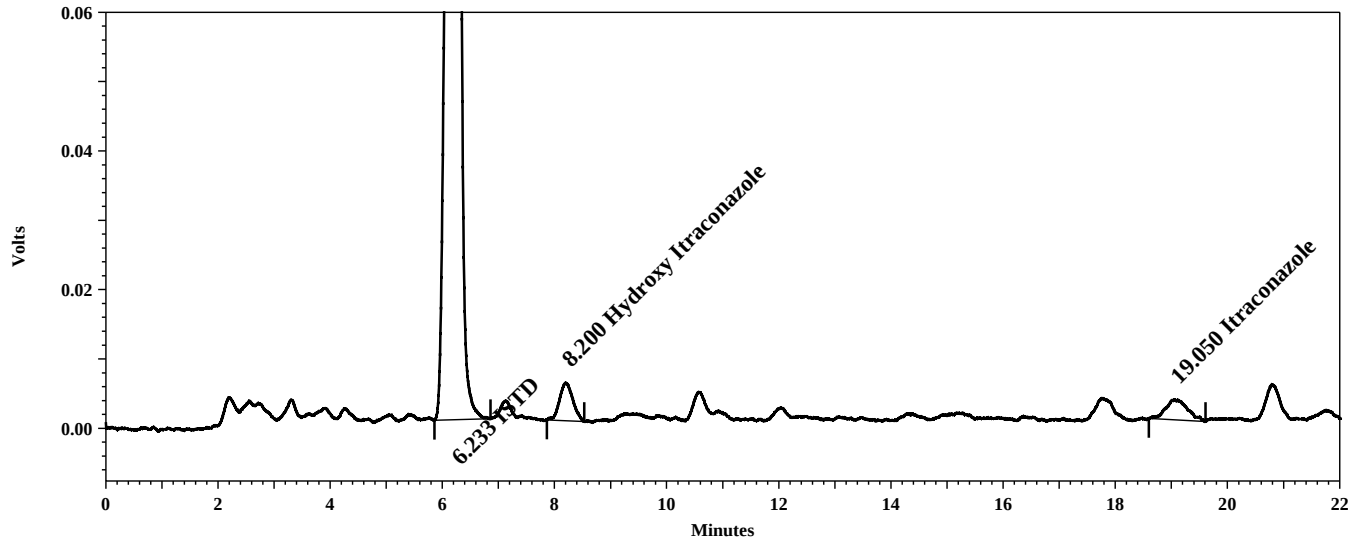

Fig. 4. Representative chromatogram of LOQ QC.

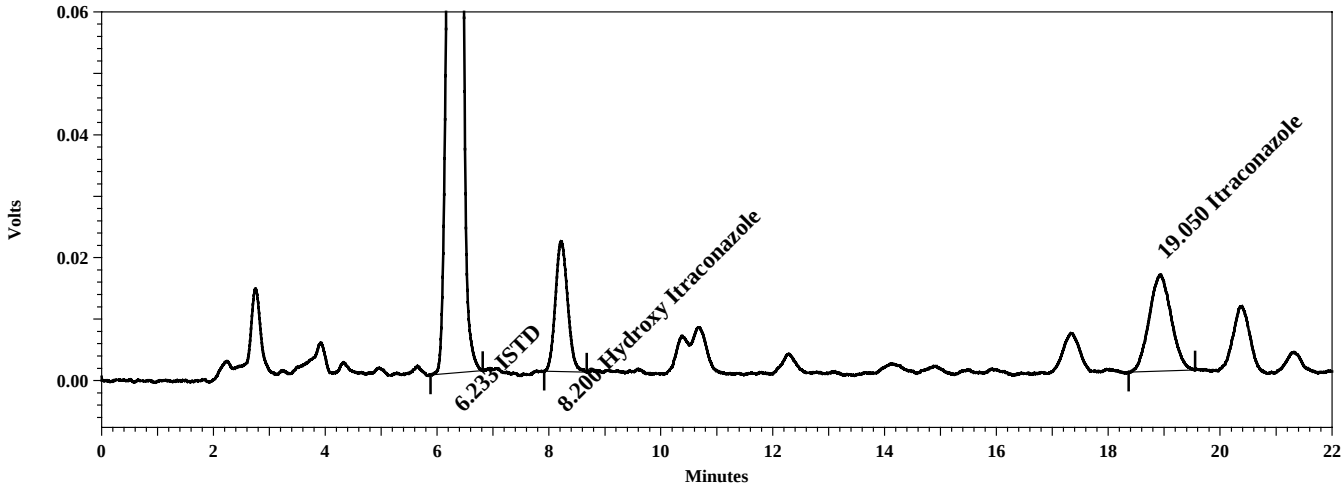

Fig. 5. Representative chromatogram of study sample (2.00 h).

Representative chromatograms of aqueous standard, plasma blank, LOQ QC, and study sample are shown in Figs. 2–5.

3.4. Stability

The concentration values obtained during the stability experiments (after comparing values obtained by analyzing stability samples and freshly prepared QC samples), i.e. autosampler stability after 50.0 h at 4 °C, freeze thaw stability after three cycles, bench top stability after 3.0 h at room temperature and long term stability after 237 days below −20 °C, suggest that there was no significant loss of itraconazole and hydroxyitraconazole concentrations (Table 5).

Table 5
Result table of stability data obtained during validation

Stability	(% Degradation)	
	Itraconazole	Hydroxyitraconazole
Short term stock solution (after 6.0 h at room temperature)	7.3	4.1
Long term stock solution (within 2 to 8 °C)	8.2 (after 14 days)	2.4 (after 16 days)
Autosampler/wet extract [*L*, *M*, and *H*] (after 50.0 h at 4 °C)	−0.1, 0.1 and 0.8	−1.4, 7.6 and 0.2
Freeze thaw (*L* and *H*) (3 cycles)	3.3 and −1.5	−2.2 and 4.1
Bench top(*L* and *H*) (after 3.0 h at room temperature)	5.6 and 3.0	0.7 and 4.0
Long term stability of drug in plasma (*L* and *H*) (after 237 days below −20 °C)	0.7 and 2.2	0.9 and 5.4

L = 15 ng/ml, *M* = 240 ng/ml, and *H* = 400 ng/ml.

3.5. Specificity

Six lots of human plasma, commercially procured, were chromatographically screened for interfering substances and did not show significant interference at the retention time of itraconazole, hydroxyitraconazole, and internal standard while comparing with freshly spiked sample at LOQ level using interference free human plasma.

3.6. Recovery

3.6.1. Itraconazole

The areas of low, medium, and high quality control samples were compared against the area of non-extracted aqueous quality control samples. The mean recoveries for itraconazole for the low, medium and high QCs were 84.54, 77.04, and 76.03%, respectively.

3.6.2. Hydroxyitraconazole

The areas of low, medium, and high quality control samples were compared against the area of non-extracted aqueous quality control samples. The mean recoveries for hydroxyitraconazole for the low, medium and high QCs were 73.30, 70.50, and 72.45%, respectively.

Table 6
Precision and accuracy of calibration curve standards and QC samples analyzed along with study samples

	Precision (%)	Accuracy (%)
Itraconazole		
Calibration curve standards	3.0–7.9	−3.5–3.3
Quality control samples	5.5–12.4	−6.3–1.0
Hydroxyitraconazole		
Calibration curve standards	3.9–7.2	−4.0–4.1
Quality control samples	8.7–12.8	−6.1–2.1

Table 7
Mean plasma concentrations (ng/ml) of itraconazole and hydroxyitraconazole after administration of two capsules each containing 100 mg of itraconazole to healthy, adult, male, human subjects under fed conditions

	Time (h)																			
	0.00	1.00	2.00	3.00	3.50	4.00	4.50	5.00	5.50	6.00	6.50	7.00	10.00	14.00	18.00	24.00	32.00	48.00	72.00	96.00	120.00
	Concentration (ng/ml)																				
Itraconazole																					
Mean	0.00	1.28	15.92	47.02	70.81	95.90	107.16	134.81	152.23	153.03	179.36	169.51	97.44	57.52	44.00	32.40	24.30	12.42	4.58	2.70	1.15
S.D.	0.000	3.284	25.429	48.342	62.015	70.150	65.074	72.538	75.275	73.899	82.629	73.316	37.376	23.039	15.499	12.020	11.979	6.474	5.803	4.235	2.759
R.S.D. (%)	–	256.5	159.7	102.8	87.6	73.1	60.7	53.8	49.4	48.3	46.1	43.3	38.4	40.1	35.2	37.1	49.3	52.1	126.7	157.1	240.4
Hydroxyitraconazole																					
Mean	0.00	0.16	18.22	64.67	95.91	124.79	145.59	181.17	208.37	214.51	240.04	237.76	215.89	172.06	148.79	136.43	90.15	42.63	9.60	2.69	0.57
S.D.	0.000	0.944	30.296	55.497	65.476	68.083	66.673	69.198	67.295	70.478	66.775	65.065	58.700	51.681	46.096	43.121	40.232	27.880	14.827	5.890	1.801
R.S.D. (%)	–	589.8	166.3	85.8	68.3	54.6	45.8	38.2	32.3	32.9	27.8	27.4	27.2	30.0	31.0	31.6	44.6	65.4	154.5	219.0	317.6

$n = 35$.

3.6.3. Internal standard

The internal standard peak areas of low, medium, and high quality control samples were compared to the internal standard peak areas of non-extracted aqueous quality control samples. Mean recovery for internal standard for the low, medium and high QCs was 91.39%.

3.7. Carryover

No significant carryover was observed when human plasma blanks were injected immediately following high standards.

3.8. Application

The applicability of this to pharmacokinetic studies was established after successful application during 35 subjects bioavailability study (Table 6). Two types of two capsules each containing 100 mg itraconazole were dosed to healthy, adult, male, human subject under fed conditions. Representative mean plasma concentrations of itraconazole and hydroxyitraconazole are given in Table 7.

4. Conclusion

The method described here for assay of itraconazole and hydroxyitraconazole simultaneously in plasma was found to be specific during a bioavailability study. This demonstrated the suitability of the analytical method for use in bioavailability studies.

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