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Quantitation of itraconazole in rat heparinized plasma by liquid chromatography–mass spectrometry

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Abstract

A liquid chromatographic–mass spectrometric (LC–MS) assay was developed and validated for the determination of itraconazole (ITZ) in rat heparinized plasma using reversed-phase HPLC combined with positive atmospheric pressure ionization (API) mass spectrometry. After protein precipitation of plasma samples (0.1 ml) with acetonitrile containing nefazodone as an internal standard (I.S.), a 50-μl aliquot of the supernatant was mixed with 100 μl of 10 m*M* ammonium formate (pH 4.0). An aliquot of 25 μl of the mixture was injected onto a BDS Hypersil C_{18} column (50×2 mm; 3 μm) at a flow-rate of 0.3 ml/min. The mobile phase comprising of 10 m*M* ammonium formate (pH 4) and acetonitrile (60:40, v/v) was used in an isocratic condition, and ITZ was detected in single ion monitoring (SIM) mode. Standard curves were linear ($r^2 \geq 0.994$) over the concentration range of 4–1000 ng/ml. The mean predicted concentrations of the quality control (QC) samples deviated by less than 10% from the corresponding nominal values; the intra-assay and inter-assay precision of the assay were within 8% relative standard deviation. Both ITZ and I.S. were stable in the injection solvent at room temperature for at least 24 h. The extraction recovery of ITZ was 96%. The validated assay was applied to a pharmacokinetic study of ITZ in rats following administration of a single dose of itraconazole (15 mg/kg).

Keywords: Itraconazole; LC–MS; Validation; Pharmacokinetics

1. Introduction

Itraconazole (ITZ), chemically 4-[4-[4-[4-[[2-(2,4-dichlorophenyl)-2-(1H-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan- 4-yl]methoxy]phenyl]-1-piperazinyl]phenyl]-2,4-dihydro-2-(1-methylpropyl)- 3H-1,2,4-triazol-3-one (Fig. 1), is a new generation triazole antifungal drug which has been shown to be highly effective in curing fungal infection following oral administration in human [1–3] and animal [4,5]. Since ITZ is a substrate/inhibitor of cytochrome P450 3A isozyme [6,7], we are interested to see the effect of pretreatment with CYP 3A inhibitors/inducers on the pharmacokinetics of ITZ using a rat model. In order to perform such a pharmacokinetic study, the development and validation of a sensitive assay using a small sample volume was necessary to monitor the levels of ITZ in rat plasma.

Previously reported high-performance liquid chromatographic (HPLC) assays for quantitation of ITZ

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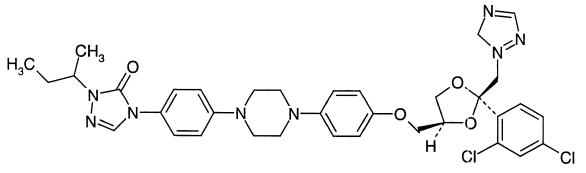

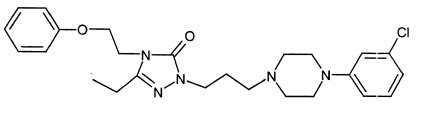

Fig. 1. Structural representation of itraconazole and nefazodone (I.S.).

required 0.5–2 ml of plasma or serum and used time-consuming liquid–liquid or solid-phase extractions [8–12]. Although simpler protein precipitation methods are available, employing either HPLC-UV or HPLC-fluorescence detection, the sensitivity (lower limited of quantitation >10 ng/ml) is not adequate for the monitoring of ITZ levels [13–15]. In addition, these assays have a long chromatographic run time. In this report, we describe a simple, rapid, and sensitive assay procedure for determination of ITZ by LC–MS using 0.1 ml of rat plasma. The method has been adequately validated to permit its application to a routine pharmacokinetic study.

2. Experimental

2.1. Chemicals and reagents

Itraconazole (10 mg/ml oral solution, Control No. 99A26/88) was obtained from Janssen Pharmaceuticals (Titusville, NJ, USA). Nefazodone, NEF (purity 99.8%), was obtained from Bristol-Myers Squibb (Wallingford, CT, USA). HPLC grade acetonitrile was obtained from Burdick and Jackson (Muskeson, MI, USA). Water was purified by Mill-Q System from Millipore (Milford, MA, USA). Ammonium formate (Avocado Research Chemicals, Wordhill, MA, USA), formic acid (J.T. Baker, Phillipsburg, NJ, USA) were of analytical grade. Control rat heparinized plasma was purchased from Lampire Biological Laboratory (Pipersville, PA, USA). All other reagents were of analytical grade and were used without further purification.

2.2. HPLC and API-MS condition

HPLC was performed on a BDS hypersil 3 μm ODS column (50 mm×2 mm) and guard column (10×2 mm) (Keystone, Wilmington, NC, USA) using an Alliance 2690 HPLC system (Waters, Milford, MA, USA). The mobile phase consisted of acetonitrile and 10 m*M* ammonium formate (pH 4, adjust by formic acid) (60:40, v/v). The flow-rate was 0.3 ml/min.

Mass spectrometric detection was carried out using a PE Sciex API 100 single stage quadrupole instrument (Perkin-Elmer, Foster City, CA, USA) operating in the positive API mode. The mass spectrometer was programmed to admit the protonated molecules at the mass to charge ratios (m/z) of 705.2 (ITZ), and 470.4 (NEF). The positive turboionspray voltage (V) was 4450 mV. The orifice and ring voltages were set at 56 and 395 mV, respectively. The dwell time was 200 ms. Curtain gas and nebulizing gas (nitrogen) pressure were set at 8.26 and 4.82 kPa, respectively. The flow-rate of heated gas (Gas 2) was operated at 5 l/min. TurboIonSpray temperature was set at 350°C. Analytical data were acquired by the PE Sciex Software (LC Tune 1.3) and the peak areas measured using MacQuan software (MacQuan 1.6).

2.3. Standard solutions

Stock solutions of ITZ and I.S. were prepared in HPLC mobile phase (1000 μg/ml) and stored at −20°C. A working I.S. solution was prepared daily at a concentration of 200 ng/ml in acetonitrile and was used to spike the samples prior to extraction.

2.4. Standard curve and quality control samples

A serial dilution technique was employed to obtain the final standard concentrations of 4–1000 ng/ml of ITZ in rat heparinized plasma. These spiked samples, containing seven different concentrations (4, 10, 25,

100, 250, 500, and 1000 ng/ml), were used to construct the standard curve. Quality control (QC) sample pools were prepared from independently prepared stock solutions in rat plasma to contain concentrations of ITZ within the standard curve range. In addition, a QC sample pool above the upper limit of the standard curve was prepared in blank plasma to serve as a dilution QC sample.

2.5. Sample preparation

To a 0.1-ml aliquot of the rat plasma standard, blank, QC, or study sample in a clean screw cap tube, was added 0.1 ml of acetonitrile containing 200 ng/ml of I.S. to precipitate the protein and then vortexed for 2 min. After centrifugation at 2000 *g* at 4°C for 5 min, 50 μl of supernatant were transferred to a clear auto sampler vial containing 100 μl of 10 m*M* ammonium formate (pH 4.0), vortexed, and centrifuged at 2000 *g* at 4°C for 3 min. A 25-μl volume of the sample was injected onto the LC–MS system.

2.6. Validation of the assay

2.6.1. Calibration

Calibration curves were constructed by plotting peak area ratio of the analyte to the internal standard against the analyte's concentration. The weighted $(1/x)$ linear regression was fitted over the concentration range (4–1000 ng/ml).

2.6.2. Accuracy and precision

The accuracy and precision of the assay were determined by assaying QC samples (10, 200, and 800 ng/ml) in six replicates on three different days. In addition, a fourth QC sample (dilution QC) was spiked above the highest standard (2000 ng/ml) and diluted with blank plasma prior to sample preparation. The dilution QC samples were analyzed in a single run. The inter-assay precision was evaluated by one-way ANOVA to obtain the treatment mean square (TMS), error mean square (EMS), and grand mean (GM). Inter-assay precision, expressed as %RSD, was defined for each of the concentration as:

$$\%\text{RSD} = 100[(\text{TMS} - \text{EMS})/N]^{0.5}/\text{GM}$$

Alternatively, if EMS is greater than TMS, the inter-assay precision can be calculated as the standard deviation (%RSD) of the individual sequence means about the grand mean.

2.6.3. Lower limit of quantitation (LLQ)

Control plasma samples were obtained from six individual rat plasma specimens. For each individual rat specimen, a blank plasma and a spiked plasma containing 4 ng/ml of ITZ were prepared and analyzed. The performance of the assay on accuracy at the LLQ was calculated as the percentage deviation (%DEV) of the mean observed concentration from the nominal concentration for each analyte in the six spiked individual samples. The precision of the assay at the LLQ was expressed as the relative standard deviation (%RSD) of the observed concentration for the analyte in the six spiked individual samples.

2.6.4. Extraction recovery

Two sets of standards, in the concentration range of 4–1000 ng/ml were prepared in rat heparinized plasma and the mobile phase, respectively. Plasma standards were processed and chromatographed as previously described; while standards prepared in mobile phase were injected onto the column directly. The extraction recovery was calculated by the following equation.

$$\%\text{Recovery} = \frac{\text{Peak area slope of standard curve prepared in plasma}}{\text{Peak area slope of standard curve prepared in mobile phase}}$$

2.6.5. Autosampler stability

The autosampler stability of ITZ and I.S. in the injection solvent was determined by periodically injecting replicate preparations of processed samples at 0, 16, and 24 h. The peak area obtained at the 0 h for ITZ and NEF was used as the reference in calculating the relative ratios for each analyte at the various time intervals.

3. Results and discussion

3.1. Specificity

Representative SIM chromatograms of plasma obtained from predose and postdose rats are pre-

sented in Figs. 2 and 3, respectively. No significant interfering peaks were detected at the retention times of the peaks of interest, as well as in the ion channel, in predose sample. The nominal retention times for NEF and ITZ were 1.16 and 2.48 min, respectively.

3.2. Linearity

A weighted linear regression of the peak area ratios versus standard concentrations was performed using a weight of 1/concentration. The observed peak area ratios were linear over the concentration range of 4–1000 ng/ml in rat heparinized plasma (Fig. 4). The values of r^2 (≥ 0.994) and the consistency in slope values (0.00581 ± 0.003), demonstrated that the standard curve had a reliable response over the studied concentration range.

3.3. Lower limit of quantitation (LLQ)

The predicted mean concentration in LLQ samples for ITZ was 4.09 ng/ml, which deviated less than $\pm 4.01\%$ from the respective nominal value. The precision estimates for the LLQ samples was 7.5%

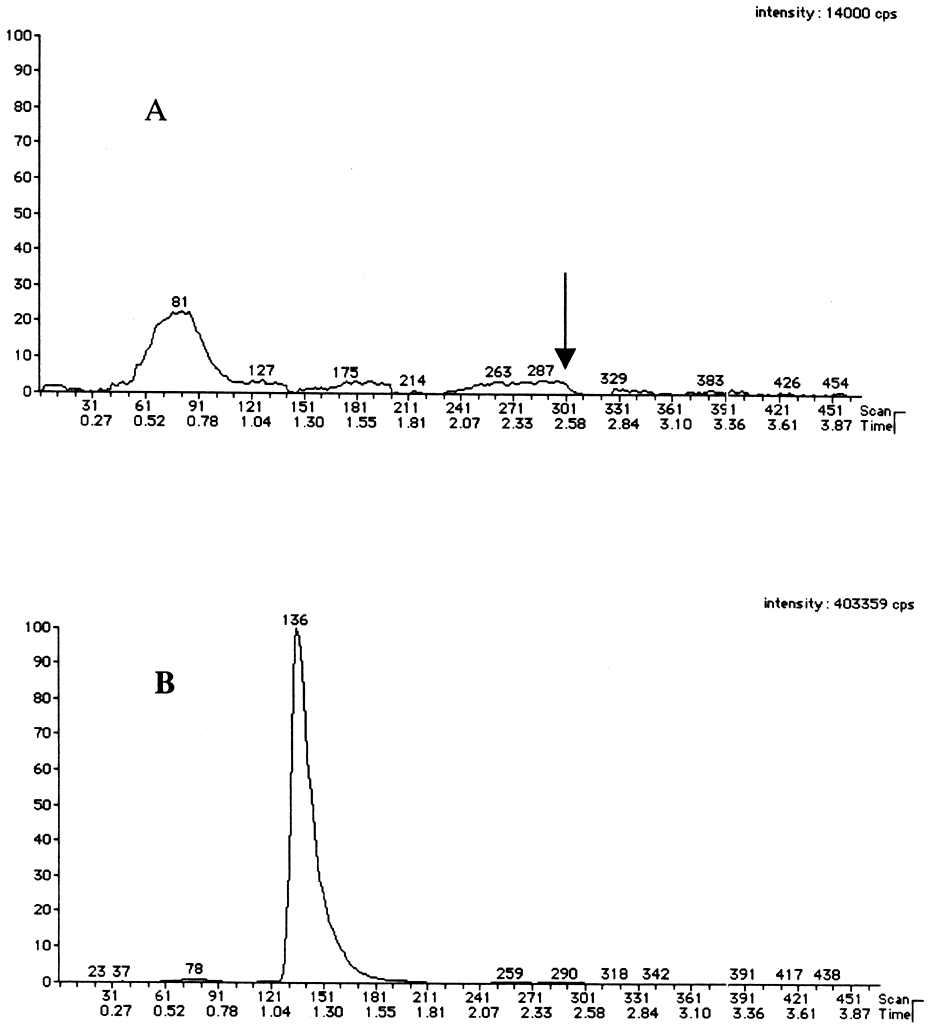

Fig. 2. Typical single ion monitoring chromatograms obtained from rat pre-dose plasma (A) ITZ and (B) internal standard (arrow indicates the position of ITZ peak in the ion channel).

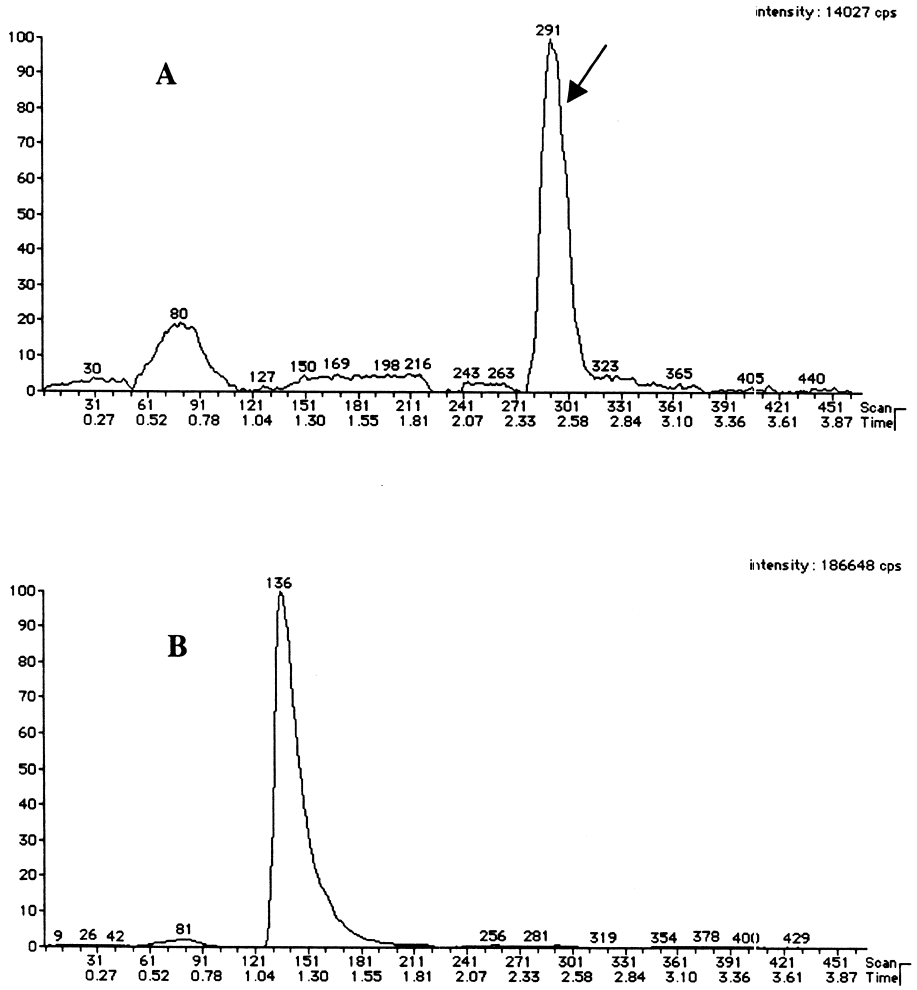

Fig. 3. Typical single ion monitoring chromatograms obtained from rat plasma after oral administration of itraconazole: (A) ITZ, 99 ng/ml and (B) internal standard.

RSD. Therefore, the LLQ for ITZ in rat heparinized plasma was established at 4.0 ng/ml.

3.4. Intra- and inter-assay accuracy and precision

The intra- and inter-assay accuracy and precision values for QC samples at four different concentrations of ITZ are provided in Table 1. The intra- and inter-assay precision values (%RSD) at the various concentrations for ITZ were $\leq$8.03%. The accuracy (%DEV) values for all four concentrations deviated by $\leq$9.66% from the corresponding nominal concentrations.

3.5. Extraction recovery

The results of the comparison of neat standards versus plasma extracted standards in the concentration range 4–1000 ng/ml for ITZ indicated that the extraction of ITZ from rat heparinized plasma was 96.1%.

3.6. Autosampler stability

The absolute peak area at three concentration levels of ITZ and I.S. was generally found to be within $\pm$11.0% from the corresponding peak areas at

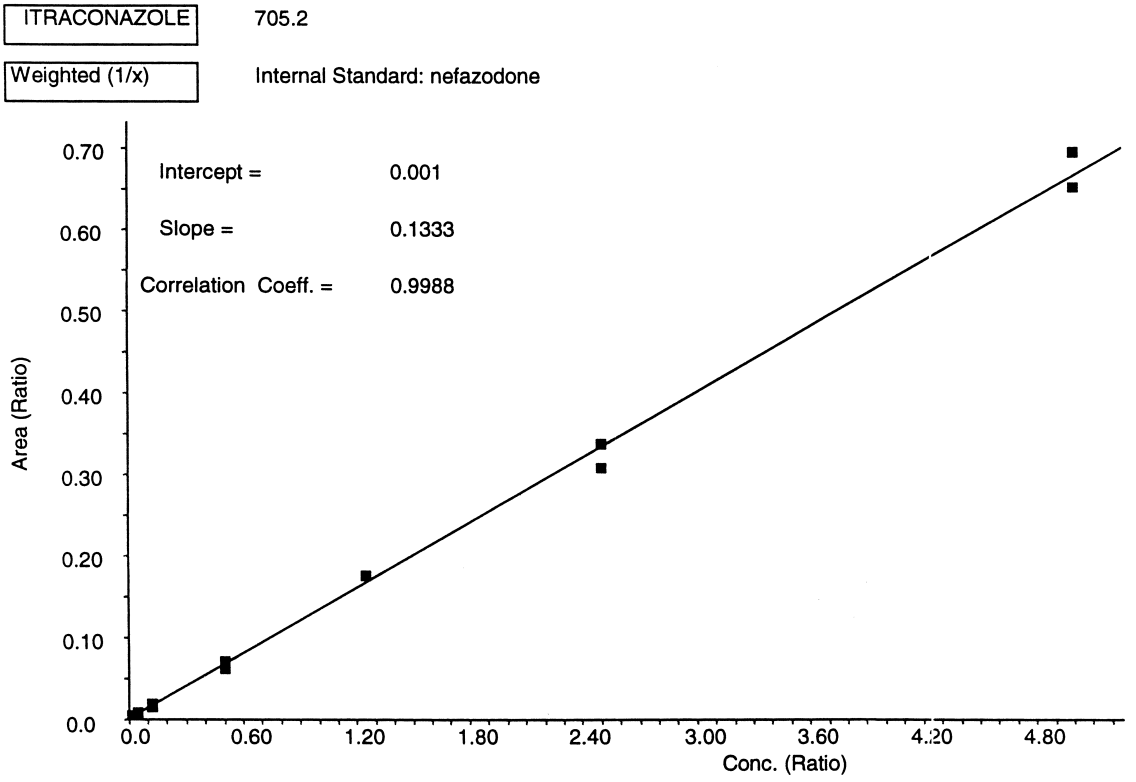

Fig. 4. Calibration curve for ITZ from 4 to 1000 ng/ml. A plot of area (ratio) (i.e., area of ITZ/area of I.S.) versus concentration (ratio) (i.e., nominal conc. of ITZ/100 ng/ml of I.S.).

time zero during the course of this stability experiment (i.e., 24 h) (Table 2).

3.7. Application

The method was applied to determine the levels of ITZ in rats ($n=5$) following the oral administration of a single 15 mg/kg dose of ITZ. The mean (SD) plasma concentration versus time profile for ITZ is depicted in Fig. 5. ITZ was rapidly absorbed in rats ($T_{max}=1$ h) and exhibited a biphase decline with a mean terminal half-life value of approximate 3.1 h. The mean (SD) pharmacokinetic parameters are tabulated in Table 3.

Table 1
Intra- and inter-assay accuracy and precision results for itraconazole

Nominal conc. (ng/ml)	Mean observed conc. (ng/ml)	Accuracy (%DEV)	Precision (%RSD)	
			Within run	Between run
10	10.91	9.06	7.56	0.00[a]
200	203.5	1.75	8.03	6.95
800	838.1	4.77	4.70	7.12
2000[b]	2193.3	9.66	7.50	7.14

[a] No significant additional variation was observed as a result of performing the assay in different runs.
[b] Dilution QC samples (analyzed in a single run) with 5-fold dilution.

Table 2
Reconstituted autosampler stability for itraconazole and nefazodone

Conc. (ng/ml)	Time (h)	Itraconazole		Nefazodone[a]	
		Peak area	%DEV[b]	Peak area	%DEV[b]
10	0	14 227	0.00	4 490 323	0.0
	16	14 979	5.29	4 510 806	0.5
	24	14 001	−1.59	4 892 457	9.0
100	0	162 409	0.00	4 407 393	0.0
	16	157 664	−2.92	3 921 986	−11.0
	24	168 523	3.76	4 734 292	7.4
500	0	923 772	0.00	4 795 785	0.0
	16	839 907	−9.08	4 399 694	−8.3
	24	885 912	−4.10	5 080 677	5.9

[a] The concentration for nefazodone (I.S.) was 200 ng/ml.
[b] $\frac{\text{Peak area } (T=t) - \text{Peak area } (T=0)}{\text{Peak area } (T=0)} \times 100$

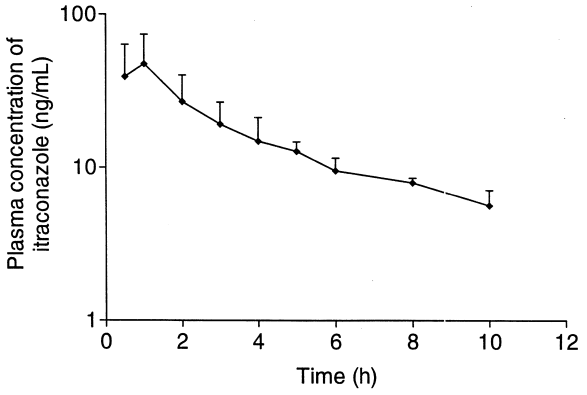

Fig. 5. Mean (±standard deviation) plasma concentration versus time profile of itraconazole in rats ($n=5$) following a single 15 mg/kg oral dose administration.

Table 3
Mean (±standard deviation) pharmacokinetic parameters of Itraconazole in rats ($n=5$) following a single 15 mg/kg oral dose administration

C_{max}[a] (ng/ml)	T_{max}[b] (h)	$AUC_{(0-T)}$[c] (h.ng/ml)	AUC_{inf}[d] (h.ng/ml)	$t_{1/2}$[e] (h)
50.9 (23.8)	1.00 (0.5, 3)	145 (63)	171 (68)	3.11 (1.46)

[a] C_{max}, peak plasma concentration.
[b] T_{max}, median time for attainment of $C_{max.}$(min, max value reported).
[c] $AUC_{(0-T)}$, area under the plasma concentration–time curve up to time T, the last quantifiable time point.
[d] AUC_{inf}, area under the plasma concentration–time curve up to time infinity.
[e] $t_{1/2}$, terminal elimination half-life.

3.8. Discussion

During the method development, we noticed that if the supernatant following protein precipitation with acetonitrile was directly injected on the LC–MS system, the peak shape was broad and lead to poor sensitivity. But if we simply diluted the supernatant with two volumes of aqueous phase (10 m*M* ammonium formate), and then injected the samples, the peak shape became more sharp and sensitivity was much improved. Although the processed sample has been diluted by a factor of three before injection, it did not compromise sensitivity of this assay because the supernatant was directly transferred to a vial and injected onto the column. As compared to the previous method, the following steps: liquid–liquid extraction, evaporation, subsequent transfer, and reconstitution are totally eliminated in the sample preparation, and as a consequence the sample preparation time is considerably reduced.

4. Conclusions

A sensitive, selective, accurate, and precise HPLC procedure with single ion monitoring by single quadrupole mass spectrometer was developed and validated for determination of itraconazole in rat heparinized plasma. The reported method offers several advantages such as a rapid and simple extraction scheme, improved sensitivity, a short

chromatographic run time, and the ability to work with very small sample volume, as compared to previous methods. This makes the method suitable for use in settings where low doses are administered, and/or there are limitation to the amount of blood can be collected.

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