

High-Performance Liquid Chromatographic-Tandem Mass Spectrometric Determination of Itraconazole in Human Plasma for Bioavailability and Bioequivalence Studies

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A highly sensitive high-performance liquid chromatographic-tandem mass spectrometric method (HPLC-MS-MS) has been developed to quantify itraconazole in human plasma for the purpose of pharmacokinetic studies. Sample preparation was carried out by liquid-liquid extraction using loratadine as an internal standard. Chromatographic separation used a YMC C₁₈ column, giving an extremely fast total run time of 3 min. The method was validated and used for the bioequivalence study of itraconazole tablets in healthy male volunteers ($n = 31$). The lower limit of detection proved to be 0.2 ng/mL for itraconazole.

Key Words : High-performance liquid chromatography-tandem mass spectrometry, Itraconazole, Bioequivalence

Introduction

Itraconazole (Fig. 1) is a triazole antifungal agent with a broad spectrum of activity. It acts primarily by inhibiting the biosynthesis of ergosterol, an essential component of fungal cell membranes.¹⁻⁴ It is used in the treatment of a variety of fungal infections. The pharmacokinetics of orally administered itraconazole in humans are characterized by considerable interindividual variation in drug absorption, extensive tissue distribution, with the concentrations in tissue being many times higher than those in plasma, and an elimination half-life of approximately 24 h. Itraconazole is known to be extensively metabolized in humans, yielding over 30 metabolites, including the antifungally active metabolite hydroxy-itraconazole.

Several methods have been reported to quantify itraconazole in biological fluids including HPLC and LC-MS-MS techniques. Methods with a sensitivity of 10 ng/mL having sample preparation using solid-phase extraction,⁵ a sensitivity of 20 ng/mL having sample preparation using three-step liquid-liquid extraction⁶ and a sensitivity of 25 ng/mL having sample preparation using a protein precipitation are available.⁷ More methods are also available for the determination of itraconazole with lower sensitivity using LC-MS-MS as detection technique.^{8,9} However, a method for rapid and precise determination of concentrations of itracon-

azole in human plasma simultaneously, which can be used for bioavailability and bioequivalence studies has not been reported yet utilizing high-performance liquid chromatographic-tandem mass spectrometric technique. The aim of this study was, therefore, to develop determination method of itraconazole in human plasma. The developed method was validated and used for a bioequivalence study, of two tablet formulations of itraconazole in 31 healthy male volunteers. The study protocol was approved by the Korean Food and Drug Administration.

Experimental Procedures

Materials and reagents. Itraconazole and test tablets were purchased from Han All Pharmaceutical Company (Seoul, Korea). Sporanox[®] capsules were obtained from Janssen Pharmaceutical Company (Seoul, Korea). Loratadine was provided by Sigma-Aldrich Company (MO, USA). Acetonitrile, Heptane, Isoamylalcohol and ammonium formate (all HPLC grade) were from Fisher Scientific Korea Limited (Seoul, Korea). All other reagents were of analytical grade. Drug-free human plasma (blank plasma) was obtained from healthy male volunteers by centrifugation of whole blood treated with the anticoagulant, heparin, and stored at approximately -20 °C until needed.

Liquid chromatography. High-performance liquid chromatography was performed using: Agilent HPLC systems 1100 series (Palo Alto, CA, USA). The column used was a YMC C₁₈ (5.0 × 2.0 mm I.D., 3 μm, Japan). The mobile phase consisted of acetonitrile: 10 mM ammonium formate = 85 : 15 (v/v) and was set at a flow rate of 0.2 mL/min.

Tandem mass spectrometry. Mass spectrometric detection was performed using: Applied Biosystems MDS SCIEX API 3000 triple-quadrupole mass spectrometry (Concord,

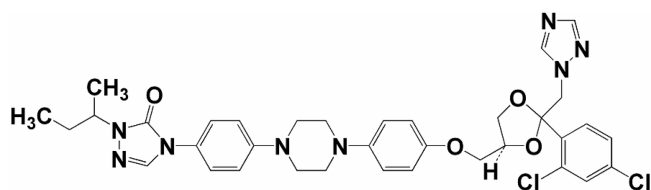


Figure 1. Structure of itraconazole.

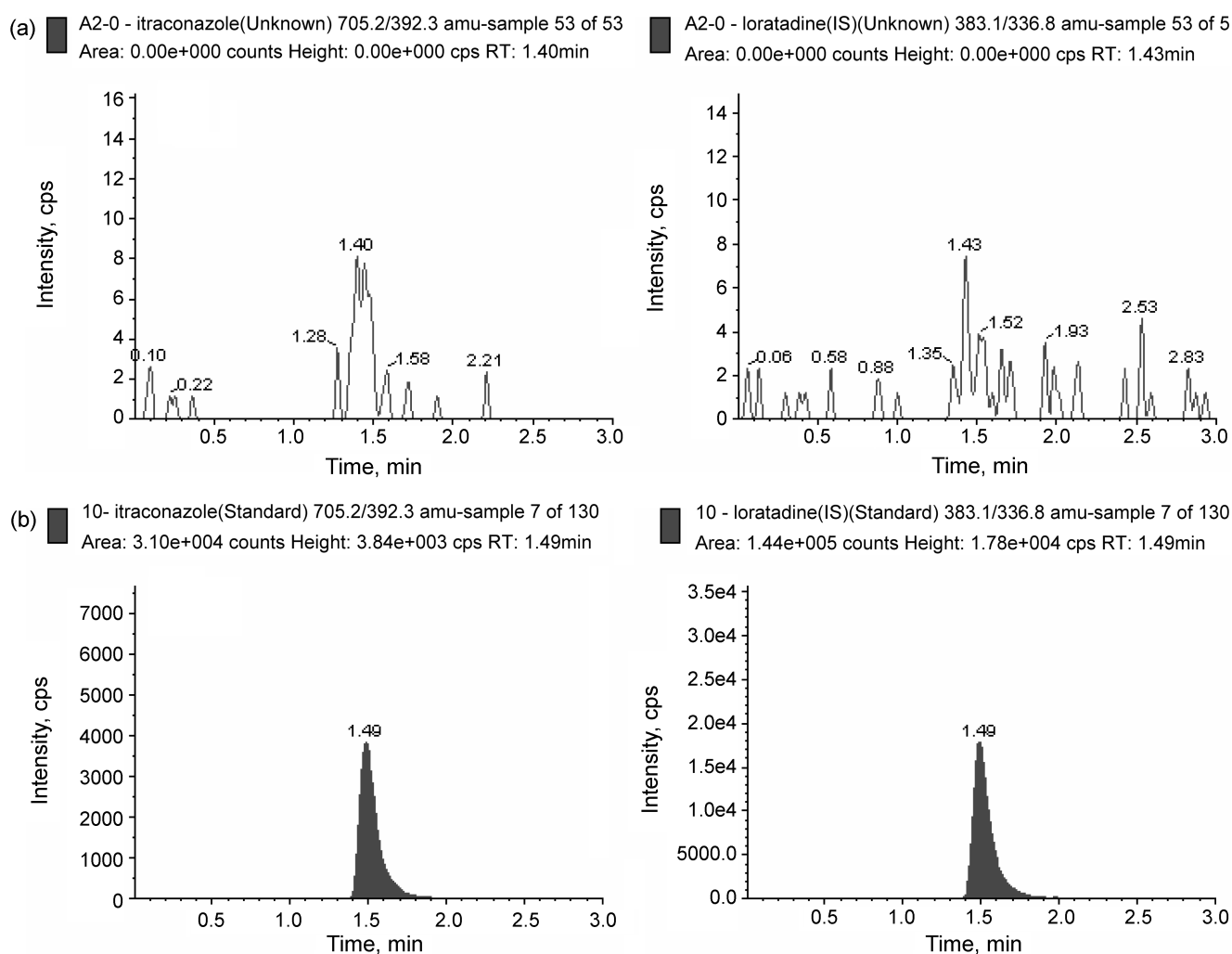


Figure 2. Chromatograms of (a) blank plasma sample and (b) itraconazole (10 ng/mL) and loratadine.

Canada). Electrospray ionization-mass spectrometry was performed in the positive mode using the following operating parameters: nebulizing gas, nitrogen; collision gas, nitrogen; ion spray temperature, 350 °C; and an ion spray voltage of 5.5 kV. System control and data evaluation were carried out using Analyst[®] (ver. 1.3). Multiple reaction monitoring was used to quantify itraconazole (m/z 705.2 \rightarrow 392.3) and loratadine (m/z 383.1 \rightarrow 336.8). Mass spectra of blank plasma and itraconazole are shown in Figure 2a and 2b.

Preparation of stock solutions, calibration standards and quality control samples. Primary stock solution of itraconazole (100 $\mu\text{g}/\text{mL}$) was prepared in methanol. The 100 $\mu\text{g}/\text{mL}$ solution was further diluted with methanol to 0.2, 0.5, 1, 2, 5, 10, 20, 50 and 100 ng/mL to prepare spiking standard solutions. Loratadine (internal standard) working stock solution was made upto 200 ng/mL in methanol. All solutions were stored at 4 °C until analysis. Calibration standards were freshly prepared before each measurement by adding 20 μL of the spiking standard solutions of 0.2, 0.5, 1, 2, 5, 10, 20, 50 and 100 ng/mL to 0.5 mL of blank human plasma.

Sample pre-treatment. A 20 μL of loratadine stock solution (200 ng/mL) was added to 0.5 mL of each plasma sample and vortex-mixed. The plasma was then maintained to be basic condition by addition of 50 μL of 1 M Na_2CO_3 . Itraconazole and loratadine were extracted with 1200 μL of heptane-isoamylalcohol (98 : 2, v/v) for 5 min on a shaker. The organic and aqueous layers were separated by centrifugation at 13,000 rpm for 10 min. The organic layer was transferred to another clean tube and evaporated. The residue was reconstituted in 100 μL of mobile phase. Samples were vortex-mixed for 30 s and centrifuged for 1 min at 3,000 rpm prior to loading onto the automated injector tray. The LC-MS-MS assays were performed with 10 μL injection.

Bioequivalence study design. The developed method was used to evaluate the bioequivalence of two tablet formulations of itraconazole in healthy volunteers: Han All Itraconazole tablets (test formulation from Han All Pharmaceutical Company, Seoul, Korea, lot 0599005) and Sporanox[®] tablets (reference formulation from Janssen Pharmaceutical Company, Seoul, Korea, lot 9931).

The bioequivalence protocol used was approved by the

Korean Food and Drug Administration. Thirty-one healthy male volunteers (one volunteer was dropped out due to drug intolerance), aged between 21 and 25 years, were selected for this study after clinically assessing their health status evaluation such as hematology, biochemistry, electrolytes, and urinalysis testing. The volunteers had the following clinical characteristics: age, 23.66 ± 1.15 years; height, 175.03 ± 3.12 cm; body weight, 66.72 ± 5.28 kg.

The study was based on a single dose, randomized, two-treatment, two-period crossover design. During phase 1 period, the volunteers were hospitalized at 18:00 h and had a normal evening meal, and then after an overnight fast they were administered (at 08:00 h) a single dose (itraconazole 100 mg) with 240 mL of water. Food and drinks were not allowed until 4 h after administration. Lunch and dinner were served at 4 and 9 h after administration. After a wash period of 14 days, the study was repeated in the same manner (phase 2) to complete the crossover design.

Heparinized blood samples (10 mL) were collected from a suitable forearm vein using an indwelling catheter into heparin-containing tubes before (0 h) and 1, 2, 3, 4, 5, 6, 7, 9, 12, 24, 48 and 72 h after dosing. The blood samples were centrifuged at 3,000 rpm for 10 min, and plasma samples were separated and stored at -70 °C until required for analysis.

Pharmacokinetic parameters including AUC_{72h} (the area under the plasma concentration versus time curve from time 0 to 72 h), C_{max} (peak plasma concentration), T_{max} (time to C_{max}), and $t_{1/2}$ (elimination half-life) were calculated using K-BE TEST 2002 supplied by the Korean Food and Drug Administration.

Results and Discussion

Assay specificity, selectivity, linearity and lower limit of quantification (LOQ). An assay specificity was assessed by extracting samples of six different batches of blank plasma and then comparing the resulting chromatograms for

Table 1. Intra- and inter-day coefficient of variation and accuracy for determination of itraconazole in human plasma (n=5)

Concentrations (ng/mL)	CV (Coefficient of Variation, %)		Accuracy (%)	
	Intra-Day (n = 5)	Inter-Day (n = 5)	Intra-Day (n = 5)	Inter-Day (n = 5)
0.2	12.95	11.68	111.30	114.40
0.5	12.42	11.60	96.60	96.84
5	12.55	14.88	88.60	86.40
50	10.56	11.21	85.16	95.16
100	11.72	13.70	85.52	94.06

plasma samples spiked with loratadine (IS), itraconazole at the lowest (0.2 ng/mL) or the highest concentration (100 ng/mL) of the calibration standards. The chromatograms were also inspected visually for interfering chromatographic peaks caused by endogenous substances. Figure 3 shows representative chromatograms of itraconazole (41.3 ng/mL) and loratadine (IS), obtained from a healthy volunteer's plasma sample at 2 h after the oral administration (Fig. 3).

Calibration curves were prepared using seven standards in the 0.2-100 ng/mL range. The slope and intercept values of two replicate calibration curves gave the following equation was $y = 0.0203x + 5.46e^{-5}$. The average regression coefficient was $r = 0.9990$ and LOQ for itraconazole was 0.2 ng/mL.

Intra-day repeatability and inter-day reproducibility. Precision and accuracy were determined by repeating the analysis of the standards five times in a single day at the concentrations of 0.2, 0.5, 5, 50 and 100 ng/mL (Table 1). Intra-day precision values were ranged from 10.56 to 12.95%, while accuracies were between 85.16 and 114.40%. Precision and accuracy values were determined on five different days at four different concentration levels. Even at the LOQ, the precision and accuracy values did not exceed 15%.

Bioequivalence study. The developed method was

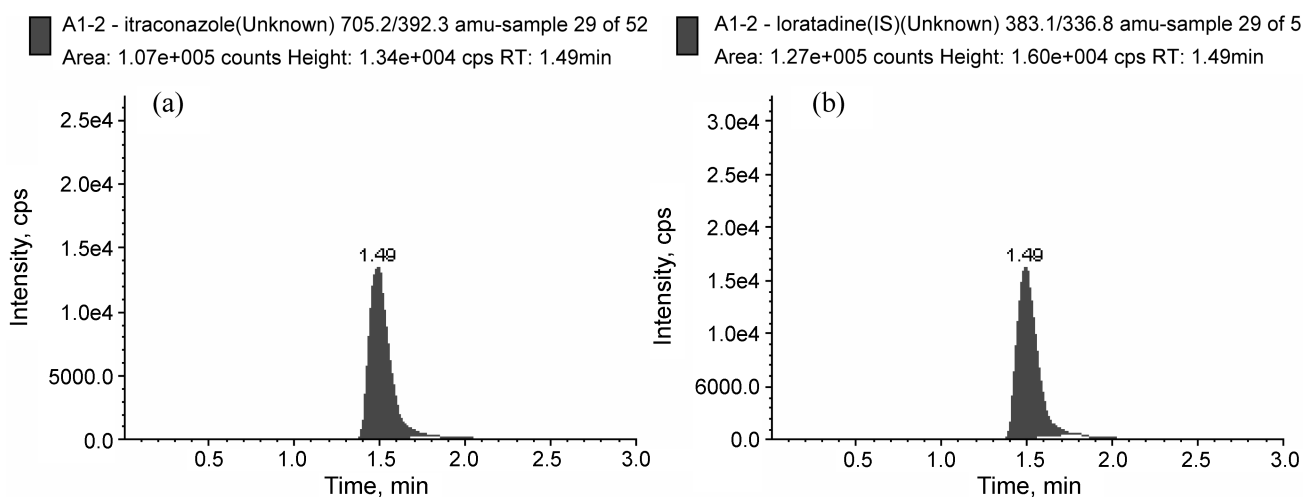


Figure 3. Chromatograms of a healthy volunteer's plasma sample 2 h after the oral administration. Panel (a) and (b) shows itraconazole and loratadine (internal standard), respectively. Calculated plasma concentration of itraconazole shown above is 41.3 ng/mL.

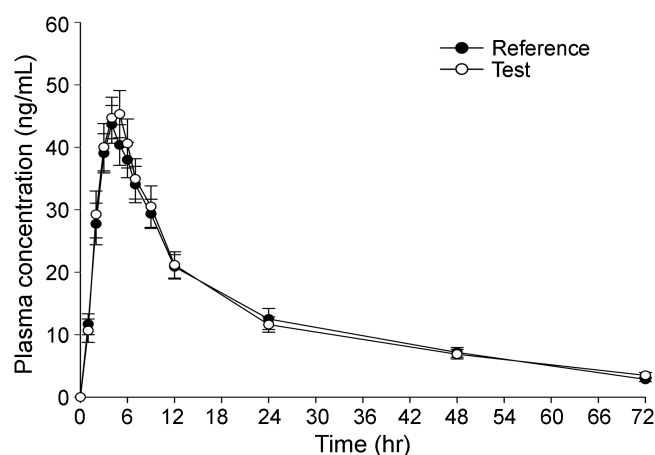


Figure 4. Plasma drug concentration-time curves of test and reference itraconazole formulations in healthy male volunteers ($n = 31$).

Table 2. Pharmacokinetic parameters for itraconazole of an oral dose of 100 mg of test (Han All Itraconazole tablet) and reference (Spornox[®] capsule) formulations, obtained from healthy human volunteers ($n = 31$)

Pharmacokinetic parameters	Itraconazole	
	test	Reference
T_{max} (h)	4.00 ± 1.36	4.35 ± 1.23
C_{max} (ng/mL)	49.90 ± 15.00	47.02 ± 13.54
AUC_{0-72} (ng h/mL)	891.00 ± 286.30	887.50 ± 320.40
$t_{1/2}$ (h)	24.13 ± 8.86	21.41 ± 4.71

successfully used for the bioequivalence study involved in thirty-one healthy male volunteers and pharmacokinetic parameters were determined up to 72 h after the oral administration. Plasma drug concentration-time curves are shown in Figure 4. Calculated kinetic parameters were listed in Table 2. All pharmacokinetic parameters were similar to those reported previously.

There were no significant differences for the kinetic parameters between the two formulations. Using data obtained

Table 3. Bioequivalence statistics

Dependent variable		Reference tablet	Test tablet	90% Confidence limit
Itraconazole	$\ln(AUC)$	6.73	6.74	94.47-108.35
	$\ln(C_{max})$	3.81	3.87	99.71-112.63

with logarithmically transformed, the 90% confidence intervals of test to reference ratio of the AUC_{0-72} , were within the bioequivalence range of 80-125% set by the Korean Food and Drug Administration, and that of C_{max} was within 80-125%. Therefore, two products were concluded to be bioequivalent (Table 3).

In conclusion, we have successfully developed and validated an HPLC-MS-MS method for determination of itraconazole in human plasma. The method uses liquid-liquid extraction of the drugs from human plasma and electrospray ionization mass spectrometry after reverse phase chromatographic separation. This assay is considered to be simple, accurate and reproducible. Also, this method has been successfully applied in a bioequivalence study of the itraconazole. The present method may also be used as a stability-indicating method to analyze itraconazole in pharmaceutical products.

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