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Journal of Chromatography B, 852 (2007) 174–179
JOURNAL OF CHROMATOGRAPHY B
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An optimized analytical method of fluconazole in human plasma by high-performance liquid chromatography with ultraviolet detection and its application to a bioequivalence study

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Received 25 August 2006; accepted 9 January 2007
Available online 18 January 2007

Abstract

A sensitive and accurate HPLC-UV method for the quantification of fluconazole (FLA) level in human plasma has been developed. The sample was prepared by one-step liquid–liquid extraction (LLE) of FLA from plasma using dichloromethane. Phenacetin was used as the internal standard. The chromatographic retention times of FLA and phenacetin were 4.6 and 8.3 min, respectively. The lower limit of quantitation (LLOQ) was 0.05 μg/mL, and no interferences were detected in the chromatograms. The devised HPLC-UV method was validated by evaluating its intra- and inter-day precisions and accuracies in a linear concentration range between 0.05 and 10.00 μg/mL. The devised method was successfully applied to a bioequivalence studies involving the oral administration of a single 150 mg FLA tablet and 3 × 50 mg FLA capsules in healthy Korean male volunteers.

Keywords: Fluconazole; High performance liquid chromatography (HPLC); Validation; Bioequivalence; Human plasma

1. Introduction

Fluconazole (FLA) [2-(2,4-difluorophenyl)-1, 3-*bis*-(1H-1, 2,4-triazole-1-yl)-2-propanol] (Fig. 1) is a triazole agent which is one of the most commonly prescribed systemic antifungals [1]. FLA is almost completely absorbed (>90%) from the gastrointestinal tract after oral administration, and about 11–12% of the drug in plasma is protein bound. The recommended dosage of FLA is generally 100–200 mg once daily, and the time to attain peak concentration in plasma is 1.7–4.3 h after single-dose drug administration. FLA has a long half-life (32 ± 5 h) and is excreted predominantly unchanged in urine (75 ± 9%) [2,3].

Several analytical methods for determining FLA levels in human plasma and serum are described in the literature. These include assays based on gas chromatography (GC) [4] and liquid chromatographic-tandem mass spectrometry (LC–MS/MS) [5,6]. Although both methods are highly sensitive, the GC method is laborious and unsuitable for routine analysis or the processing of large numbers of plasma samples. In addition, LC–MS/MS instruments are not readily available in all laboratories because of the high cost and the expensive apparatus required.

Several HPLC methods for determining FLA levels in human plasma have been described [7–16]. The majority utilize ultraviolet detection (UV) and a reversed-phase octadecylsilyl (C_{18}) column. However, existing methods have shortcomings in terms of pharmacokinetic studies, i.e., a narrow concentration range (LLOQ ≥ 0.2 μg/mL) [7–13], lengthy running times (>10.0 min) [7,9–11,13–16] and the lack of an internal standard (I.S.) [8–10,12,16]. Therefore, a sensitive and a short chromatographic run-times of HPLC-UV analytical method is required to quantify FLA levels in human plasma to support pharmacokinetic and bioequivalence studies.

This paper describes an HPLC-UV method to determine the plasma FLA concentrations. It utilizes liquid–liquid

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doi:10.1016/j.jchromb.2007.01.012

extraction (LLE) and can analyze over the concentration range of 0.05–10.00 μg/mL. Of the two previously reported ISs, UK-48134 [14] and phenacetin [11,13,15], the latter was used due to its commercial availability. The developed method was utilized in a bioequivalence study of a FLA tablet [1 × 150 mg] versus capsules [3 × 50 mg] administered orally to 24 healthy male Korean volunteers.

2. Experimental

2.1. Chemicals and reagents

FLA (Fig. 1a) and phenacetin (I.S.; Fig. 1b) were purchased from LKT Laboratories Inc. (St. Paul, MN, USA) and the Sigma–Aldrich (St. Louis, MO, USA), respectively. Dichloromethane and acetonitrile (HPLC grade) were obtained from J.T. Baker (Philipsburg, NJ, USA). Water was purified using a Milli-Q purification system (Millipore Corp., Bedford, MA, USA). All other chemicals and solvents were of the highest analytical grade available. The reference medication was Diflucan® [50 mg fluconazole capsule, Pfizer Pharm Korea Co. Ltd.] and the test medication was Neoconal® [150 mg fluconazole tablet, Taejoon Pharm. Co. Ltd. (Seoul, Korea)].

2.2. Calibration standards and quality control (QC) samples

A stock solution of FLA was prepared in water at 1000 μg/mL. Working solutions of FLA were prepared from stock solution by serial dilution with water to concentrations of 5, 10, 20, 50, 100, 200, 500, 1000 μg/mL. Phenacetin (I.S.) was also dissolved in water to prepare a stock solution (500 μg/mL) and this solution was further diluted with water to provide a working I.S. solution of concentration 50 μg/mL. Stock and working solutions were stored at 4 °C. Calibration standard samples were prepared by adding 100 μL of the FLA working solutions to 9.90 mL of drug-free human plasma to achieve final FLA concentrations of 0.05, 0.10, 0.20, 0.50, 1.00, 2.00, 5.00 and 10.00 μg/mL. QC samples were also prepared at three different concentrations (0.15 μg/mL (low), 4.00 μg/mL (medium) and 8.00 μg/mL (high)). These were stored as 500 μL aliquots at −70 °C.

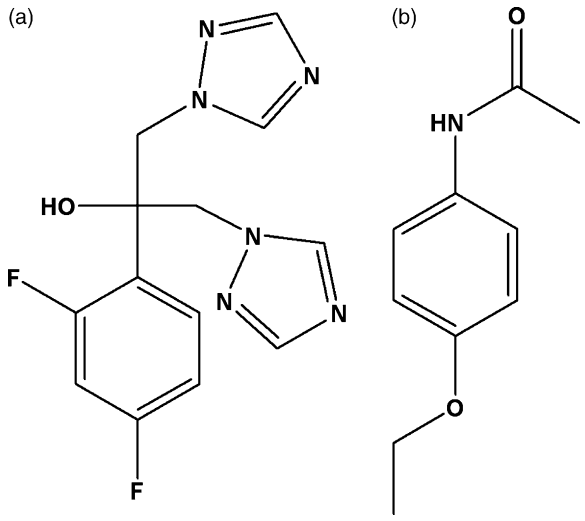

Fig. 1. Structures of: (a) fluconazole and (b) phenacetin (I.S.).

2.3. Chromatographic conditions

The HPLC system consisted of a Waters model 515 HPLC pump, a model 717 plus autosampler and a model 486 tunable absorbance detector set to 210 nm (Waters, Milford, MA, USA). Separations were achieved at 30 °C using a CAPCELL PAK UG 120 column (C_{18} reverse-phase, 250 mm × 4.6 mm I.D., 5 μm, Shiseido, Tokyo, Japan). The mobile phase used for analysis consisted of a mixture of acetonitrile and 10 mM sodium phosphate buffer (30:70, v/v). The mobile phase was adjusted to pH 5.7 with phosphoric acid, filtered through a 0.45 μm filter, and delivered at a rate of 1.0 mL/min. Chromatographic data were collected and analyzed using Empower Chromatography data software (Waters, Version 5.0).

2.4. Preparation of plasma samples

After thawing at room temperature, an aliquot of each sample (500 μL) was pipetted into a glass tube and 10 μL of I.S. working solution (50 μg/mL) was added. After vortexing briefly, 20 μL of 5 M sodium hydroxide and 5 mL of dichloromethane were added to each sample, shaken for 10 min, and then centrifuged for 10 min at 3000 rpm. The upper aqueous layer was then removed and 4 mL of the organic layer was evaporated to dryness using a nitrogen flow in a TurboVap LV (Caliper Life Sciences, Mountain View, CA, USA) evaporation system at 45 °C. The residue obtained was dissolved in 100 μL of mobile phase and vortexed for 10 min. After centrifugation (3000 rpm, 20 min), samples were transferred to autosampler vials and 50 μL aliquots were injected into the HPLC system.

2.5. Validation of the analytical method

2.5.1. Specificity

Blank samples obtained from six different volunteers, which were collected under controlled conditions, were subjected to the preparation procedure described above and evaluated to determine the extent to which endogenous plasma components may interfere with the chromatograph determinations of FLA or I.S. levels.

2.5.2. Calibration curves

Standard calibration curves were obtained using blank samples and eight prepared calibration samples covering the FLA range (0.05–10.00 μg/mL) by determining the peak area ratios of FLA versus I.S. FLA concentrations were calculated using an FLA/I.S. area ratio versus calibration curve. The linearity of the calibration curve was also examined, and was found to have a correlation coefficient (r^2) of 0.99 or better. The limit of detection (LOD) was defined a signal to noise ratio (S/N) of 3 and the LLOQ was defined as the concentration corresponding to an S/N of 10.

2.5.3. Precision and accuracy

Intra- and inter-day assay precisions were determined as relative standard deviations (R.S.D.), and intra- and inter-day assay accuracies were expressed as percentages of theoretical concentration, as accuracy (%) = (found concentration/theoretical concentration) × 100. Intra-day assays involved five replicates per day and inter-day assays were performed on 4 separate days. The acceptance criterion recommended by Food and Drug Administration (USFDA) for each back-calculated standard concentration is a 15% deviation from the theoretical value except at the LLOQ, which was set at 20% [17].

2.5.4. Recovery

Recovery of FLA in plasma was evaluated by comparing mean detector responses to different QC samples post-extraction with those prepared by adding FLA to post-extracted drug free plasma at the same nominal concentrations. The recovery of I.S. from plasma was evaluated using the same process.

2.5.5. Stability

The stability of FLA in human plasma was assessed by analyzing three QC samples, containing low (0.15 μg/mL), medium (4.00 μg/mL) and high (8.00 μg/mL) concentrations of FLA that had been freeze/thaw or stored for different time and temperatures. Results were compared with those of freshly prepared samples, and percentage concentration deviation was calculated. The protocol for the stability study included: (a) freeze–thaw stability, which was determined after three freeze (−70 °C)–thaw cycles on consecutive days, (b) short-term stability, which was determined by exposing samples to room temperature for 24 h, (c) long-term stability, which was determined after keeping the plasma samples frozen at −70 °C for 30 days, (d) post-preparative stability, which was determined after keeping the samples in auto-sampler at 4 °C for 24 h and (e) working solution stability, which was determined by exposing working solutions containing FLA or I.S. to room temperature for 6 h. The protocol was based on criteria recommended by USFDA [16].

2.6. Subjects and clinical procedure

The proposed analytical method was applied to a bioequivalence study. This study was carried out on a group of 24 healthy Korean male volunteers aged 23.9 ± 2.6 years old (range: 21–32 years) with an average body weight of 70.2 ± 7.5 kg (range: 56–85 kg) and an height of 175.0 ± 4.8 cm (range: 169–185 cm). All were informed beforehand of the content of the study and provided written consent. Based on medical history, clinical examinations, and laboratory investigations, no subject had any history or evidence of renal, hepatic, or gastrointestinal disease or drug allergy.

This study was an open-labeled, randomized, 2 × 2 crossover trial with a 2-week washout interval. Subjects were requested not to take any medication for 2 weeks before the study and to fast for at least 12 h (overnight) before each treatment. A single-dose of FLA (150 mg) consisting of three Diflucan® capsules (or one Neoconal® tablet) was orally administered to each subject in the fasting state. Fasting was continued for a further 4 h after drug administration. Heparinized blood samples (7 mL) were collected from a forearm vein according to a time schedule, which included a blank before drug administration and subsequent samplings at 0.25, 0.5, 1, 1.5, 2, 4, 8, 12, 24, 48, 72 and 120 h post-dosing. Immediately after sampling, the blood obtained was placed in an ice–water bath, centrifuged for 10 min at 3000 rpm, and plasma was stored at −70 °C until required for assay. This study was approved by the Korean Food and Drug Administration (KFDA) and Kyung-Hee University Ethical Committee before obtaining written consents from all volunteers.

2.7. Pharmacokinetic data and statistical analysis

The following pharmacokinetic values were determined for the period 0–120 h; the area under the plasma concentration–time curves from time zero to 120 h or infinity ($AUC_{0–120\,h}$, $AUC_{0–\infty}$), the maximum plasma concentration (C_{max}), the time required to reach C_{max} (T_{max}), the elimination rate constant (K_e) and the half-life ($T_{1/2}$). These values were computed using WinNonlin Professional Software (Version 3.0.1). Statistical analysis of bioequivalence parameters was carried out using K-BE test 2002 program (Version 1.2.1) for ln-transformed pharmacokinetic parameters C_{max}, $AUC_{0–120\,h}$ and $AUC_{0–\infty}$ [18]. Bioequivalence was assessed using a 90.0% confidence interval (C.I.) for the ln-transformed bioequivalence parameters, at an acceptable range of 0.80–1.25 [19,20].

3. Results and discussion

3.1. HPLC chromatogram

Under the experimental conditions described above, FLA and I.S. were well separated from endogenous materials. Representative chromatograms of a blank plasma sample, a plasma sample spiked with FLA and I.S., and a volunteer sample are shown in Fig. 2. No interference was found in the chromatograms of six randomly selected human plasma samples at the retention times of FLA or I.S., which were 4.6 and 8.3 min, respectively, and the total running time for each sample was 10.0 min. Although other analytical methods using short (<15 cm) column have been reported [12,13,15], the peaks of FLA or I.S. were not well resolved against the endogenous peaks from plasma, resulting in a long chromatographic run-time for separation. This method reduced the analysis time compared to the existing FLA method using I.S. (>11.8 min) [7,9,11,13–15].

3.2. Linearity and sensitivity

The linearity of detector response was assessed for various extracted plasma standards over the range of 0.05–10.00 μg/mL. The calibration curve exhibited excellent linearity and had a high correlation coefficient (Table 1). In addition, the curve showed great back-calculated precision and accuracy (Table 2). The LOD was determined to be 0.01 μg/mL and the LLOQ was 0.05 μg/mL. These levels are lower than those of the method of previous reports for HPLC-UV methods [7–16].

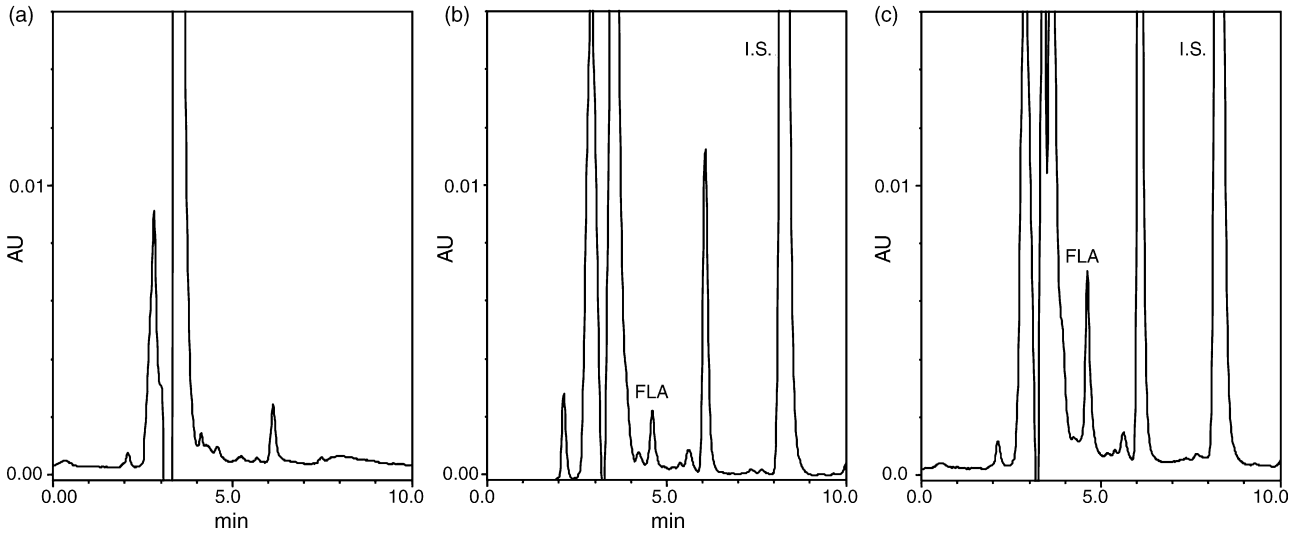

Fig. 2. Representative chromatograms of: (a) blank plasma, (b) plasma spiked with fluconazole (FLA, final plasma concentration: 0.05 μg/mL) and 20 μL of phenacetin (I.S., 40 μg/mL) and (c) plasma sampled from a volunteer (120 h after an oral administration of 150 mg fluconazole capsules; the plasma concentration of fluconazole corresponds to 0.19 μg/mL).

Table 1
Linearity obtained after regression analysis of the method for determining fluconazole in plasma samples

Number	Slope	Intercept	r^2
1	0.2842	0.0176	0.9993
2	0.2774	0.0082	0.9999
3	0.2821	−0.0021	0.9998
4	0.2902	0.0048	0.9999
5	0.3058	0.0021	0.9999
Mean	0.2879	0.0061	0.9998
S.D.	0.0110	0.0074	0.0003
S.E.	0.0049	0.0033	0.0001

3.3. Precision and accuracy

Precisions, which are represented as R.S.D. (%) at each concentration, were always <15%, with the exception of LLOQ (<20%) in the concentration range of 0.05–10.00 μg/mL. Accuracies were within 85–115%, except for LLOQ (range 80–120%). Intra-day precision ranged from 3.31 to 12.36%, with accuracy ranging from 100.48 to 117.61%, while inter-day precision ranged from 3.56 to 18.72%, with accuracy ranging from 98.38 to 107.96% (Table 3). Thus, the results obtained were reproducible and satisfied the USFDA criterion [17].

Table 2
Accuracy and precision of calibration standards of the method for determining the concentration of fluconazole in plasma samples ($n = 5$)

Theoretical concentration (μg/mL)	Concentration found (μg/mL; mean ± S.D.)	Accuracy (%)	Precision (CV%)
0.05	0.0491 ± 0.0077	98.2	15.7
0.10	0.1012 ± 0.0097	101.2	9.6
0.20	0.2100 ± 0.0197	105.0	9.4
0.50	0.5179 ± 0.0132	103.6	2.5
1.00	0.9983 ± 0.0463	99.8	4.6
2.00	2.0153 ± 0.0501	100.8	2.5
5.00	4.9252 ± 0.0325	98.5	0.7
10.00	10.0344 ± 0.0236	100.3	0.2

3.4. Recovery and stability

The recovery of FLA in the LLE procedure from 0.5 mL of plasma was measured at three different concentrations over the calibration range used. To reduce LLOQ and analysis time, LLE was performed using four different extraction solvents (hexane, diethyl ether, methyl *tert*-butyl-ether, dichloromethane). Table 4 shows the absolute recovery, expressed as a percentage, obtained for both FLA and I.S. Dichloromethane was chosen as the solvent because of the good analyte recoveries obtained. Regardless of the drug concentration, the recoveries ranged from 82.3 to 84.3% and no clear relationship was found between concentration and recovery. The recoveries of FLA and I.S. using dichloromethane were consistent and reproducible, which was satisfactory.

FLA stability values are presented in Table 5. No significant deterioration of analyte or I.S. was observed under any conditions. Thus, the described extraction procedure appears to avoid analyte degeneration.

Table 3
Intra- and inter-day assay precision and accuracy for HPLC assay of fluconazole in human plasma ($n = 5$ per test)

Theoretical concentration (μg/mL)	Precision (CV%)		Accuracy (%)	
	Intra-day	Inter-day	Intra-day	Inter-day
0.05	4.03	18.72	117.61	105.93
0.10	3.31	4.69	111.01	107.96
0.50	12.36	5.25	110.13	102.25
1.00	11.28	5.65	113.34	107.11
5.00	5.16	3.56	105.77	100.24
10.00	9.16	3.92	100.48	98.38

Table 4
Recovery data for fluconazole and I.S. ($n = 3$)

Theoretical concentration (μg/mL)	0.15	4.00	8.00	I.S.
n-Hexane (%)	15.86 ± 0.92	0.72 ± 0.03	0.42 ± 0.02	6.31 ± 0.48
Diethyl ether (%)	49.64 ± 3.96	33.36 ± 1.40	31.23 ± 2.04	63.18 ± 5.21
Methyl *tert*-butyl ether (%)	52.46 ± 2.85	43.20 ± 2.43	45.86 ± 1.80	79.58 ± 2.17
Dichloromethane (%)	82.30 ± 2.85	84.27 ± 5.72	83.57 ± 2.87	51.61 ± 7.82

Table 5
Stability data for fluconazole and I.S. ($n = 3$)

Theoretical concentration (μg/mL)	0.15 (low)	4.00 (medium)	8.00 (high)	I.S.
Freeze–thaw stability (three cycles, −70 °C, %)	96.25 ± 7.87	94.84 ± 9.44	102.08 ± 11.78	
Short-term stability (24 h, room temperature, %)	99.74 ± 3.46	81.01 ± 6.08	81.80 ± 3.64	
Long-term stability (30 days, −70 °C, %)	100.04 ± 4.67	99.86 ± 4.37	102.65 ± 8.18	
Post-preparative stability (24 h, 4 °C, %)	103.82 ± 7.82	102.07 ± 3.57	97.75 ± 3.26	103.91 ± 4.08
Working solution stability (8 h, room temperature, %)	101.22 ± 0.08	100.22 ± 0.12	100.28 ± 0.46	99.20 ± 1.54

Table 6
Mean pharmacokinetic parameters and 90.0% confidence interval for fluconazole after the administration of an oral dose of 150 mg of Diflucan® capsules (reference medication) and Neoconal® tablet (test medication) to healthy Korean male volunteers

Pharmacokinetic parameters	Diflucan®	Neoconal®	90.0% confidence interval
$AUC_{0–120\,h}$ (μg h/mL)	147.616 ± 34.520	145.061 ± 35.921	93.75–102.63
$AUC_{0–\infty}$ (μg h/mL)	161.112 ± 42.042	162.155 ± 50.380	93.97–105.95
C_{max} (μg/mL)	4.327 ± 0.956	3.923 ± 1.075	83.00–97.10
T_{max} (h)	1.417 ± 1.644	1.417 ± 1.477	
K_e (h^{-1})	0.022 ± 0.013	0.020 ± 0.008	
$T_{1/2}$ (h)	32.047 ± 2.901	35.170 ± 11.106	

3.5. Application to bioequivalence study

The devised HPLC method was applied to a bioequivalence study of 1 × 150 mg FLA tablet versus 3 × 50 mg FLA capsules in 24 healthy male Korean volunteers. The mean (±S.D.) plasma concentration–time profiles of FLA after these administrations are shown in Fig. 3. These profiles were then used to determine the comparative pharmacokinetic parameters of FLA (Table 6) for the two formulations. The absorption of FLA was rapid and unaffected by formulation type. Maximum absorption (C_{max}) was comparable for the two formulations and was achieved in approximately 1.5 h post-dosing. Comparable values were also obtained for AUC measurements. However, considerable inter-subject variability was shown by the 90% C.I. for C_{max} and AUCs. No significant sequence effect was observed for any bioavailability parameter, indicating that the crossover design performed as intended.

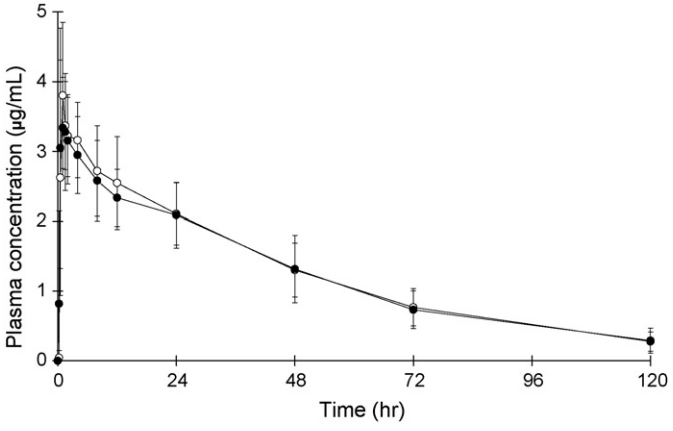

Fig. 3. Mean plasma concentration–time profile of fluconazole after the oral administration of 150 mg of fluconazole to 24 healthy Korean male volunteers. Points represent mean ± S.D. (◯, Diflucan® capsule; ●, Neoconal® tablet).

The test/reference confidence limit ratios for $AUC_{0–120\,h}$, $AUC_{0–\infty}$ and C_{max} were 93.75–102.63, 93.97–105.95 and 83.00–97.10%, respectively, for the 3 × 50 mg capsules versus the 1 × 150 mg tablets (Table 6). The 90.0% C.I.s for the ratio of test medication to reference were within the range of 80.0–125.0%, which is acceptable according to USFDA and KFDA [19,20]. Moreover, the pharmacokinetic values obtained during the present study were similar to those of previous reports from Thailand, Saudi Arabia and Serbia and Montenegro [5,8,11,16], indicating that the generic differences involved do not affect the disposition of FLA. Thus, the bioequivalence study results indicated that the absorption of the 1 × 150 mg FLA tablet is comparable to that of the 3 × 50 mg capsules.

4. Conclusion

Here, we describe a rapid and convenient method for the determination of FLA levels in human plasma. The developed method was fully validated and subsequently successfully applied to a bioequivalence study. The bioequivalence of the two

different formulations after oral administration in 24 healthy male volunteers was examined by monitoring FLA levels in plasma. The statistical analysis results based on comparisons of the three pivotal parameters ($AUC_{0–120\,h}$, $AUC_{0–\infty}$ and C_{max}) confirmed the bioequivalence of the two formulations and suggested that they can be prescribed interchangeably.

Acknowledgements

This work was supported by the Korean Science & Engineering Foundation (grant no. R13-2002-020-01002-0) and by the Seoul Research and Business Development Program (10524).

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