DEVELOPMENT AND APPLICATION OF SIMPLE HPLC-UV METHOD FOR FLUCONAZOLE QUANTIFICATION IN HUMAN PLASMA

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ABSTRACT

A simple and rapid high performance liquid chromatography method to quantify fluconazole in human plasma was described. The assay method involved a single step liquid-liquid extraction using dichloromethane. Tinidazole was used as internal standard. The separation was carried out using reversed phase C-18 column with UV detection at 260 nm. The mobile phase was comprised of buffer-acetonitrile (80:20, v/v) adjusted to pH 5.0 with glacial acetic acid. The mean recovery was 102.8% ± 5.53 for fluconazole and 78.27% ± 1.97 for tinidazole. The standard calibration curve was linear between 0.125 μg/mL and 10.0 μg/mL, with a correlation coefficient of 0.9958 ± 0.003. The drug in plasma was stable for 6 months when kept at -20°C. Tinidazole was stable in stock solution for 8 hours. The retention time was 8.3 min for fluconazole and 6.7 min for tinidazole, with a total sample run time of less than 10 min. The method was successfully applied to quantify fluconazole in plasma samples obtained from bioequivalence pilot study involving twelve healthy human volunteers.

Keywords:

INTRODUCTION

Fluconazole [2-(2,4-difluorophenyl)-1, 3-*bis*-(1H-1, 2,4-triazole-1-yl)-2-propanol] is a triazole anti-fungal drug used in the treatment of oropharyngeal, esophageal, or vulvovaginal candidiasis. It is also useful for the treatment of other serious systemic candida infections[1]. Fluconazole is also used to prevent yeast infections in patients who are likely to become infected because they are being treated with chemotherapy or radiation therapy before a bone marrow transplant[2]. The drug has an oral bioavailability of more than 90%[3]. Fluconazole is a highly selective inhibitor of fungal cytochrome P450 sterol C-14 (x-demethylation). Hence, the drug acts by inhibiting the synthesis of ergosterol and thus blocking the formation of the cell membrane[4].

Analytical methods for quantification of fluconazole in human plasma have been reported[5-13]. Nevertheless, there are limitations with the reported methods. These include lengthy running time[5], lack of sensitivity[6-7] and the use of expensive gas-chromatography and liquid chromatography-mass spectroscopy/mass-spectroscopy (LC-MS/MS) instruments which are not readily available in most of the analytical laboratories[8-11,13]. In the method of Kim et al[12], the recovery of fluconazole was approximately 82% and the fluconazole peak was interfered by the peak of plasma endogenous compound.

The purpose of this study was to develop a simple and rapid high performance liquid chromatography method to quantify fluconazole in human plasma. The method was applied in bioequivalence pilot study of fluconazole involving twelve healthy volunteers. The method showed improved recovery and better separation than the method of Kim et al[12]. Tinidazole was used as an internal standard. The molecular structure of fluconazole and tinidazole are shown in Figures 1 and 2.
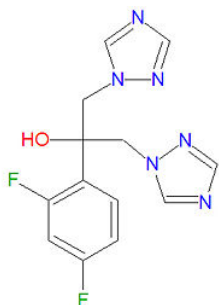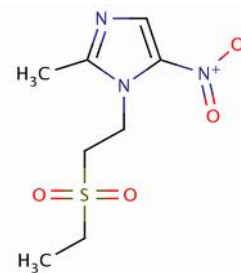

Fig. 1: Molecular structure of fluconazole.

Fig. 2: Molecular structure of tinidazole.

MATERIALS AND METHOD

Reagents and Chemicals

Fluconazole and tinidazole working standards were supplied by Yung Shin Pharmaceutical, (Selangor, Malaysia). Dichloromethane and sodium hydroxide were purchased from R&M Marketting, (Essex, England). Methanol and acetonitrile (HPLC grade) were obtained from J.T. Baker (Philipsburg, USA). Diflucan and Fukole capsule was supplied by Yung Shin Pharmaceutical Industries (Selangor, Malaysia).

Equipment

The HPLC system was comprised of a Shimadzu (VP series, Kyoto, Japan) pump (LC-10AT vp/FCV-10AL-vp) with solvent cabinet, an auto-injector (SIL-10AD vp), UV/VIS detector (SPD-20AD vp), and a computer software (VP-CLASS). Chromatographic separation was achieved using C18 (250 x 4.6 mm ID, 5μm) Agilent Eclipse Plus analytical column (Agilent, USA) fitted with guard column (Zorbax Eclipse Plus) packed with replaceable C-18 cartridge (12.5 x 4.6 mm ID, 5μm).

Sample preparation

A 1 mL aliquot of plasma was measured accurately into a glass tube, followed by the addition of 50 μL of 20 μg/mL tinidazole (internal standard), 25 μL of 6M sodium hydroxide and 5 mL of dichloromethane. The content was vortexed for 1 min and centrifuged at 4000 rpm for 15 min. The organic layer was transferred into a reactivial and evaporated to dryness at 45 °C under a gentle stream of nitrogen gas. The residue was reconstituted with 200 μL of mobile phase and vortexed for 1 min. The solution was then transferred into glass vial and transferred to the autosampler tray. 100 μL of sample was injected into the column.

Chromatography

The mobile phase consisted of a mixture of 0.01M sodium acetate buffer and acetonitrile (80:20, v/v) adjusted to pH 5.0 with glacial acetic acid. The analysis was run at a flow-rate of 1.2 mL/min. The concentration of fluconazole in the plasma was determined at a detection wavelength of 260 nm. The mobile phase was filtered with 0.45 μm nylon membrane (Whatman, UK) under vacuum suction. The samples were quantified using peak area ratio of fluconazole to internal standard.

Preparation of standard solutions

Fluconazole stock solution of 0.1 mg/mL was prepared by dissolving 5mg of fluconazole in 50 mL of methanol. Internal standard stock solution of 0.1 mg/mL was prepared by dissolving 5mg of tinidazole in 50 mL of methanol. It was further diluted with methanol to obtain a concentration of 20 μg/mL.

Preparation of standard plasma samples

The standard plasma samples were prepared by spiking drug-free plasma with known amount of fluconazole at concentrations from 125 ng/mL to 10 μg/mL[14].

Linearity

The linearity of fluconazole standards was evaluated by analysing a set of standards ranging from 0.125 to 10.0 μg/mL. The set of standard was replicated 5 times to get the mean linearity. Linear regression analysis of the calibration data was performed using the equation $y = mx + b$ where y was the peak area ratio of fluconazole over tinidazole, x the concentration of fluconazole, m and b respectively the slope and intercept of the curve[15]. Unknown concentrations were computed from the linear regression equation of the peak ratio of fluconazole over tinidazole against fluconazole concentration for the calibration curve.

Limit of quantification (LOQ)

The lowest standard on the calibration curve was taken as the limit of quantification. The analyte response at LOQ was at a signal to noise ratio of 5:1.

Accuracy, precision and recovery

The accuracy and precision of fluconazole were evaluated using 0.125 μg/mL (LOQ) and three quality control samples, at 0.5, 2.0 and 8.0 μg/mL. Accuracy describes the closeness of mean test results obtained to the true value of analyte. Six samples of each concentration were prepared and the analysis was run for 6 days for inter-day accuracy. For intra-day accuracy, six replicates of each analyte concentration were injected within the same day. Accuracy is presented in % CV and according to FDA regulations; the % CV of each concentration must always within the range of ± 15%, except for LOQ, which is within 20%.

Precision describes the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of a single homogenous volume of biological matrix. The between-day precision was determined in six replicates over six days. The within-day precision was determined at the same concentrations, in six replicates within the same day. Precision is presented in % CV and according to FDA regulation; the % CV of each concentration must always within the range of ± 15%, except for LOQ, which is within 20%.

The recovery for fluconazole and tinidazole (5 ug/mL) was calculated by comparing the peak area obtained from the plasma sample after treatment with that of the aqueous drug solution of corresponding concentration.

Stability study

Freeze and thaw stability

The analyte stability was determined after three freeze and thaw cycles. Three aliquots at each of the low and high concentrations were stored at -20℃ for 24 hours and thaw unassisted at room temperature of 26℃. When completely thawed, the samples were

refrozen for 24 hours under the same conditions. The freeze-thaw cycles were repeated two more times, and analyzed on the third cycle.

Short-term temperature stability

Three aliquots of each of the low and high concentrations were thawed at room temperature of 26℃ and kept at this temperature for 8 hours.

Stock solution stability of drug and internal standard

The stability of stock solutions of drug and the internal standard was evaluated at room temperature of 26 ℃ for 8 hours.

Long-term stability

Long-term stability was assessed by storing samples at -20℃ for 6 months. The stability of fluconazole in human plasma when stored at −20℃ was evaluated by assaying three aliquots of each of the lower and higher concentrations on day 1 and last day of 6-month storage time.

Bioequivalent pilot study

The study protocol was approved by the Joint Ethics Committee of School of Pharmaceutical Sciences, USM and Hospital Lam Wah Ee on Bioequivalent Studies. This was a single-dose, fasting, open-label, randomized, two-sequence, two-period, two-treatment crossover study involving twelve volunteers. Two oral capsule formulations, Diflucan® (reference formulation) and Fukole® (test formulation), both containing 150 mg of fluconazole were compared. The volunteers were administered two capsules of both products. After a washout period of two weeks, the volunteers were crossed over and received the alternate products. Venous blood samples were collected prior to dosing and at 0.25, 0.50, 0.75, 1, 2, 3, 4, 6, 12, 24, 36, 48, 60, 72, 96, 120 and 144 hours after drug administration. The plasma samples were centrifuged at 3500 rpm for 15 min. The supernatant was transferred to blank tube and stored at -70°C until further analysis.

RESULTS AND DISCUSSION

HPLC Chromatogram

Using the chromatographic condition as described, fluconazole and tinidazole were well separated. Fluconazole had a retention time of 8.34 min, whereas tinidazole was 6.75 min. Figure 3 shows the chromatogram of blank plasma, while Fig. 4 shows the chromatogram of blank plasma spiked with 10 μg/mL of fluconazole and 20 μg/mL of tinidazole. No interference was found in the chromatograms of six randomly selected human plasma samples at the retention times of fluconazole and tinidazole. The total run time was 9.5 minute. The method was simple, rapid and hence suitable to be applied to analyze large batch of blood samples.

In the method of Kim et al. [12], the analyte peak was interfered by the peak of endogenous compound. The fluconazole peak was not completely separated from the plasma endogenous compound peak. In the present study, the fluconazole peak was not interfered by the peak of plasma endogenous compound. This could be attributed to the use of a higher molarity of sodium hydroxide and different mobile phase in the present study.

Linearity

The results of linearity of 5 standard calibration curves are presented in Table 1. The mean standard calibration curve was linear from the range of 125 ng/mL - 10.0 μg/mL, with mean r^2 of 0.9958. The linear regression equation could be described by, $y = 0.2058 x + 0.0234$.

Accuracy, precision and linearity

The results of accuracy are presented in Table 2. The between-day accuracy values ranged between from -0.885 to -5.47% for the three quality control samples and -10.67% for LOQ. The within-day accuracy value ranged between -8.77 to 6.32% for the three quality control samples and 19.15% for LOQ.
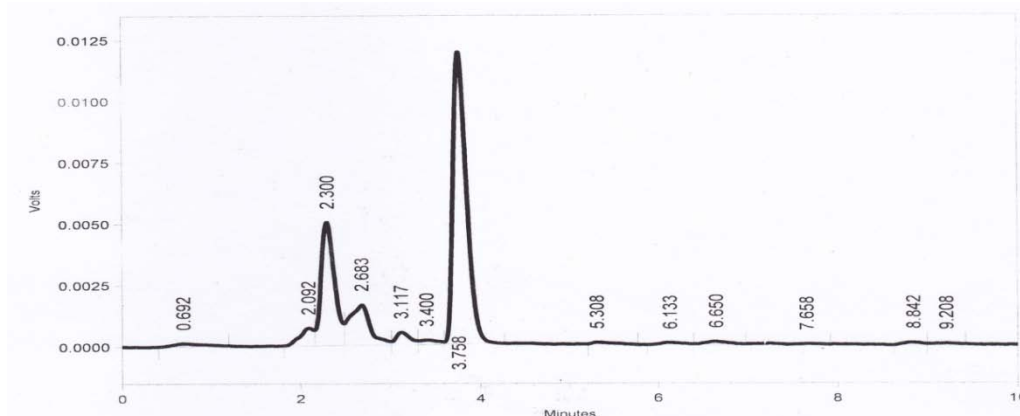
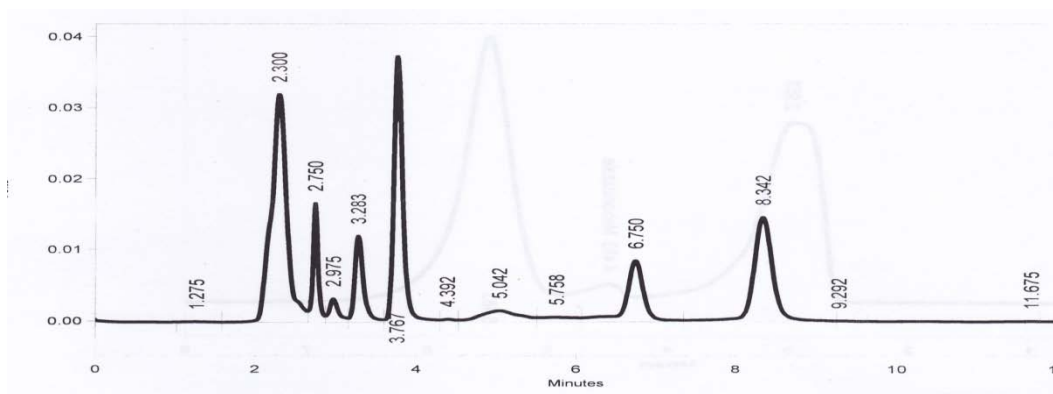**Fig. 3: Chromatogram of blank human plasma.**

Fig. 4: Chromatogram of human plasma spiked with 10 µg/mL of fluconazole (retention time, 8.34 min) and 20 µg/mL of tinidazole (retention time, 6.75 min)

Table 1: Results of Linearity.

S. No.	Slope	Intercept	r²
1	0.0219	0.0036	0.9988
2	0.2574	0.0549	0.9986
3	0.2556	0.0167	0.9962
4	0.2517	0.0389	0.9922
5	0.2422	0.0022	0.9930
Mean	0.2058	0.0233	0.9958
Stdev	0.1029	0.0230	0.0031

Table 2: Results of precision and accuracy

Concentration (µg/ml)	Between-day Mean ± Stdev (%)	Precision (%)	Accuracy (%Error)	Within-day Mean ± Stdev (%)	Precision (%)	Accuracy (%Error)
8.00	7.93 ± 0.27	3.35	-0.89	8.03 ± 0.15	1.83	0.41
2.00	1.90 ± 0.11	5.89	-5.10	1.83 ± 0.09	4.64	-8.77
0.25	0.24 ± 0.03	12.26	-5.47	0.27 ± 0.02	5.49	6.32
0.125	0.11 ± 0.02	18.07	-10.67	0.15 ± 0.01	7.54	19.15

Mean ± Stdev, N = 6.

The results of precision are presented in Table 2. The between-day precision values were between 3.35 to 12.26% for the three quality control samples and 18.07% for LOQ. The within-day precision ranged between 1.83 to 5.49% and 7.54% for LOQ.

The results of extraction recovery are presented in Table 3. The recovery values of the three quality control samples ranged between 103.24% to 104.53% and 99.53% for LOQ. The data of precision, accuracy and recovery showed that the assay method is highly reproducible.

Stability Study

The stability data are presented in Table 4. For freeze-thaw stability at low and high concentration quality control samples, the % remaining was 112.78% and 101.52% respectively. For short term stability, the % remaining values were 97.35% and 98.34% respectively, revealing that the samples were stable when kept under room temperature for 8 hours. For long term stability, the % remaining values were 104.66% and 99.56% respectively, suggesting that the samples were stable when kept frozen for 6 months at -20°C.

Table 3: Result of recovery.

Drug	Concentration (µg/mL)	Mean Recovery ± Stdev (%)
Fluconazole	0.125	99.53 ± 7.06
	0.25	103.88 ± 4.03
	2.00	103.24 ± 4.97
	8.00	104.53 ± 6.07
Tinidazole	0.50	78.27 ± 1.97

Mean ± Stdev, N = 6.

Table 4: Results of stability study.

Stability study	Concentration (µg/mL)	Mean ± Stdev (% remaining)
Freeze-thaw stability 3 cycles	0.25	112.78 ± 1.28
	8.00	101.52 ± 2.87
Short-term stability	0.25	97.35 ± 6.67
	8.00	98.34 ± 3.45
Fluconazole stock solution stability	100.00	104.83 ± 2.76
Tinidazole stock solution stability	20.00	103.43 ± 3.85
Long-term stability	0.25	104.66 ± 10.66
	8.00	99.56 ± 0.40

N = 3

Bioavailability study

The validated HPLC assay was successfully applied in bioequivalence pilot study involving twelve healthy volunteers. The mean plasma concentration profiles after oral administration of the two products, Fukole and Diflucan, are presented in Figure 5. The average Cmax, AUC$_{0-48}$ and Tmax for Diflucan were 6.65 ± 1.13 µg/mL, 272.72 ± 52.42 µg*hr / mL, and 1.52 ± 0.86 hour respectively, while for Fukole were 6.16 ± 1.80 µg/mL, 255.12 ± 85.57 µg*hr / mL, and 1.56 ± 0.99 hour respectively. The Cmax value obtained in the pilot study was calculated to be 48 times higher than the LOQ value. In addition, at 144 hours (which was approximately 5 half-life of fluconazole) after drug dosing, the drug was detected in the plasma at concentration above LOQ. Hence, the assay method could be applied for the analysis of fluconazole in pharmacokinetic and bioequivalence studies.
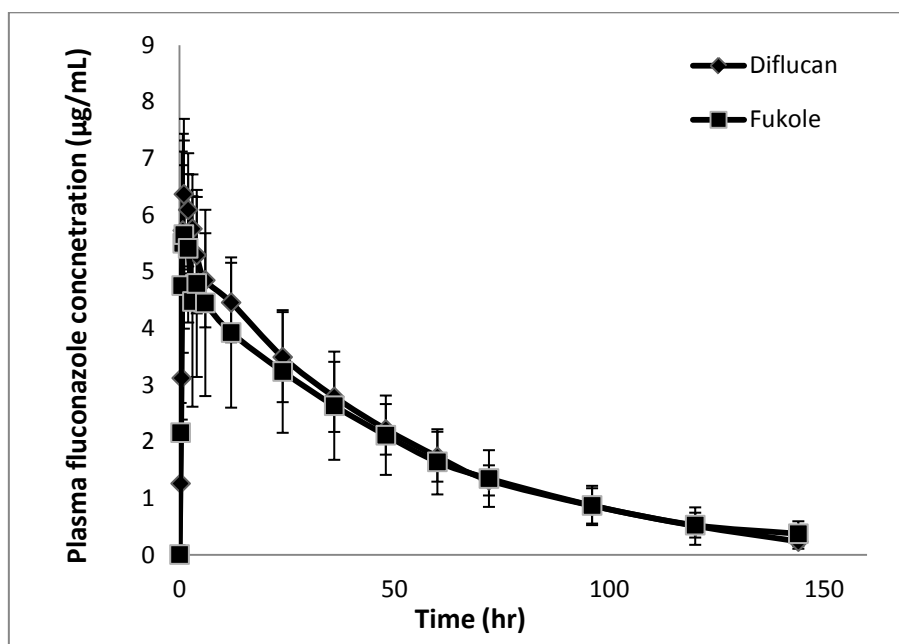

Fig. 5: Mean plasma fluconazole concentration profiles after dosing with Difflucan and Fukole capsules. Mean ± SD, N = 12.

CONCLUSION

In conclusion, this report presented a rapid, simple, and fully validated HPLC assay method to quantify fluconazole in human plasma using tinidazole as internal standard. The HPLC assay method was successfully applied to quantify fluconoazole in bioequivalence pilot study.

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