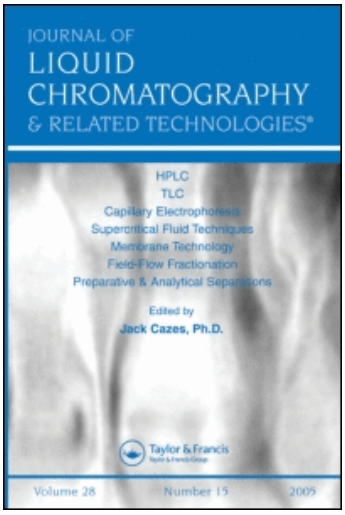

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HPLC Assay of Fluconazole and Its Application to Patients with Early Septic Shock

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HPLC Assay of Fluconazole and Its Application to Patients with Early Septic Shock

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Abstract: This work describes a precise, simple, and accurate HPLC method for the determination of fluconazole in plasma samples with enough sensitivity to monitor patients suffering from early septic shock for a month. The separation was done using an HPLC system with UV absorbance detector (set at 262 nm). It was performed using a μ Bondapak C_{18} column ($3.9 \times 150\,mm^2$) packed with 10 μm spherical particles. The mobile phase consisted of acetonitrile (20%) in 0.05 M ammonium acetate containing 0.1% triethylamine; acetic acid was used to adjust the pH to 7. Sample run time was 15 min. Metoclopramide was used as the assay internal standard (IS). Using the chromatographic conditions described, fluconazole and metoclopramide were well resolved with mean retention times of 6.7 and 10.2 min, respectively. Linear response ($r > 0.996$) was observed over the range of 0.2–15 μg/mL of fluconazole. There was no significant difference ($p > 0.05$) between inter- and intra-day studies for fluconazole. The mean relative standard deviation (RSD%) of the results of within-day precision and accuracy of the drug was $\leq$7.6%, which confirmed the reproducibility of the assay method. The applicability of the assay was demonstrated in measuring fluconazole pharmacokinetics in human plasma after daily intravenous infusion of 200 mg fluconazole to patient with early septic shock. Re-analysis of samples several weeks after the initial analysis showed no loss in fluconazole. Patient with early septic shock showed a mean $t_{1/2}$ of

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fluconazole of 12.3 ± 9 h after a single dose and 41.3 ± 20 h after multiple administrations.

Keywords: Fluconazole, HPLC, pharmacokinetics, metoclopramide, methods validations, patients, early septic shock

INTRODUCTION

Fluconazole is a fluorine-substituted bis-triazole antimycotic agent with demonstrated activity against a host of superficial and invasive fungal infection. It has been approved for oral and parental use in the treatment of fungal infection.[1] It is given for fungal infection in AIDS patients and mycosis.[2] Fluconazole is a water soluble, low molecular weight compound with limited plasma binding and long elimination half-life. The time to reach steady state with once daily dosage is approximately 7 days, with an elimination half-life of 30 h.[1,2]

Ketoconazole has been shown to be a useful prophylaxis in the development of the acute respiratory distress syndrome (ARDS) and to be able to reduce mortality.[3] Ketoconazole has been shown to prevent ARDS and decrease the mortality in high risk, septic patients.[4] The initial work on ketoconazole has been in a recent protocol study.[5] The precise action of ketoconazole remains speculative in regard to the reduction of mortality and ARDS. Ketoconazole, although an imidazole molecule used as an antifungal agent, has been shown to selectively block thromboxane synthesetase.[6] Because ketoconazole cannot be administered intravenously, Jacobs et al.[7] decided to test a closely related drug, fluconazole, that is suitable for intravenous administration and less hepatotoxic in patients suffering from early septic shock.[7] Unlike ketoconazole, fluconazole is not a thromboxane inhibitor.[8,9] Fluconazole has been shown (in vitro) to increase the bactericidal activity of neutrophils although having minimal inherent bactericidal activity. Zervos et al.[10] demonstrated that fluconazole has no effect on cytokine protein or mRNA levels. It has also been demonstrated that fluconazole is a weak inhibitor of inducible nitric oxide activity in mouse and human cells.[11] Therefore, fluconazole might be a useful drug to reduce organ dysfunction in patients in early septic shock. Also, fluconazole might protect these patients with early septic shock from deterioration in their organ dysfunction compared with patients not receiving fluconazole.[7]

Pharmacokinetics of fluconazole has been examined after a single dose and repeated doses administered to healthy subjects,[12–14] immunocompromised patients,[15] obese, burn, septic patients,[16] and in bone marrow transplant (BMT) patients.[17] However, limited data are available following multiple doses and, to our knowledge, none existing in patients with septic shock. To investigate the fluconazole in this septic shock population, an

assay to adjust the dose and to calculate the pharmacokinetic parameters of the drug for this population is necessary.

Several assay methods for fluconazole quantitative determination in body fluids are available.[18–24] Some used gas chromatography (GC) with electron capture detection[18] or nitrogen selective detection[19] and others utilized bioassays;[20–22] and some groups used high-performance liquid chromatography (HPLC).[23,24] The reported methods have some disadvantages. In the GC method, the column has to be pre-treated with benzoyl chloride to avoid fluconazole being adsorbed to the column and, additionally, a time consuming extraction procedure is employed. There was no facility in our laboratory for the reported bioassays. On the other hand, the cited HPLC method has the disadvantage that some assays used high buffer concentrations, high concentration of IS, or solid phase extraction, which resulted in high RSD% when applied in our laboratory.

Multiorgan dysfunction, often in association with severe sepsis or septic shock, is associated with high morbidity and mortality (40–100%) and long hospital stays.[25,26] Mortality is related to the degree of organ dysfunction involved.[25,27] The exact pathphysiology of organ dysfunction related to sepsis is still not well established. Fluconazole given intravenously has recently been demonstrated to reduce the development of multiorgan dysfunction and mortality in early septic shock <24 h.[7] The mechanism of action of fluconazole in reducing multiorgan dysfunction may be due to its ability to increase the bactericidal activity of neutrophils through a non-cytokines mediated pathway.[10] The approved protocol for the fluconazole study in Riyadh Military Hospital was in need of a biological assay to measure fluconazole in patients with early septic shock, to determine its pharmacokinetics in this patient population. Therefore, this study describes a precise, reproducible, simple, and rapid HPLC method for determination of fluconazole in plasma samples, with enough sensitivity to monitor patients suffering from early septic shock. It should be mentioned that the complete patients' pharmacokinetics and pharmacodynamics studies will be published elsewhere in the near future.

EXPERIMENTAL

Materials

Sample, Reagents, and Solvents

Fluconazole powder was obtained from Pfizer (Amboise, France). Metoclopramide was kindly supplied by Saudi Pharmaceutical Industries and Medical Appliances Corporation (SPIMACO, Saudi Arabia). All other reagents and chemicals were analytical grade, and used as received.

Instrumentation

A Waters HPLC system was equipped with a Waters 484 variable UV absorbance detector, and a Waters 717 plus autosampler. Waters 501 solvent delivery system was used to operate the gradient flow through a μ Bondapak C18 column (3.9 × 150 mm) packed with 10 μm spherical particles. Flow rate was monitored by Waters automated gradient controller. The initial flow rate was 1.2 mL/min for 6 min and the flow was increased to 2 mL/min within 0.5 min after 5 min flow rate was reduced to its initial value. Sample run time was 15 min. Chromatograms were recorded on a Waters 746 Data Module integrator chart. The HPLC system was operated at ambient temperature.

Methods

Analytical Conditions

The mobile phase consisted of acetonitrile (20%) in 0.05 M ammonium acetate containing 0.1% triethylamine; acetic acid was used to adjust the pH to 7 before the addition of acetonitrile. The mobile phase was prepared fresh each day. Degassing was achieved by filtration through a 0.22 μm Millipore membrane filter and sonication for 10 min. The injection volume was 75–100 μL. The detection was made at a wavelength of 262 nm. All analyses were performed at ambient temperature.

Preparation of Standard Solutions

Stock solutions of fluconazole and metoclopramide were prepared in methanol and stored in 4 mL amber glass vials at −20°C until used. Metoclopramide was used as the assay IS. Daily dilutions were made in HPLC quality water to give fluconazole concentrations of 0.2–15 μg/mL in human plasma, and a constant concentration of 0.4 μg/mL of the IS.

Drug Analysis

One milliliter of blank human plasma was spiked with 0.2–15 μg of fluconazole and 0.4 μg (25 μL) of IS in a 15 mL screw-capped test tube fitted with a polyteflon-lined cap. Fifty microliters of isopropyl alcohol was added and the tube was vortexed for about 10 s, then 100 μL of 0.1 N NaOH was added and the tube was vortexed for about 20 s. The solution was mixed with 5 mL of dichloromethane, vortexed at high speed for 2 min, and centrifuged at 4000 rpm for 5 min. The aqueous layer was aspirated to a waste and the organic layer was transferred to a clean tube. The tube containing the organic layer was placed in a water bath (50°C) and evaporated to dryness

under a stream of nitrogen. The residue was reconstituted in 150 μL of mobile phase prior to injection into the chromatograph for analysis.

Determination of Accuracy, Precision, Reproducibility, and Recovery

To assess the accuracy and precision of the within-day assay, six extractions of plasma samples of fluconazole, at the following concentrations of 0.2–15 μg/mL, were performed on a single day. The reproducibility of the assay (within-day and between-day) was evaluated by comparing the linear regression analysis of three standard plots obtained from spiked human plasma samples, at three different days, over a 2 month period. The recovery of each drug was assessed by extracting plasma specimens spiked to contain drug concentrations from 0.2 to 15 μg/mL. The peak area ratio (fluconazole/IS) was then compared with the peak area ratio for aqueous standards containing equivalent amounts of the drug and IS without extraction.

Patients and Blood Samples Collection

Patients are included in the study according to the inclusion and exclusion criteria approved by the Ethics Committee in Riyadh Military Hospital (Table 1). Patients received 200 mg/day fluconazole via intravenous infusion over 1 h. Blood samples (5 mL) were collected in heparenized tubes at the specified intervals, according to an approved protocol and upon the clinician's request. Plasma samples were immediately separated by centrifugation at 4000 rpm for 15 min and stored at −70 till assayed as mentioned previously. It should be mentioned that none of the patients received metoclopramide.

Data and Statistical Analysis

All results are expressed as mean ± SD. The RSD% was calculated for all values. The elimination half-life ($t_{1/2}$) was determined from the last two points after a single dose, and at a steady state, within the dosing intervals.

Table 1. Inclusion and exclusion criteria for patient's collection of blood samples

Inclusive criteria	Exclusive criteria
Admitted to ICU	Pregnant women
Known to have peritonitis	Children <16-year-old
With early septic shock (<24 h)	Cirrhosis of the liver
	An underlying malignancy

C_{max} is the maximum concentration at a steady state, C_{min} is the minimum concentration at a steady state, and accumulation index (R_{ac}) = $C_{max}/C_{max,1}$, where $C_{max,1}$ is the maximum concentration after the first dose.

The student *t*-test was used to examine the concentration difference at each day, and one-way analysis of variance (ANOVA) was employed to evaluate the reproducibility of the assay. The level of confidence was 95%.

RESULTS AND DISCUSSION

Figure 1 shows representative chromatograms of extracted drug-free plasma taken from a patient just before receiving the first dose of fluconazole, blank (A), the same plasma samples spiked with 5 μg/mL fluconazole and 0.4 μg/mL IS (B), and plasma taken from a patient 1 h after day 7 of the daily dosing of fluconazole intravenous infusion of 200 mg and also spiked with 0.4 μg/mL IS (C). A comparison of Figure 1A with 1B indicates that fluconazole peaks are free from matrix interference. Therefore, concomitant drug administrations showed no interference with fluconazole detection in all studied patients ($n = 18$ patients). Using the chromatographic conditions described, fluconazole and metoclopramide were well resolved with mean retention times of 6.7 and 10.2 min, respectively.

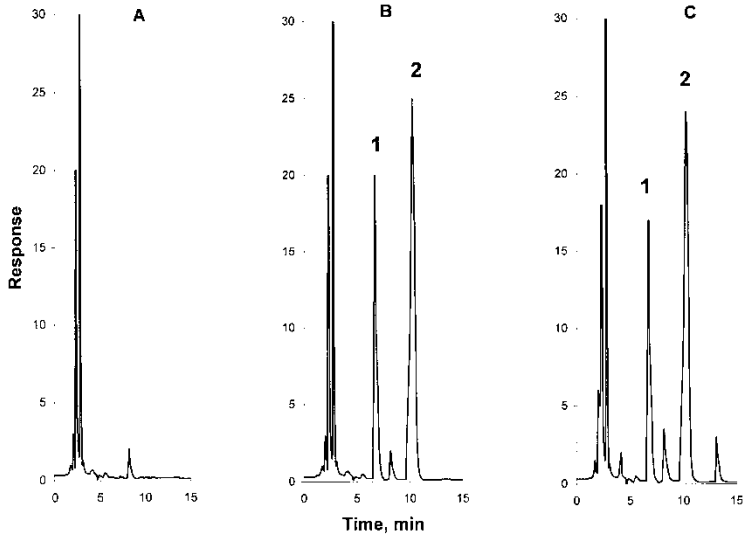

Figure 1. Chromatograms of extracted drug-free plasma, blank plasma (A), the same plasma samples spiked with 5 μg/mL fluconazole and 0.4 μg/mL IS (B), and plasma taken from a patient 1 h after day 7 of the daily dosing of fluconazole intravenous infusion of 200 mg and also spiked with 0.4 μg/mL IS (C).

Table 2. Within-day precision and accuracy of fluconazole recovery from spiked human plasma samples ($n = 6$)

Spiked concentration (μg/mL)	Mean measured concentration (μg/mL)	Standard deviation	Recovery (%)	Relative standard deviation (%)
0.5	0.65	0.07	103	10.7
5.0	5.42	0.46	102	8.5
15.0	13.67	0.54	97	3.9

Least-squares regression calibration curve was found to be linear at plasma concentrations between 0.2 and 15 μg/mL of fluconazole. The mean linear regression equation of the peak area ratios (Y) vs. drug concentrations (X) of fluconazole was typically of the form $Y = 0.082X + 0.04$. The mean correlation coefficients, r, was generally >0.997. The detection limit of the assay, based on extraction of 1 mL of plasma, was 50 ng/mL at a signal to noise ratio of >3.

Within-day precision and accuracy of the method were determined from replicate analysis ($n = 6$) of three spiked plasma test standards at concentrations within the linear range of the assay for each drug (Table 2). The mean percentage recovery of 0.2–15 μg/mL ($n = 6$) of fluconazole was 101 ± 5% (RSD% = 7.7). Table 2 shows the recoveries of three different concentrations of fluconazole. Extraction efficacy was found not to vary significantly among different concentrations of fluconazole.

The reproducibility of the assay was evaluated by comparing the linear regressions of three standard plots prepared at three different days over a 3 month period for each drug. The results of this evaluation are summarized in Table 3. The mean correlation coefficient was ≥ 0.996 with RSD% of the slopes of the three lines $\leq 7.6\%$. Analysis of variance of the data (Table 4) indicated that there is no significant difference ($p > 0.05$) in the slopes, within-day and between-day, of the calibration curves. The results confirmed the reproducibility of the assay method.

Table 3. Reproducibility of data from standard plots in human plasma ($n = 6$)

Standard plot[a]	Slope[b]	Intercept[b]	Correlation coefficient[b]
1	0.0898	0.017	0.998
2	0.0675	0.091	0.996
3	0.0894	0.009	0.998

[a]Obtained from assays on three different days.
[b]The mean of six determinations.

Table 4. One-way ANOVA for the reproducibility of the assay (within-day and between-day)

Source of variation	Sum of squares	Degree of freedom	Mean squares	*F* value[a]
Total	0.008763	17		
Between	0.002555	2	0.001277	3.09
Within	0.00621	15	0.000414	

[a]*F* (95%) tabulated = 3.68, no significant difference at $p > 0.05$.

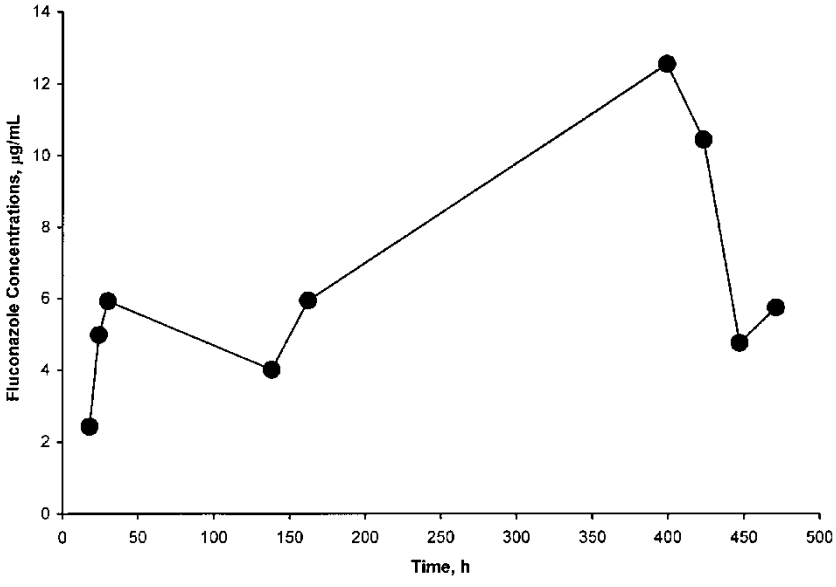

Figure 2. Plasma concentrations time profile of fluconazole in a patient with early septic shock receiving multiple 200 mg daily doses of intravenous infusion for 19 days.

Table 5. Pharmacokinetic parameters of fluconazole after intravenous infusion to patients with early septic shock

Parameter	Value
$t_{1/2}$ after single dose (h)	12.3 ± 9.0
$t_{1/2}$ after repeated doses (h)	41.3 ± 20
C_{min}, at steady state (µg/mL)	1.02 ± 0.78
C_{max}, at steady state (µg/mL)	7.3 ± 4.8
R_{ac}	2.9 ± 0.6

Figure 2 depicts a typical plot of fluconazole plasma concentrations time profile in a patient with early septic shock after multiple 200 mg daily doses of intravenous infusion for 19 days. The $t_{1/2}$ of fluconazole in this patient after a single dose was 3.2 h, and at steady state was 49.5 h with R_{ac} of 5.8. This figure demonstrates the usefulness of the method for analysis of fluconazole in these patients. Table 5 depicts the pharmacokinetic parameters of fluconazole after intravenous infusion to patients with early septic shock. It should be mentioned that re-analysis of patients' plasma samples after several weeks of the initial analysis showed no significant change in fluconazole concentrations.

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