CHAPTER 3

METHODOLOGY OF STUDY

3.1. Materials

3.1.1. Drugs

Ketoconazole used in this study was Nizoral® (lot No. 192044) from OLIC (Thailand) limited, Ayudthaya, Thailand, under the contact with Janssen Pharmaceutica LTD.

Ascorbic acid powder USP/BP (AR grade) was purchased from Srichand United Dispensary Co., LTD.

3.1.2. Reagents and Chemical Substances

Acetonitrile and Methanol, HPLC grade, were purchased from J.T. Baker, NJ, USA. Disodium hydrogen orthophosphate anhydrous was purchased from BDH Laboratory supplies, Poole, England. Ketoconazole is working standard, 99.8 %.

3.2. Equipment

3.2.1. HPLC model

- Waters 600 controller (Waters Cooperation Company Ltd. Milford, USA)
- Waters 470 spectrofluorometer detector (Waters Cooperation Company Ltd. Milford, USA)
- Waters 717 auto-sampler injection valve (Waters Cooperation Company Ltd. Milford, USA)

- Waters 746 integrator (Waters Cooperation Company Ltd. Milford, USA)
- μ-Bondapack ® column: a reverse phase column C_{18}, 10 μm particle, 30 cm length x 3.9 mm internal diameter. (Waters Cooperation Company Ltd. Milford, USA)
- μ-Bondapack ® C_{18} guard column: packed with resolved C8

3.2.2. Instruments

- Vortex mixer
- Centrifuge machine
- pH meter
- Micropipette (200 and 1000 μl)
- Pipette tip
- Needle (22 G)
- Heparin lock
- NG tube (diameter 4.0 mm-12F)
- test tube with cap
- Disposable syringe (5 ml)
- Eppendorf microcentrifuge tube (1.5 ml)
- PTFE filter, pore size 0.45 μm

3.3. Methodology

3.3.1. Determination of ketoconazole in plasma

3.3.1.1. Sample Preparation

The 250-μl of plasma sample was transferred into an eppendorf microcentrifuge tube and equal volume of acetonitrile was added for deproteinization. The mixture was vortexed for 30 seconds using vortex mixer and centrifuged at

10,000 rpm for 15 minutes. The supernatant was transferred into a new tube and 50 µl was injected into the column.

3.3.1.2. Chromatographic Conditions

Plasma ketoconazole concentrations were determined by high-performance liquid chromatography (HPLC). The assay was modified from Yeun and Peh study (1998) using the following parameters:

Column : reverse phase column (µ-Bondapack C18, 10 µm particle, 300 mm length, and 3.9 mm internal diameter)

Guard column : µ-Bondapack C18 pack with resolve C8, 1.5 cm

Mobile phase : Mixture of Acetonitrile : 0.05 M disodium hydrogen orthophosphate (60:40 % V/V) and pH was adjusted to 6.0 with glacial acetic acid

Flow rate : 1.5 ml/min

Infection volume : 50 µl

Detector : Fluorescent, an excitation wavelength of 260 nm and an emission wavelength of 375 nm.

Temperature : room temperature (~ 25 °C)

3.3.1.3. Mobile Phase Preparation

Mobile phase consisted of acetonitrile and 0.05 M disodium hydrogen orthophosphate anhydrous (60:40 % by volume). The mixture was adjusted to pH 6.0 with glacial acetic acid and filtered through PTFE filter, pore size 0.45 µm, and helium degassed prior to use.

3.3.1.4. Standard Curve

Plasma containing a known quantity of ketoconazole was run in parallel with the samples on each day of analysis. Standard curve was prepared by diluted stock

solution (1000 µg/ml) to serial concentrations of 8, 2, 0.5 and 0.1 µg/ml with drug-free plasma. All samples were proceeded following the procedures as described in 3.3.1.1 and 3.3.1.2. Standard calibration curves were conducted by the least-square linear regression of the ketoconazole concentrations and peak area ketoconazole. Unknown concentrations of ketoconazole in patient's plasma were calculated from the standard curves by reverse prediction.

3.3.1.5. Recovery Study

Analytical recovery of plasma ketoconazole was determined by comparing the peak area of deproteinized drug in plasma with the peak area of the deproteinized equivalent drug in mobile phase. A good recovery should be more than 90 % and percent coefficient of variation (%CV) less than 5 %.

3.3.1.6. Limit of Quantification

Limit of quantification was obtained by adding known amount of ketoconazole to drug-free plasma (0.05 – 8 µg/ml) and deproteinization as described above. The peak areas of ketoconazole were calculated and plotted the correlation between the concentration of ketoconazole and peak area. The lowest concentration of ketoconazole with that could still be linearly correlated was regarded as lower limit of quantification.

3.3.1.7. Precision of the Assay Procedure

Intraday (within-day) and interday (between-day) were established by adding a series known amount of ketoconazole to drug-free plasma (1, 2, 8 µg/ml). The precision was calculated as percentage of coefficient of variation (%CV). It should be less than 5 % for intraday and 10 % for interday.

$$\% CV = \frac{SD}{X} \times 100$$

Where : SD = standard variation of mean

X = mean value

3.3.2. Sample Size Calculation

The study was conducted to determine the influence of vitamin C on ketoconazole absorption in AIDS patients. However, there were no reports on correlation of ketoconazole and vitamin C in AIDS patients. So, the data from the study of Lake-Bakaar *et al.* (1988a) on ketoconazole absorption in ten AIDS's patients was used to calculate sample size. They found that the $AUC_{0\text{-}24h}$ (mean $\pm$ SE) of ketoconazole absorption statistically significant increased from 5.5 $\pm$ 2.8 mg.h/L without acid to 12.4 $\pm$ 2.8 mg.h/L with acid ($p < 0.05$).

$$\text{Standard deviation} \quad (SD) = SE\sqrt{n}$$
$$= 2.8\sqrt{10}$$
$$= 8.85 \text{ mg.h/L}$$

The different AUC of ketoconazole absorption (d) = 12.4 – 5.5 = 6.9 mg.h/L

Type I error 5% ($\alpha = 0.05$), $Z_\alpha = 1.96$, and type II error 10% ($\beta = 0.10$), $Z_\beta = 1.28$

$$n = \frac{(Z_\alpha + Z_\beta)^2 \, SD^2}{d^2}$$
$$= \frac{(1.96 + 1.28)^2 (8.85)^2}{(6.9)^2}$$
$$= 17.27$$
$$\approx 18$$

The randomized crossover design was chosen in this study, so it could decrease intra-subjects variation of ketoconazole absorption between the two treatments. Hence, the sample size was reduced to nine and 25 % more patients

should be performed. Therefore, the number of sample size in this study was twelve of AIDS patients.

3.3.3. Pharmacokinetic Study

3.3.3.1. Patient Selection

Twelve patients with AIDS, who had CD_4 T-lymphocyte absolute cell count less than 200 cell/mm^3 (obtained within the proceeding 2 months) and the age over 18 years, participated in the randomized crossover study. Written informed consent was obtained for all patients, and the study protocol was approved by the ethic committee, Songklanagarind Hospital.

Patients were excluded from the study according to the following criteria:

- renal or hepatic impairment, as the level shown in *Appendix A*.
- diarrhea or vomiting during the study period
- currently received the agents known to influence gastric acidity, as shown in *Appendix B*.
- received ketoconazole within a week prior the study.
- known history of azole antifungal agents or vitamin C hypersensitivity.

3.3.3.2. Study Design

Patients were randomized to receive two treatment sequences in a two-way crossover study design, with a week washout period separating each study treatment.

Treatment A, which patient served as the control group, 200 mg of ketoconazole was administered with glucose solution (5 g of glucose in 30 ml of water) and 120 ml of water.

Treatment B, which patient served as the study group, 200 mg of ketoconazole was administered with freshly prepared vitamin C solution (1.5 g of vitamin C and 5 g of glucose in 30 ml of water) and 120 ml of water.

Patients were hospitalized for 2 days at Prince of Songklanagarind hospital for 24 hours of blood sampling during the treatment. They fasted from 12 PM on the night prior to the study until 4 hours after the ketoconazole administration. Thirty minutes prior ketoconazole administration, NG tube (diameter 4.0 mm-12F) was inserted into the stomach; manual auscultation and aspiration was done to verify the position of the tube. After that 5-ml of gastric content was aspirated for pH measurement by pH meter and the tube was removed.

Blood samples (5 ml) were obtained from an indwelling venous catheter before ketoconazole administration (at predose of ketoconazole) and at 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 6, 8, 12 and 24 h following. Each sample was centrifuged at 2,500 rpm for 15 min, and plasma was harvested and stored at $-80^{\circ}C$ until the time of analysis.

3.3.3.3. Pharmacokinetic analysis

Plasma ketoconazole concentration-time data were fitted using Winnonlin (version 1.1) by non-compartmental model with first-order absorption and first-order elimination. The highest measured plasma drug concentration represented the C_{max}, while T_{max} was the time for the occurrence of C_{max}. The AUC was estimated by using the linear trapezoidal rule from 0 to *t* (AUC_{0-t}), where *t* is the time to the last measurable ketoconazole concentration. Addition of the area approximated by the last measurable concentration-time point/K_{el} was used to extrapolated to infinity ($AUC_{0-\alpha}$). The elimination half-life was calculated as 0.693/ K_{el}.

2.3.3.4. Statistical analysis

A two-way analysis of variance for repeated measurement was applied to assess significantly differences in $AUC_{0-\alpha}$, C_{max} and T_{max} between the treatment. The data did not show normal distribution; therefore, non-parametric statistic test was used for data assessment. Wilcoxon's signed rank test was treated for pairwise comparisons. The priority value of significance was set a *p* value of less than 0.05.