CHAPTER 3

METHODOLOGY OF STUDY

3.1. Materials

3.1.1. Drugs

Ketoconazole used in this study was Nizoral®(Lot No.B420006) from OLIC(Thailand) limited, Ayudthaya, Thailand, under the contract with Janssen Pharmaceutica LTD.

Efavirenz (Storcrin®, Lot No.G0119) was purchased from B.L.H.Trading Co.,LTD., Bangkok, Thailand, under the contract with Bristol-Mayers Squibb Holding Pharma limited, Puerto Rico, USA.

3.1.2. Reagents and Chemical substances

Acetonitrile and methanol, HPLC grade, were purchased from J.T.Baker, NJ, USA. Disodium hydrogen orthophosphate anhydrous and glacial acetic acid were purchased from Merck Darmstadt, Germany.

Standard powder of ketoconazole was purchased from Sigma Chemical Co., St.Louis, MO, USA.

Water was purified for HPLC by the MiLLi Q Water Purification System, Millipore, Milford, USA.

3.2. Equipment

3.2.1. HPLC model

- Waters 2695 pump, autosampler (Waters Associates, Milford, USA.)
- Waters 2475 spectrofluorometer detector (Waters Associates, Milford, USA.)
- Empower software (Waters Associates, Milford, USA.)

- μ-Bondapak® C_{18} column : a reverse phase column C_{18}, 10 μm particle, 30 cm length x 3.9 mm internal diameter. (Waters Associates, Milford, USA.)
- μ-Bondapak® C_{18} guard column : packed with resolved C_{18} (Waters Associates, Milford, USA.)

3.2.2. Instruments

- Vortex mixer
- Centrifuge machine
- pH meter
- Micropipette (200 and 1,000 μl)
- Pipette tip
- Disposable needle (22G)
- Haparin lock
- Test tube with cap
- Disposable syringe (3 ml and 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 appendorf microcentrifuge tube and equal volume of acetonitrile was added for deprotienizaton. The mixture was vortexed for 30 seconds using vortex mixer and centrifuge at 5,000 g for 15 minutes. The supernatant was transferred into a new vial 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 parameter :

Column : reverse phase column (μ-Bondapak C_{18}, 10 μm particle, 300 mm length, 3.9 mm internal diameter)

Guard column : μ-Bondapak C_{18} pack with resolve C_{18}, 1.5 cm

Mobile phase : Mixture of Acetonitrile : 0.05 M disodium hydrogen orthophosphate (45:55 %v/v) and pH was adjusted to 6.0 with glacial acetic acid

Flow rate : 1.5 ml/min

Injection volume : 50 μl

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

Temperature : room temperature (~25^{o}C)

3.3.1.3. Mobile phase preparation

Mobile phase consisted of acetonitrile and 0.05 M disodium hydrogen orthophosphate anhydrous (45:55 % 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 degassed by sonification for 15 minutes prior to using.

3.3.1.4. Standard curve

Plasma contained 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 (1,000 μg/ml) to serial concentrations 16, 8, 2, 0.4 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 concentration 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 deprotienized 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) should be less than 5%.

3.3.1.6. Lower limit of quantification

Lower limit of quantification was obtained by adding known amount of ketoconazole to drug-free plasma (0.01-16 μg/ml) and deproteinized as above. The peak areas of ketoconazole were calculated and plotted for the correlation between the concentration of ketoconazole and peak area. The lowest concentration of ketoconazole which was still linearly correlated was regarded as lower limit of quantification (LLOQ).

3.3.1.7. Precision of the assay procedure

Intraday (within-day) and interday (between-day) were established by adding a series of known amount of ketoconazole to drug-free plasma (0.4, 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.

$$\%\ CV = [SD / \overline{X}] \times 100$$

Where : SD = standard deviation of the mean

$\overline{X}$ = mean value

3.3.2. Sample size calculation

The study was conducted to determine the influence of efavirenz on pharmacokinetics of ketoconazole in HIV-infected patients. However, there were no report on correlation of ketoconazole and efavirenz in HIV-infected patients. So, the study of Clarke et al. (2000) on the influence of efavirenz on the pharmacokinetics of methadone in 11 HIV-infected patients was used to calculate sample size. They found

that the AUC_{0-24} of methadone was also significantly reduced from 12,341 to 5,309 $ng.ml^{-1}.h$ in the presence of efavirenz, p = 0.012 (SD= 5,599 $ng.ml^{-1}.h$).

The different AUC of ketoconazole (d) = 12,341-5,309 = 7,032 $ng.ml^{-1}.h$

Type I error 5% (α=0.05), Z_α= 1.645, and Type II error 10% (β=0.10), Z_β= 1.282

$$N = \frac{2(Z_\alpha + Z_\beta)^2(SD)^2}{d^2}$$

$$= \frac{2(1.645+1.282)^2(5599)^2}{(7032)^2}$$

$$= 10.86$$

$$\approx 11$$

A total sample size of 11 patients should be enough to detect a significant pharmacokinetic difference in AUC for ketoconazole in the presence or absence of efavirenz. Hence, the number of sample size of the HIV-infected patients in this study was twelve.

3.3.3. Pharmacokinetic study

3.3.3.1. Patient selection

Inclusion criteria :

- Twelve HIV-infected patients, who had CD_4 T-lymphocyte absolute cell count less than 350 cell/mm^3 (obtained within the proceeding 2 months) and the age were over 18 years, participated in the 1-sequence, 2-period pharmacokinetics interaction study. Written informed consent was obtained from all patients, and the study protocol was approved by the ethic committee, Songklanagarind Hospital.

Exclusion criteria:

Patients were excluded from the study in the following cases :

- renal or hepatic impairment
- diarrhea or vomiting during the study period
- currently received the agents known to influence on ketoconazole pharmacokinetics.
- received ketoconazole, itraconazole, fluconazole or antiretroviral drugs within 1 month prior to the study
- known history of azole antifungal agents or antiretroviral agents hypersensitivity

3.3.3.2. Study design

The study was a 1-sequence and 2-periods without washout period pharmacokinetic interaction study.

Patients were hospitalized for 2 days at Songklanagarind hospital for blood sampling during each phase of the study. They were fasted from 12 PM on the night prior to the study and until 4 hours after the ketoconazole administration.

Phase 1, Patients served as control group, 400 mg of ketoconazole was administered with 200 ml water under supervision on day 1.

Blood samples (approximately 5 ml) were obtained from an indwelling venous catheter before ketoconazole administration and at 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 6, 8, 12 and 24 h after administration of ketoconazole. Each sample was added to the heparinized tube and centrifuged at 1000g for 10 min, and plasma was harvested and stored at –80^{o}C until the time of analysis.

Phase 2, Patients served as study group, each patient took 600 mg of efavirenz once daily. The drug was taken at bedtime (9.00 PM) to attenuate possible central nervous system effect of this drug (Adkins, 1998). Efavirenz was combined with 300 mg of

lamivudine and 40 mg (or 30 mg when B.W. was lower than 60 kgs.) of stavudine twice a day, they were instructed to ingest the drugs in the morning and evening at 12-h interval. There is no evidence that any of this NRTI drugs interact with CYP3A4 (Berry, 1999). Lamivudine, stavudine and efavirenz were taken in day 2 and continue throughout the course of treatment. On day 16 of the study, patient received 400 mg of ketoconazole with 200 ml water. Blood samples were collected as previously mentioned.

3.3.3.3. Pharmacokinetic analysis

Plasma ketoconazole concentration-time data were fitted using WinNonlin Version 4.1 (Pharsight, Mountain View, CA) by non-compartment model with first-order absorption and the first-order elimination. The maximun plasma concentration (C_{max}) and the time for occurrence of C_{max} (T_{max}) were determined by visual inspection of individual plasma concentration-time profiles. The area under the concentration-time curve between 0 and 24 h (AUC_{0-24}) was estimated by using the linear trapezoidal rule.

3.3.3.4. Statistical analysis

All results were expressed as mean $\pm$ standard deviation (SD). The data show normal distribution, therefore, parametric statistic test was used for data assessment. Paired t-test was treated for pairwise comparisons. The priority value of significance was set at *p*-value of less than 0.05.