CHAPTER III

MATERIALS AND METHODS

MATERIAL

A. Test products

Nine different 200-mg tablet brands of ketoconazole referred to, in this study, as A, B, C, D, E, F, G, H and I were bought from various drugstores. One was the innovator's product that was assigned as the reference against the others. Other information of test products were accessible in appendix A.

B. Reagent

1. Standard ketoconazole powder, potency 100.06%, (Condry product Schweizechall PTE Ltd.) Lot. 945/89
2. Quinidine hydrochloride (Sigma company, USA.) Lot. 85C0285
3. Acetonitrile AR (Merck) Lot. 8248408
4. Methanol AR (Merck) Lot. K11532509
5. Diethylamine for synthesis (Merck) Lot. 7247154
6. Sodium hydroxide AR (Merck) Lot.735C642598
7. Monobasic potassium phosphate GR (Merck) Lot.720A168646
8. Sodium chloride extra pure (Vidhayasom) Lot.630

9. Concentrated hydrochloric acid 37% GR (Merck) Lot. 800K63614917
10. Heparin 5,000 I.U./ml (Leo Pharmaceutical Products, Ballerup-Denmark) Lot. 3390025
11. Dichloromethane AR (Merck) Lot. 908K11456150
12. Diazepam (Sigma company, U.S.A.) Lot. 83C0516
13. Ammonium acetate AR (Merck) Lot. 707CC536116
14. Ortho-phosphoric acid 85% GR (Merck) Lot.816K10108973

C. Apparatus

1. Analytical balance (Sartorius 1615MP, West Germany)
2. Disintegration tester (GC-21, Hanson Research Corp., Northridge, Calif., U.S.A.)
3. Dissolution apparatus (72 RL, Hanson Research Corp., Northridge, Calif., U.S.A.)
4. Spectrophotometer (Spectronic 2000, Bausch and Lomb, N.Y., U.S.A.)
5. High pressure liquid chromatography (LC-3A, Shimadzu, Japan)
6. Digital pH meter(PBS 730 EL-Hama Instruments)
7. Vortex mixer (Vortex-genuine, Scientific Industries Inc., Bohemia, N.Y., U.S.A.)
8. Waterbath (Memmert, Edelstaph Rost Frei)
9. Digital computer (IBM Compatible 16 Bit, Micro source)
10. Refrigerate centrifuge (Sigma 302K Lab.

Centrifuge Gmbtt., Germany)

11. Shaker (KSB, Edmund Buhler, Germany)

METHODS

A. *In Vitro* Studies

All nine brands of ketoconazole tablets were subjected to standard *in vitro* test as specified in the United Stated Pharmacopoeia XXII (The United State Pharmacopoeia Convention, 1990), including content of active ingredient, uniformity of dosage units, disintegration test , and other non-official tests. These are as follows.

1. Weight Variation

Twenty tablets of each of the nine brands of ketoconazole tablets were sampling and accurately weighed tablet by tablet. The average weight and standard deviation were calculated.

2. Content of Active Ingredient

The amount of ketoconazole in tablets was determined by HPLC, using a modification of the United Stated Pharmacopoeia XXII method (The United State Pharmacopoeia Convention, 1990), as follows.

Sample preparation :

Weigh and finely powder not less than 20 ketoconazole tablets. Accurately weighed a portion of the powder which is equivalent to about 200 mg of ketoconazole and transfer it to a 200-ml Erlenmyer flask, add 50.0 ml of methanol-methylene chloride(1:1), shake for 30 min., and centrifuge at 3000 rpm. for 10 min. Transfer 5.0 ml of the clear supernatent solution so obtained to a 50-ml volumetric flask, add 5.0 ml of internal standard solution, diluted with mobile phase to volume, and mix.

Internal standard solution :

Dissolved diazepam in methanol-methylene chloride to obtain a solution containing about 0.6 mg/ml.

Standard solution :

Transfer 10.0 ml (20.0 mg) of standard solution containing ketoconazole 2 mg/ml to a 50-ml volumetric flask, add 5.0 ml of internal standard solution, dilute with mobile phase to volume, and mix.

Procedure : Separately inject equal volumes (15 mcl) of the standard and sample solutions into the following chromatograph.

Chromatographic System

Apparatus	HPLC LC-3A, Shimadzu, Japan
Column	μ- Bondapak C_{18}, Stainless steel column, Water association Pty. Ltd.,USA. (30*3.9 mm. i.d.) particle size 10μm.
Mobile phase	74:26 mixture of a 1 in 500 solution of diethylamine in methanol and ammonium acetate solution (1 in 200)
Flow rate	1.5 ml/min
UV detector	225 nm
Attenuation	2^5 mv/ full scale
Column temperature	ambient
Pressure	160 kg/cm^2
Injection volume	15 mcl

The United State Pharmacopoeia System Suitability Test

Resolution between ketoconazole and internal standard must not less than 2.0 and coefficient of variation (C.V.) of peak height ratio following five replicate injections of standard solution must not be more than 2.0%. The resolution is determined by the formula :

$$2\,(t_2-t_1)\,/\,W_1+W_2 \qquad \text{(eq. 1)}$$

in which t_2 and t_1 are the retention times of the two peaks and W_1, W_2 are the corresponding widths of the bases of the peaks, obtained by extrapolating the relatively straight sides of the peaks to the baseline.

Calculation : Calculate the quantity, in mg, of ketoconazole in the portion of tablets taken by the formula:

$$10\ W_S\ (R_u/R_S) \qquad \text{(eq. 2)}$$

in which W_S is the weight, in mg, of ketoconazole taken for standard preparation R_u and R_S are the ratios of the peak heights of ketoconazole to those of diazepam from the sample solution and the standard solution, respectively.

3. Uniformity of Dosage Units

According to the United State Pharmacopoeia, ten ketoconazole tablets were individually assayed by HPLC, using the modified United State Pharmacopoeia method as described. The amount of ketoconazole in each tablet and coefficient of variation of percent labeled amount were calculated (The United State Pharmacopoeia Convention, 1990).

4. Disintegration Test

The disintegration test of tablet products were studied using United State Pharmacopoeia XXII method, as follows.

4.1 Uncoated Tablets

Place 1 tablet in each of six tubes of the basket , add a disk in each tube , and operate the apparatus, using water maintained at 37 ± 2 °C as the immersion fluid.

The times used to complete disintegration of each of six tablets were recorded. Then the mean disintegration time and standard deviation of each brand were calculated.

4.2 Film-coated Tablets

Place 1 tablet in each of six tubes of the basket and immerse the basket in water at room temperature for 5 min. Then add a disc to each tube, and operate the apparatus, using simulated gastric fluid maintained at 37 ±2 °C as immersion fluid. The disintegration times and its standard deviation were then calculated.

In this study, coated-tablets were performed not only in simulated gastric fluid but also in the carbon dioxide-free water as in the case of uncoated tablets.

5. Dissolution Test

For each brand, the dissolution test was carried out on six individual tablets using 900 ml of simulated gastric fluid without pepsin and 900 ml of simulated intestinal fluid without pancreatin, maintained at 37±0.5 °C as described in the United State Pharmacopoeia dissolution type 2. The paddle was stirred at 50 rpm.

In acidic medium, samples of the dissolution medium, 5 ml, were taken at 0, 2.5, 5.0, 7.5, 10.0, 12.5, 15.0, 20.0, 25.0, 30.0, 35.0, 40.0, 45.0, 50.0 and 60.0 min. The sample concentrations were determined by UV spectrophotometer at 231 nm using the dissolution medium

as blank, in comparison with the standard curve I.

In simulated intestinal fluid without pancreatin, 5 ml of dissolution medium samples were taken at 0, 5.0, 10.0, 15.0, 20.0, 25.0, 30.0, 45.0, 60.0, 90.0, 120.0, 150.0, 180.0, 210.0 and every 30 min interval until the plateau level was achieved. The absorbances of the samples were determined at 231 nm, using the dissolution medium as blank, in comparison with the standard curve II.

Standard curve I

Standard solution with known concentrations of 4.0 , 6.0, 8.0, 10.0, 12.0, 14.0, 16.0, 18.0 and 20.0 mcg/ml of ketoconazole in the simulated gastric fluid without pepsin were determined in a spectrophotometer at 231 nm, using the dissolution medium as blank. Absorbance obtained versus their known concentrations were fitted to a straight line using linear regression.

Standard curve II

Standard solution of ketoconazole with concentrations of 2.0, 4.0, 6.0, 8.0, 10.0, 12.0, 14.0 and 16.0 mcg/ml in the simulated intestinal fluid without pancreatin were analyzed using UV spectrophotometer at 231 nm. Absorbance obtained versus their known concentrations were fitted to a straight line using linear regression.

The dissolution rates in either medium were determined by Sigma-minus method.

In Vitro Evaluation

The results from the *in vitro* data for nine brands of ketoconazole tablets were determined whether the products met the specification for assay , content uniformity of dosage units, and disintegration test as stipulated by the United State Pharmacopoeia XXII (The United State Pharmacopoeia Convention, 1990). Statistical analysis for the differences of disintegration time and dissolution rate between innovator's product and the locally-made products was done by the analysis of variance (ANOVA) and t-test using a computerized statistical program SAP. The correlation coefficient test was also performed to assess the relationship between dissolution rate and disintegration time.

B. *In Vivo* Studies

Test Products

Products to be compared for their bioequivalence consisted of five different brands of 200-mg ketoconazole tablets conforming to the United State Pharmacopoeia standard, including an original brand which was assigned as the reference against the other four generic brands having highest, medium and lowest *in vitro* dissolution rate constants in the simulated gastric fluid without pepsin, respectively.

Subjects

Twelve subjects were selected from Thai-healthy male volunteers aging between 20-40 years and weighing 50-70 kg. The volunteers were required to be free of serious cardiovascular, hepatic, renal, gastrointestinal diseases, drug abuses, and/or alcoholic dependence as assess by reviewing of their medical history. All subjects were drug-free for at least two weeks prior to and until completion the study. They were also asked to refrain from alcoholic and caffeine-containing beverages 48 hr prior to each dosing and until the collection of the last blood sample.

Each subject gave his written informed consent before participating in the study. All subjects were provided with detail information of the possible side effects of ketoconazole and other hazards that might be encountered.

Study Design

The administration of the five brands to the subjects was carried out by means of a single-dose crossover design with at least a one-week drug free interval between any two successive administrations. The subjects were randomly divided into five groups and to one of the five sequences of administration. After fasting over night, each subject ingested a 200-mg tablet of ketoconazole with 200-ml of water and he was permitted to

have a normal diet 2 hr. after drug administration.

Plasma samples

Before drug administration, an dwelling catheter was placed in a forearm vein of each subject for blood sampling. Patency of the catheter was maintained by periodic flushing with small injections of 10 units/ml of heparin in normal saline. Blood samples (5 ml) were collected in heparinized tubes at 0, 0.5, 1.0, 1.50, 2.0, 3.0, 4.0, 6.0, 8.0, 10.0 and 12.0 hr postdose. The plasma was immediately separated by centrifugatizing at 3,000 rpm. for 10.0 min. and then stored at -10.0 °C until the time of analysis.

Analysis of Plasma Samples

Plasma concentrations of ketoconazole were quantitated by an HPLC method modified from Badcock(1984) , as following.

1.0 ml of plasma sample

↓ added 1.5 ml of acetonitrile containing quinidine 4.8 mcg/ml(internal standard)

centrifuged at 3,000 rpm. for 10min

↓

injected 200 mcl of supernatant into chromatograph

Chromatographic condition for analysis of plasma concentration

Apparatus	HPLC LC-3A, Shimadzu, Japan
Column	μ- Bondapak C_{18}, Stainless steel column, Water association Pty. Ltd.,USA. (30*3.9 mm i.d.) particle size 10μm.
Mobile phase	77:23:0.07 mixture of methanol, water, and diethylamine
Flow rate	1.4 ml/min
UV detector	254 nm
Attenuation	2^1 mv/ full scale
Column temperature	ambient
Pressure	140 kg/cm^2
Injection volume	200 mcl

The plasma ketoconazole concentrations were quantitated by comparison of the peak height ratio of the drug to the internal standard with the standard curve.

Standard Curve

Ketoconazole plasma standard were prepared by spiking human plasma with ketoconazole solution to give final concentrations of 0.0, 0.20, 0.50, 1.0, 2.0, 4.0, 6.0 and 8.0 mcg/ml and assigned as the procedure previously described. Standard curves were generated by using the least squares regression of the peak height ratio against the concentrations of the spiked plasma.

Assay Validation

The modified Badcock's method was validated under the condition used by the following means.

Within run precision was determined by analyzing of three sets of the calibration curves (7 different concentrations per curve) with were precipitated protein at the same day. Peak height ratio of ketoconazole to quinidine was compared, and the percent coefficient of variation (%CV) for each concentration was determined.

Between run precision was determined by comparing the peak height ratios for four standard curves injected on four different days, then %C.V. of each concentration was calculated.

To assess the recoveries of ketoconazole, different calibration curves which were injected on four different days were determined. Peak heights of ketoconazole were compared with those of aqueous solution (900 mcl) fortified with known amounts of ketoconazole (100 mcl) to achieve the concentrations of 0.20, 0.50, 1.0, 2.0, 4.0, 6.0 and 8.0 mcg/ml and then mixed with 1.5 ml of acetonitrile.

The recoveries of internal standard were assessed at the concentration of 4.8 mcg/ml only using the same procedure as described. Percent recoveries of each were calculated by the formula :

$$\text{percent recovery} = \frac{\text{peak height of sample injected} * 100}{\text{peak height of standard solution}} \qquad \text{(eq. 3)}$$

Pharmacokinetic Analysis

The pharmacokinetic analysis of individual plasma ketoconazole levels from each treatment was performed using the CSTRIP, a Fortran IV computer program for obtaining initial polyexponential parameter estimates (Sedmen and Wagner, 1976). The analysis indicated that a biexponential equation :

$$C_t = A(e^{-Kel \cdot t} - e^{-Ka \cdot t}) \qquad \text{(eq. 4)}$$

without a lag time could be best described the concentration -time curves for ketoconazole, where

C_t is the plasma drug concentration at any time

t is time

K_a, K_{el} and A are initial parameter estimates obtained from CSTRIP program analysis

A is the ordinate intercept constant

K_a is the absorption rate constant

K_{el} is the systemic elimination constant

The peak concentration(C_{max}), the time to reach the peak (t_{max}), the plasma half-life of ketoconazole ($t_{1/2}$) and the area under the curve (AUC_0^∞) were determined by the following relationships.

$$C_{max} = A(e^{-Kel \cdot tmax} - e^{-Ka \cdot tmax}) \qquad \text{(eq. 5)}$$

$$t_{max} = \frac{1}{K_a - K_{el}} \frac{\ln K_a}{K_{el}} \qquad \text{(eq. 6)}$$

$$t_{1/2} = \frac{\ln 2}{K_{el}} \qquad \text{(eq. 7)}$$

$$AUC_0^{\infty} = A\left(\frac{1}{K_{el}} - \frac{1}{K_a}\right) \qquad \text{(eq. 8)}$$

Bioavailability Analysis and Statisticals

The difference in C_{max}, t_{max} and AUC_o^{∞} between innovator's product and the selected locally-formulated brands were analyzed by one-way analysis of variance (ANOVA). When significant treatment effects were observed from ANOVA, differences between treatments were examined by means of t-test. The test brands are considered to be bioequivalent to innovator's product when their C_{max}, t_{max} and AUC show no statistically significant differences (α = 0.05) from innovator's product.

In Vitro-In Vivo **Correlation Study**

A Pearson's correlation coefficient test and t-test were used to investigate the relationship between the *in vitro* parameters, which were dissolution rate constant, disintegration time, and the *in vivo* parameters which were C_{max}, t_{max}, AUC_0^{∞}. Null hypothesis for t-test is slope of linear regression line in correlative study equals zero. The strength of correlation will be accepted to be large enough and to be able to provide reliable prediction of ketoconazole bioavailability if correlation coefficient is more than 0.90 or less than -0.90 and slope of regression line is not equal zero. (alternative hypothesis of t-test is accepted.)