CHAPTER III

MATERIALS AND METHODS

Materials

A. Test Products

Three commercial brands of roxithromycin tablets were bought from various drugstores. One was the innovator's product that was assigned as the reference standard against the other two local manufactured brands.

The letters A, B and C were given to represent the brand names of each product. Other informations of these products were all accessible in Appendix A.

B. Reagents

1. Working standard roxithromycin powder (Siam Pharmaceutical Co.,Ltd.) potency: 97.74%, Lot No. ROX-47
2. Working standard clarithromycin powder (Siam Pharmaceutical Co.,Ltd.) potency: 98.75%, Lot No. CLA-05
3. Acetonitrile HPLC grade (J.T. and Baker, England)
   Lot No. 1648
4. Methanol HPLC grade (Lab-Scan, Thailand)
   Lot No. 97070068
5. Monobasic potassium phosphate AR. (E-Merck, Germany)
   Lot No. K23775573
6. Phosphoric acid (E-Merck, Germany)
   Lot No. 816K10108973

7. Heparin injection BP (Porcine mucous, Denmark) 5,000 i.u./mL
   Lot No. 2437612
8. Sodium hydroxide (E-Merck, Germany)
   Lot No. B695998
9. Absolute ethanol (Lab-Scan, Thailand)
   Lot No. 97090059
10. Antibiotic medium No. 1 (Difco Laboratories, USA)
    Lot No. 110782JB

C. Apparatus

1. Analytical balance (Sartorius, 1615MP; S/N 3209026, Germany)
2. Digital pH meter (Orion, Germany)
3. High performance liquid chromatography(LC-10AD, Shimadzu,Japan)
4. Sonicator (Bransonic 221, USA)
5. Dissolution apparatus (VK 7000, Vankel Industries, Inc., USA)
6. Vortex mixer (Vortex-Genie, Scientific Industries, Inc., USA)
7. Centrifuge (Sigma 302K, Sigma Lab, Centrifuge Gmbtt, Germany)
8. Incubator
9. Digital computer
10. Micropipet (Socorex, Switzerland)
11. Disintegrator Tester (Erweka ZT 31, Germany)
12. Glassware

Methods

A. In Vitro Studies

Three commercial brands of 150 mg roxithromycin tablets, were evaluated following the tests as stated in the pharmacopoeia. The tests were:

1. Uniformity of Dosage Units (The United States Pharmacopoeia 1995)

Ten tablets of 150 mg roxithromycin tablets from each brand were individually assayed for the percent labeled amount of roxithromycin in each tablet by HPLC method. The mean and standard deviation of percent labeled amount were calculated.

2. Content of Active Ingredient

The amount of roxithromycin in tablet was determined according to the method modified from that of Oliveira, Bergold, and Schanpoval (1996). It was described as follow.

Standard preparation: 100, 200, 400, 500, 600, 800, and 1000 μL of roxithromycin stock solution (1 mg/mL in methanol) were pipetted and transferred to individual 10 mL volumetric flasks and 1 mL of clarithromycin stock solution (1 mg/mL in methanol) was then added to all flasks, diluted with mobile phase to volume and mix. The final concentrations of roxithromycin in standard solutions were 10, 20, 40, 50, 60, 80 and 100 μg/mL, respectively and the internal standard (clarithromycin) concentration was 100 μg/mL. The standard solutions were injected into the HPLC system.

Assay preparation: The contents of 20 tablets of each brand were carefully ground and mixed uniformly. A weighted amount of this powder, equivalent to 50 mg of roxithromycin was transferred to 50 mL volumetric flask with 20 mL of methanol and shaken for 15 minutes. The mark was completed with the same solvent. After filtration, pipetted 0.5 mL of solution accurately to a 10 mL

volumetric flask and added 1 mL of clarithromycin solution (internal standard, 1 mg/mL in methanol), diluted with mobile phase to volume and mix. The final concentration of roxithromycin was about 50 μg/mL and the internal standard (clarithromycin) concentration was 100 μg/mL (Repeat for three sets of preparation of each brand).

Chromatographic condition

Apparatus : Shimadzu® LC-10A HPLC pump, equipped with Spectro Monitor 4100 variable wavelength UV detector and Shimadzu® C-R1A File No.2 integrator.

Column : μ-Bondapak®(C_{18}), stainless steel column, 3.9×300 mm, 125 A 10 μm of dimethyloctadecylsilyl bond amorphous silica. (Waters Associates Pty-Ltd., Milford, MA, USA)

UV detector : 210 nm

Flow rate : 1.7 mL/min.

Chart speed : 2 mm/min.

Pressure : 120 kg/cm^2

Attenuation : 2^4 mv/full scale

Operating temperature: 50°C

Injection volume: 50 μL

3. Assay Validation

The modified Oliveira et al. method was validated under the following conditions

3.1 Within-run precision

This precision was determined by analyzing the three sets of the calibration curves at the same day. Peak height ratio of roxithromycin to clarithromycin was compared and the percent coefficient of variation (%C.V.) for each concentration was determined (Table 2).

3.2 Between-run precision

This precision was determined by comparing the three sets of the calibration curves on three different days and the percent coefficient of variation (%C.V.) for each concentration was calculated (Table 3).

3.3 Accuracy

Accuracy in term of percent recovery was done by computing the ratio of inversely estimated concentration obtained using linear regression equation of a calibration curve and known concentration of each concentration (Table 4).

Calibration Curve

Stock solutions of roxithromycin (1 mg/mL) and clarithromycin (1 mg/mL) were prepared in methanol. 100, 200, 400, 500, 600, 800, and 1000 μL of roxithromycin stock solution were pipetted and transferred to individual 10 mL volumetric flasks and 1 mL of clarithromycin stock solution was then added to all flasks, diluted with mobile phase to volume and mix. The final concentrations of roxithromycin in standard solutions were 10, 20, 40, 50, 60, 80 and 100 μg/mL, respectively and the internal standard (clarithromycin) concentration was 100 μg/mL. The standard solutions were injected into the HPLC system (Appendix B : Table 31, Figure 17).

4. Disintegration Test

The disintegration test of roxithromycin tablets were determined according to the United States Pharmacopoeia XXIII method. The procedure was described as follow:

A tablet was introduced into each of the six tubes of the basket. A disk was then added to each tube and the apparatus was operated using water maintained at 37±1 °C as immersion fluid. The time that the apparatus started to move until all of the tablet have disintegrated and passed through the baskets was the disintegration

time. The average and standard deviation of disintegration time of each brand were calculated.

5. Dissolution Test

The dissolution test method for roxithromycin tablet was not available in any pharmacopoeias. The dissolution test was initiated using varied pH of various types of buffer solution (900 mL) with the USP dissolution apparatus type II (paddle method). The purpose of the procedure was to achieve a plateau level of percent roxithromycin dissolved within one hour. The test was tried out and found that phosphate buffer pH 7.4±0.1 was the most appropriate dissolution medium. The procedure was described as follow:

Nine hundred millilitres of phosphate buffer pH 7.4±0.1 was placed in the vessel and equilibrated at 37±0.5 °C. A tablet was introduced into each of six vessels, the apparatus was then immediately operated and the paddle maintained stirring at speed of 50 rpm. Three millilitres of dissolution medium was taken from each vessel at appropriate time intervals and the same quantity of the medium was added immediately after each sampling to keep the volume of dissolution medium constant during the period of the test. The sampling medium was then added with 1 mL of clarithromycin solution (internal standard, 1 mg/mL in methanol) and diluted with the dissolution medium to 10 mL of volumetric flask and a 50 µL was injected into the HPLC system. The amount of the drug dissolved at various time intervals was quantified using the calibration curve.

Calibration Curve

Stock solutions of roxithromycin (1 mg/mL) and clarithromycin (1 mg/mL) were prepared in methanol. 100, 200, 400, 500, 600, 800, and 1000 µL of roxithromycin stock solution were pipetted to individual 10 mL volumetric flasks and 1 mL of clarithromycin stock solution was added to all flasks, diluted with the dissolution medium to volume and mix. The final concentrations of roxithromycin in standard solutions were 10, 20, 40, 50, 60, 80 and 100 µg/mL, respectively and the

internal standard (clarithromycin) concentration was 100 μg/mL. All samples were analyzed following the same procedure as previously described. The ratio of the peak height of roxithromycin to the internal standard versus the known roxithromycin concentrations were fitted to the straight line using linear regression (Appendix B : Table 32, Figure 18).

6. In Vitro Evaluation

The physical characteristics of all three brands of roxithromycin tablets were examined and evaluated to determine whether each brand passed the general standard of USP XXIII requirements. A one way analysis of variance (ANOVA) and/or least significant different (L.S.D.) were performed to assess the difference of the dissolution rate constants between the values of the innovator's product and those of the other manufactured brands.

B. In Vivo Studies

1. Test Products

Ail three commercial brands of 150 mg roxithromycin tablets, were used in this study.

2. Subjects

Twelve healthy Thai male volunteers with the ages ranged from 20 to 40 years participated in this study. Demographic data are presented in Appendix D. Prior to study, all subjects must pass the physical examination and clinical laboratory tests. The methods of the study were clearly explained to all subjects. They gave written informed consents before participating the study and they were asked to take no medication, alcoholic preparations and cigarettes for at least one week preceeding the study and during the experimental period.

3. Dose and Drug Administration

One tablet of 150 mg roxithromycin was given orally with 200 mL of water in a single dose. All subjects received each dose in the morning after overnight fast. No food or drink (other than water) was permitted until 2 hours after dosing.

4. Experimental Design

The study was conducted in a randomized complete crossover design. Each subject received the drug in a randomized order with 1 week washout period between each administration as showed in Table 1.

5. Sample Collection

Five millilitres of blood samples were collected from a forearm vein using a disposable syringe. They were immediately transferred into heparinized tubes. Blood sample was collected before drug administration and at 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 12, and 24 hours after dosing. The blood was immediately centrifuged at 2,500 rpm., for 10 minutes and the plasma was separated and kept at -20°C until subsequent analysis.

6. Determination of Roxithromycin in Plasma

Plasma roxithromycin concentrations were determined by microbio--logical agar diffusion assay using a method modified from those described in the Code of Federal Regulations (CFR 21,1994) and by Campa et al.(1990) and Barry (1986), which was good correlated to the high-performance liquid chromatography method (Puri and Lassman, 1987). The procedure was described as follows:

6.1 Preparation of sample and standard

A 0.5 mL absolute ethanol was added to 500 μL plasma sample and the mixture was left overnight at 4°C. Each sample was centrifuged at 3000 rpm for 15 min and the clear supernatants were collected. The pellets were extracted twice

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Table 1 Dosing Schedule

Subject	week		
No.	1	2	3
1	A	B	C
2	B	C	A
3	C	A	B
4	A	B	C
5	B	C	A
6	C	A	B
7	A	B	C
8	B	C	A
9	C	A	B
10	A	B	C
11	B	C	A
12	C	A	B

A, B, C : represent the brand name of 150 mg roxithromycin tablets.

with absolute ethanol (1:4 v/v). The supernatants were pooled and evaporated to dryness by warming samples at 40°C with Rotary evaporator. The residue was reconstituted in 500 μL of phosphate buffer (pH 8) and 50 μL aliquot was filled into the inoculated plates.

Standard roxithromycin was added to 500 μL of pooled drug free plasma to make the final concentrations of 0.50, 1.00, 3.00, 5.00, 7.00, and 10.00 μg/mL. Then, they were treated like sample (Appendix B : Table 33, Figure 19).

6.2 Preparation of inoculum

A single colony from freshly grown overnight culture of *Micrococcus luteus* ATCC 9341 was inoculated on the surface of Antibiotic medium No.1 agar slant and incubated at 37°C for 24 hours. The surface growth culture was collected in 5 mL of sterile saline. In this study, a 13 mm diameter absorption cell will give 25 percent light transmission at a wavelength of 580 nm.

6.3 Preparation of inoculated plates

Preparation of base layer was done by pipetting a 21 mL of molten agar into each Petri dish. Distribute the agar evenly in each dish on a flat, level surface. After the agar hardens, place a cover on each plate. To prepare the seed layer, add the inoculum of the test organism suspension (*Micrococcus luteus* ATCC 9341) about 2% to a sufficient amount of agar, which has been melted and cooled to 48-50° C. Swirl the flask to obtain a homogeneous suspension, and add 4 mL of the inoculated media to each of the plates containing the uninoculated base agar. Spread evenly over the agar surface, cover, and allow to harden on a flat, level surface. After the agar had hardened, 6 cylinders were placed on the inoculated agar surface so that they were at approximately 60° intervals on a 2.8 cm radius.

6.4 Procedure for assay

All standard and test samples were assayed triplicately by filling the cylinders with 50 μL of the extract and incubating for 18 hours at 37°C in incubator. The inhibition zone diameters were measured using vernier caliper.

6.5 Estimation of potency

Potency of roxithromycin in sample was estimated from calibration curve plotting the concentrations of roxithromycin in plasma in logarithmic scale versus inhibition zone diameters (mm) in ordinary scale.

7. Assay Validation

The modified method of Campa, et al., and Barry was validated under the following conditions :

7.1 Within-run precision

This precision was determined by analyzing the three sets of the calibration curves at the same day. The inhibition zone diameters were compared and the percent coefficient of variation (%C.V.) for each concentration was determined (Table 10). The accepted percent coefficient of variation should be less than 15.

7.2 Between-run precision

This precision was determined by comparing the three sets of the calibration curves on three different days and the percent coefficient of variation (%C.V.) for each concentration was calculated (Table 11). The accepted percent coefficient of variation should be less than 15.

7.3 Accuracy

Accuracy in term of percent recovery was done by computing the ratio of inversely estimated concentration obtained using linear regression equation of a calibration curve and known concentration of each concentration (Table 12).

8. Pharmacokinetic Analysis

The pharmacokinetic analysis of individual plasma roxithromycin concentrations from each treatment was established using graphical method.

The peak plasma concentration (C_{max}), and the time to reach the peak plasma concentration (t_{max}) of roxithromycin were directly obtained from the data, meanwhile, the area under the concentration-time curve (AUC) was calculated using the trapezoidal rule. Other pharmacokinetic parameters, the absorption rate constant (K_a) was obtained by calculation following the Wagner-Nelson method, the elimination rate constant (K_e) was obtained from slope of the concentration-time curve in semilog scale, and the elimination half-life ($t_{1/2}$) was calculated using an equation, $t_{1/2} = 0.693/ K_e$.

9. Evaluation of Bioequivalence

The bioequivalence of all three brands of 150 mg roxithromycin tablets were evaluated using the three relevant pharmacokinetic parameters, C_{max}, t_{max} and AUC.

9.1 Statistical test

The differences of these three pharmacokinetic parameters among the three brands were determined by one way analysis of variance (ANOVA) at the significant level of α=0.05. If the test was statistically significant difference, t-test will be utilized to test which pair (test product vs reference product) was significant difference.

9.2 Construction of 90% confidence interval

A 90% confidence interval of individual parameter ratio was constructed using an equation

$$\mu_{T,obs} - \mu_{R,obs} \pm t_{0.95,\nu}\, s(2/n)^{1/2}$$

where $\mu_{T,obs}$ = observed mean for the test treatment

$\mu_{R,obs}$ = observed mean for the reference product

ν = the degree of freedom associated with the "error" mean square

$t_{0.95,\nu}$ = the point that isolates probability of 0.05 in the upper tail of the Student's *t* distribution with ν degrees of freedom

s = the square root of the "error" mean square from the crossover design analysis of variance

n = the total number of subjects participating in the crossover design

The upper and the lower limits of the confidence interval were divided by the average value of the reference product and 1 was then added to each limit to obtained the ratio of 90% confidence interval.

The tested brands were considered to be bioequivalent to the innovator's product, when the corresponding pharmacokinetic parameters showed no statistically significant difference from those of the innovator's product and the ratios of individual parameter of tested products relative to reference product were within 80-125% of 90% confidence interval.