CHAPTER III

MATERIALS AND METHODS

MATERIALS

A. Test products

Four brands of 250 mg ciprofloxacin film-coated tablets referred to, in this study, as A, B, C and D were bought from various drug stores. One was the innovator's product that was assigned as the reference product against the others. Other information of test products were accessible in Appendix A.

B. Reagent

1. Standard ciprofloxacin hydrochloride monohydrate, potency 98.03%, Biolab Lot B 03/90
2. Pipemedic acid (Sigma) Lot. 19F 0302
3. Methanol AR (Merck) Lot. 004K 13511209
4. Triethylamine AR (Merck) Lot. 6230418
5. 25% Tetrabutyl ammonium hydroxide in MeOH AR (Fluka) Lot. 36740789
6. 85% O-Phosphoric acid AR (Emerck) Lot. 11931 K12677273
7. Monobasic potassium phosphate AR (Merck) Lot. 943 A 459773
8. Acetonitrile HPLC (Riedel-De-Haen) Lot. 00500 2.51322
9. Heparin 5000 I.U./ml(Leo pharmaceutical Products), Lot. 3990044

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C. Apparatus

1. Analytical balance (Sartorius 1615 MP, West Germany)

2. Disintegration tester (Manesty Machines Ltd., England)

3. Dissolution apparatus (72 RL, Hanson Research Corp., Northridge, Calif., USA.)

4. Spectrophotometer (Spectronic 2000, Bausch and Lomb, N.Y., USA.)

5. High pressure liquid chromatography (LC-3A, Shimadzu, Japan)

6. Digital pH meter (Orion model SA 520, Orion research, USA.)

7. Vortex mixer (Vortex-Genuine, Scientific Industries Inc., Bohemia, N.Y., USA.)

8. Waterbath (Memmert, Edelstaph Rose Frei, West Germany)

9. Sonicator (Bransonic, Branson Cleaning Equipment Company, USA.)

10. Refrigerated centrifuge (Sigma 302 K Lab. Centrifuge Gmbitt., West Germany)

11. Digital computer (IBM Compatible 16 Bit, Microsource Thailand)

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METHODS

A. In Vitro Studies

Four brands of 250 mg ciprofloxacin film-coated tablets, were evaluated using both official and non-official tests of the United States Pharmacopoeia XXII, the United States Pharmacopoeia XXII Supplement 1, and/or the British Pharmacopoeia 1988 for film-coated tablets. The test included;

1. Weight variation

Twenty tablets of each brand of ciprofloxacin tablets were sampling and accurately weighed tablet by tablet according to the British Pharmacopoeia 1988 (London Her Magesty's Stationery Office, 1988). The average weight and standard deviation were calculated.

2. Content of Active Ingredient

Assay Preparation :

Transfer 5 ciprofloxacin tablets to a 500-ml volumetric flask, add about 400 ml of water and sonicate for about 20 minutes. Dilute with water to volume, and mix. Transfer 10.0 ml of this filtrated solution to a 100 ml volumetric flask, dilute with water to volume, mix.

Standard Preparation :

Dissolve an accurately weighed quantity of ciprofloxacin hydrochloride monohydrate quantitatively in water to obtain a solution having a known concentration of about 0.3 mg/ml.

Separately inject equal volumes (about 10 mcl) of the standard preparation and the assay preparation into the chromatograph. Calculate the quantity, in mg, of ciprofloxacin in each tablet taken by the formula:

$$(331.35/385.82)\ (CL/D)(r_u/r_s)$$

inwhich 331.35 and 385.82 are the molecular weights of ciprofloxacin and ciprofloxacin hydrochloride monohydrate, respectively, C is the concentration, in mg per ml, of ciprofloxacin hydrochloride monohydrate in the standard preperation, L is the labeled quantity, in mg, of ciprofloxacin in each tablet, D is the concentration, in mg per ml, of ciprofloxacin in the assay preparation, based on the labeled quantity per tablet and the extent of dilution, and r_u and r_s are the ciprofloxacin peak responses obtained from the assay preparation and the standard preparation, respectively.

Chromatographic system

Apparatus : HPLC LC-3A Shimadzu, Japan

Column : Partisphere C-18, stainless steel column, Whatman, England 12.5 cm. x 4.6 mm. I.D., particle size 5 µm

Mobile phase : Phosphoric acid (2 in 1000) adjusted with triethylamine to a pH of 3.0 ± 0.1 : Acetonitrile (87:13)

Detector : UV spectrophotometer at 278 nm

Flow rate : 1.5 ml/min

Temperature : 30 ± 1.0°C

Injected volume : 10 mcl

3. Disintegration test

The disintegration tests for all brands of ciprofloxacin film-coated tablets were studied according to the British Pharmacopoeia 1988 (London Her Majesty's Stationary Office, 1988).

Procedure : A tablet was placed in each of six tubes of the basket. The disk was then added in each tube. The apparatus was operated using water maintained at 37 $\pm$ 1°C as the immersion fluid. The test was passed if all six tablets had disintegrated completety within 30 minutes. If the preparation being examined fail to comply because of adherence of the tablet to the discs, repeat the test on a further six tablets omitting the discs.

4. Dissolution Test

According to the United States Pharmacopoeia XXII (United States Pharmacopeial Convention Inc., 1990), dissolution of ciprofloxacin tablets were established using the paddle method and water as dissolution medium

Procedure : Nine hundred millilitres of water was placed in the vessel and equilibrated at 37 $\pm$ 0.5°C. A tablet was placed in each vessel. The apparatus was then immediately operated and maintained stirring speed at the rate of 50 rpm. Five millilitres of samples were taken just prior to introducing the tablets and at 5, 10, 15, 20, 25, 30, 45, 60, 90, and 120 minutes intervals after the tablet was already placed in the vessel. The equivalent amount of temperature equilibrated water was added immediately after each sampling to maintain a constant volume of dissolution medium. The percent of drug

dissolved was determined using a UV spectrophotometer at 276 nm, in comparison with a calibration curve.

Calibration curve :

Standard solution of ciprofloxacin concentrations of 0.5, 0.8, 1.0, 1.5, 2, 3, 4, 5 and 6 mcg/ml in water were prepared and they were analyzed using a UV spectrophotometer at 276 nm. Absorbances obtained versus known concentrations were fitted to a straight line using linear regression (Steel and Torrie, 1980).

The dissolution rate of ciprofloxacin from each brand was determined by sigma-minus method (Gibaldi and Perrier, 1982).

5. In Vitro Evaluation

The physical characteristics of all four brands of ciprofloxacin film-coated tablets were examined and evaluated to determine whether which brand passed the general standard of the United States Pharmacopoeia XXII and/or the British Pharmacopoeia 1988 requirements.

Analysis of variance and the student's t-test (Steel and Torrie, 1980) were used to assess the differences between the innovator's product and others for the disintegration times and the dissolution rates.

B. In Vivo Studies

1. Test Products

All four brands of 250 mg ciprofloxacin film-coated tablets were subjected for in vivo studies. One was the innovator's product which was assigned as the

assigned as the reference standard against the other three generic brands.

2. Subject

Twelve healthy male volunteers aged from 24 to 58 years (mean age 33.42 years) with a mean weight of 57.79 kg (range 50.5 to 70.0 kg) were enrolled into the study (Appendix B). The volunteers were required to be free of serious cardiovascular, hepatic, renal, gastrointestinal diseases,drug abuse and alcoholic dependence as assessed by reviewing of their medical history. The subjects abstained from alcohol containing beverages and medications for 2 weeks before and until completion the study. None of the volunteers had a history of allergic reactions to a quinolone antibiotic and related compound. The information of possible side effects of ciprofloxacin were explained to all subjects. Each subject participated in the experiment after informed consent was obtained.

3. Experimental Design

The study was conducted using a crossover design. Each subject received the drug in a randomized order with a one-week washout period between each administration as shown in Table 1. After fasting overnight, subjects were given a 250-mg tablet of ciprofloxacin and 200 ml of water in the morning. Breakfast was allowed two hours post dose.

Table 1 Treatment Schedule

Subject No.	Week			
	1	2	3	4
1	A[a]	B	C	D
2	B	D	A	C
3	C	A	D	B
4	D	C	B	A
5	A	B	C	D
6	B	D	A	C
7	C	A	D	B
8	D	C	B	A
9	A	B	C	D
10	B	D	A	C
11	C	A	D	B
12	D	C	B	A

a. Each A, B, C, and D represented the brand name of ciprofloxacin tablets

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4. Sample Collection

Blood samples were drawn from a forearm vein of each subject prior to dosing and at 0.5, 1.0, 1.5, 2.0, 2.5, 3, 5, 8 and 12 hours after drug administration. The samples were kept in heparinized tubes (one drop of 5000 I.U. /ml of heparin solution in the test tube). After centrifuging the plasma samples were separated and promptly frozen at -10°C until the time of assay.

5. Analysis of Ciprofloxacin in Plasma

Plasma ciprofloxacin concentrations were determined by HPLC method modified from Morton et al (1986) and Pauliukonis et al (1984) as follows :

Plasma sample 0.5 ml

- added 1 ml of Methanol containing pipemedic acid 1.2 mcg/ml
- Vortexed 10 seconds
- centrifuged at 4500 rpm. for 10 minutes

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Inject 100 mcl of supernatant into the HPLC

Chromatographic system

Apparatus	:	HPLC LC-3A , Shimadzu, Japan
Column	:	Partisphere C-18 , stainless steel column, Whatman, England 12.5 cm. X 4.6 mm. I.D. , particle size 5 um
Mobile phase	:	Methanol-tetrabutyl ammonium hydroxide-phosphate buffer which prepared

by adding 2.4 gm Potassium dihydrogen phosphate, 27 ml of 0.8 M Tetrabutyl ammonium hydroxide to 1 litre of deionized water and adjusted pH with phosphoric acid to 3.0 ± 0.1 : methanol (87:13)

Internal Standard : Pipemedic acid

Detector : Fluorescence excitation at 300-400 nm emission at 450-800 nm

Flow rate : 1.8 ml/min

Temperature : Ambient

Injected volume : 100 mcl

The plasma ciprofloxacin concentrations were quantitated using the calibration curve (Appendix C).

Calibration Curve

Plasma containing ciprofloxacin standard was prepared by spiking human plasma with ciprofloxacin solution to give final concentrations of 0.15, 0.2, 0.4, 0.6, 0.8, 1.0, 1.4, 1.8 and 2.2 mcg/ml. The samples were analyzed following the procedure as previously described (Morton et al., 1986; Pauliukonis et al., 1984).

Ciprofloxacin/internal standard peak height ratios versus known ciprofloxacin concentrations were fitted to a straight line using linear regression (Appendix C)

6. Assay Validation

The analysis method was validated under the condition used by the following methods.

Precision :

Within run precision of the precipitation procedure and chromatograph was determined by analyzing replicated standard curves on the same day (three sets of calibration curve). Peak height ratio of ciprofloxacin to pipemedic acid was compared over the standard curves and the percentage coefficient of variation (%cv) for each concentration was calculated.

Between run precision were determined by comparing replicated standard curves on four different days and the percentage coefficient of variation (%cv) of each concentration was calculated.

Recovery :

Five different ciprofloxacin concentrations consisted of 0.2, 0.6, 1.0, 1.4, 2.2 mcg/ml were compared with the peaks resulting from aqueous solutions which prepared by using mobile phase replaced the human plasma in the same amount. Percent recovery of ciprofloxacin and internal standard were calculated by the formula :

$$\text{percent recovery} = \frac{\text{peak height of sample injected}}{\text{Peak height of standard solution}} \times 100$$

7. Pharmacokinetic Analysis

Individual plasma ciprofloxacin time profile from each treatment was analyzed using the CSTRIP computer program (Appendix D). A, B, I, α, β, and Ka were the pharmacokinetic parameters which directly obtained from the computer output. The other parameters, AUC and $t_{1/2}$ were calculated from the two compartmental open model equations as follow :

$$AUC = A/\alpha + B/\beta + I/K_a$$

where; A, B and I are the coefficients

α is the distribution rate constant

β is the elimination rate constant

K_a is the first order absorption rate constant

$t_{1/2} = 0.693/\beta$

The peak height concentrations and the time to peak plasma concentrations were read directly from the plots of ciprofloxacin concentration and time profiles.

8. Bioavailability and Statistical Analysis

The comparative bioavailability of the four commercial brands of ciprofloxacin film-coated tablets were evaluated using the following parameters :

a) The peak plasma concentration, C_{max}

b) The time to peak plama concentration, t_{max}

c) The area under the plasma concentration time curve, AUC

d) The first order absorption rate constant K_a

A one way analysis of variance (ANOVA) was performed to test the statistically significant differences among the four treatments and the student's t-test was used to test for the difference between treatments at the significant level of $\alpha = 0.05$. The test products are considered to be bioequivalent to innovator's product if the pharmacokinetic parameters (C_{max}, t_{max} and AUC) of the three

local manufactured brands show no statistically significant difference at $\alpha = 0.05$ from that of the innovator's product.

C. In Vitro-In Vivo Correlation Study

A pearson's correlation coefficient test was used to analyze the relationship between the in vitro and in vivo parameters. The in vitro parameters were both the disintegration times and the dissolution rate constants and the in vivo parameters were the peak plasma concentration (C_{max}), the time to peak plasma concentration (t_{max}), and the area under the peak plasma concentration-time curve (AUC).

D. Assumption

1. All pharmacokinetic processes of ciprofloxacin occured in the body followed the first order kinetic.

2. Statistically significant differences were made at $\alpha = 0.05$

3. The pharmacokinetic parameters obtained from any subjects which were too low or too high from most corresponding pharmacokinetic parameter values would be rejected.

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