Chapter III

MATERIALS AND METHODS

Materials

A. Test products

Six commercial brands of Norfloxacin, 400 mg film-coated tablets were randomly purchased from drugstores. One was the innovator's product which was assigned as the reference standard. Others were five locally manufactured brands.

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

B. Reagents

1. Working standard ; Norfloxacin powder , potency 98.90 % (Pond's Chemical), Batch no. LS 801
2. Internal standard ; Pipemidic acid (Sigma) Lot no. P 79032
3. Glacial acetic acid GR (E.Merck) Lot no. 706 K 411156

4. Sodium hydroxide AR (E.Merck) Lot no. 735 C 642598
5. 85% Ortho phosphoric acid GR(E.Merck) Lot no. K 2771873
6. Acetonitrile AR (E.Merck) Lot no. 8248408
7. Monobasic sodium phosphate GR (BDH chemicals Ltd.) Lot no. 9528440 E
8. Methanol AR (E. Merck) Lot no. 903 K 11315509
9. 25% Tetrabutyl ammonium hydroxide in Methanol AR (Fluka) Lot no. 86882
10. Monobasic potassium phosphate GR (E.Merck) Lot no. 825 A 323473
11. Buffer pH 4.0 (E.Merck) Lot no. 87049679 a
12. Heparin 5000 i.u/ml (NOVO) Batch no. 4180042 Mfd 1/10/88, Exp 1/10/91.

C. **Apparatus**

1. Analytical balance (Mettler H51 AR and Sartorious 1615 MP, West Germany)
2. Disintegration tester (Manesty machines Ltd, England)
3. Dissolution apparatus (72 RL, Hanson Research Corp., U.S.A.)
4. Spectrophotometer (Spectronic 2000, Bausch & Lomb, U.S.A.)
5. High Performance Liquid Chromatography (LC-3A, Shimadzu, Japan)

6. Digital pH meter (PBS 730, EL-Hama Instruments, Israel)
7. Vortex mixer (Vortex-Genie, Scientific Industries. Inc., U.S.A.)
8. Refrigerated centrifuge (Sigma 302 K , Sigma Lab. Centrifuges Gmbtt, West-Germany)
9. Waterbath (W-O 350, Willi Memmert KG, West-Germany)
10. Digital computer (IBM Compatible 16 Bit, Micro Source, Thailand)

Methods

A. **In Vitro Studies**

Six commercial brands of norfloxacin, 400 mg film-coated tablets, were evaluated using the official and non-official tests of the United State Pharmacopoeia XXII and/or the British Pharmacopoeia 1988 for film-coated tablets. The tests included :

1. **Uniformity of Weight**

Twenty tablets from each product of norfloxacin tablets were randomly sampled and accurately weighed tablet by tablet according to the British Pharmacopoeia 1988 (London Her Majesty's Stationery Office, 1988). The average weight and standard deviation were calculated.

2. Assay for Content of Active Ingredient

The amount of norfloxacin in tablets was determined according to the United State Pharmacopoeia XXII (United States Pharmacopoeial Convention, Inc. 1990). The method was described as following :

Assay preparation :

Weigh and finely powder not less than 20 norfloxacin tablets. Transfer an accurately weighed portion of the powder equivalent to about 100 mg of norfloxacin, to a 200 ml volumetric flask. Add 80 ml of Mobile Phase (Appendix B), sonicate for 10 minutes, dilute with phosphoric acid solution (1 in 1000) to volume, and mix. Transfer 10.0 ml of this solution to a 25 ml volumetric flask, dilute with Mobile Phase to volume, mix, and filter through a filter having a porosity of 1 μm or less.

Standard preparation :

Dissolve an accurately weighed quantity of USP Norfloxacin RS quantitatively in Mobile Phase, and dilute quantitatively and stepwise if necessary, with Mobile Phase to obtain a solution having a known concentration of about 0.2 mg/ml.

Separately injects equal volumes (about 10 µl) of the Standard preparation and the Assay preparation into the liquid chromatograph. The ratio of peak area of the Standard preparation and the Assay preparation were determined. The quantity of norfloxacin (in mg.) in the portion of tablets was calculated from the formula 500 C (r_u/r_s) in which C was the concentration in mg/ml of USP Norfloxacin RS in the Standard preparation and r_u and r_s were the peak area obtained from the Assay preparation and the Standard preparation, respectively.

Operating condition

Precondition the column several hours with degassed 0.1 M monobasic sodium phosphate adjusted with phosphoric acid to a pH 4.0 .

Apparatus	: HPLC LC-3A , Shimadzu, Japan
Column	: µ Bondapak C_{18}, Stainless steel column, Water Associates Pty, Ltd., U.S.A. I.D. 3.9 mm. x 30.0 cm. particle size 10 µ
Mobile Phase	: Acetonitrile : Phosphoric acid (1 in 1000) [150:850]
Detector	: UV at 275 nm.
Flow rate	: 2 ml/min
Temperature	: 40 ± 1.0 °C
Injected Volume	: 10 µl

3. Disintegration Test

The disintegration tests for six brands of norfloxacin film coated tablets were conducted according to the British Pharmacopoeia 1988 (London Her Majesty's Stationery Office, 1988)

Procedure: A tablet was placed in each tube of the basket,then a disc was added in each tube. The apparatus was operated using water maintained at 37 $\pm$ 1 °C as the immersion fluid. The tablets passed the test if all six tablets had disintegrated completely within one hour . If any of the tablets failed to disintegrate,the test was repeated on a further six tablets and the immersion fluid was replaced by 0.1 N hydrochloric acid maintained at 37$\pm$ 1 °C . The tablets then passed the test if all six tablets , in the acid medium , had disintegrated within one hour .

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

4. Dissolution Test

According to the United State Pharmacopoeia XXII (United States Pharmacopoeial Convention Inc, 1990), dissolution of norfloxacin tablets were established using the paddle method and buffer pH 4.0 as dissolution medium (Appendix B).

Procedure: Seven hundred and fifty millilitres of dissolution medium was placed in the vessel and equilibrated at 37 ± 0.5 °C. A tablet was placed in each vessel. The apparatus was then immediately operated and maintained stirring speed at 50±2 r.p.m. Five millilitres of samples were taken from each vessel just prior to introducing the tablet and at 5, 10, 15, 20, 25, 30, 45, 60, 80, 100, and 120 minutes intervals after the tablet was already placed in the vessel (except for brand C the collecting time was extended until 270 minutes) An equivalent amount of temperature equilibrated buffer pH 4.0 was added immediately after each sampling to maintain a constant volume of dissolution medium through the test. The amount of drug dissolved was determined using a UV spectrophotometer at 313 nm. and a calibration curve.

Calibration curve:

Standard solution of norfloxacin with concentrations of 5, 6, 7, 8, 9, 10, 12, 14, 16, 18, and 20 µg/ml in buffer pH 4.0 were prepared and determined using a UV spectrophotometer at 313 nm. Absorbances obtained versus known concentrations were fitted to a straight line using linear regression (Appendix D). The calibration curve was linear between the concentrations of 5 to 20 µg/ml.

5. **Evaluation of the In Vitro Studies**

The physical characteristics of all six brands of norfloxacin tablets were examined and evaluated using general standard of the United State Pharmacopoeia and/or the British Pharmacopoeia to determine which brand met the requirements.

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

B. **In Vivo Studies**

1. **Test Products**

Four commercial brands of norfloxacin tablets with differences in their dissolution characteristics were selected. One was the innovator's product which was assigned as the reference standard. Others were those with maximum, moderate and minimum dissolution values, respectively.

2. **Subjects**

Twelve healthy male volunteers participated in the study. They were 19 to 36 years old (mean age 24.17 ± 5.02 years). They had normal body builds with mean weight and height of 61.58 ± 7.33 kg. (range 53 to 77 kg.) and 169.50 ± 5.21 cm. (range 163 to 177 cm.),

respectively (Appendix C). All subjects received a full physical examination as well as, renal and liver function tests, and a haematological profile to assure the absence of any diseases. None of the volunteers had a history of allergic reactions to a quinolone antibiotic and related compounds. The methods and conditions were clearly explained to all subjects. Subjects took no medication of any sort and/or alcoholic preparations two weeks prior to treatment and throughout the study. Informed consent was signed and obtained from each subject before entering the experiment.

3. **Drug Administration**

A single oral dose of 400 mg. norfloxacin tablet with 200 ml of water was given to all subjects in the morning following an overnight fast. No food and/or soft drink was allowed until two hours post dose.

4. **Experimental Design**

The study was conducted in 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.

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 norfloxacin tablets

5. **Sample Collection**

Blood samples were withdrawn from a forearm vein of each subject. The vein was kept patent by small flushing doses of heparinized saline (100 i.u./ml). Blood samples (5 ml) were obtained from the forearm cannula after discarding the first ml of blood prior to

dosing and to 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 6.0, 8.0 and 12.0 hour after administration. The samples were kept into heparinized tubes (one drop of 5000 i.u./ml of heparin solution in the test tube) After centrifugation at 3000 r.p.m. for 10 minutes, the plasma samples were seperated and stored at -10 °C until subsequent analysis.

6. **Determination of Norfloxacin in Plasma**

Plasma norfloxacin concentrations were determined by high performance liquid chromatography using a modified method as described by Pauliukonis et al (1984) and Morton et al (1986). The procedure was developed as follows :

Plasma sample 1 ml

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- added 1 ml of Methanol (contain 2.5 µg of pipemidic acid)
- vortexed 30 seconds then centrifuged at 4500 r.p.m. for 10 minutes

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inject 20 µl of supernatant into the HPLC

Operating Condition

Apparatus : HPLC LC-3A, Shimadzu, Japan

Column : µBondapak C_{18},
Stainless steel column,
Water Associates Pty, Ltd., U.S.A.
pre-column 5 cm. x 2.0 mm. i.d.,
analytical column 30 cm. x

3.9 mm. i.d. particle size 10 μ

Mobile phase : Methanol-Tetrabutyl ammonium hydroxide-Phosphate buffer (pH 3.0) which was prepared by adding 1.67 ml of 85% o-phosphoric acid, 20 ml of tetrabytyl ammonium hydroxide (0.8 M) and 240 ml of methanol to 1 liter of deionized water. The mobile phase was filtered and degassed before use.

Internal Standard : Pipemidic acid

Fluorescence detector : $\lambda_{excitation}$ at 300-400 nm
$\lambda_{emission}$ at 450-800 nm

Flow rate : 2 ml/min

Chart speed : 2 mm/min

The norfloxacin concentrations in plasma samples were quantified from the calibration curve (Appendix D).

Calibration curve :

Known amounts of standard norfoxacin were added to 1 ml of pooled drug free plasma to prepare the concentrations of 0.1, 0.2, 0.5, 0.7, 1.0, 1.5, 2.0 and 2.5 μg/ml. These samples were analyzed following the

same procedure as previously described (Pauliukonis et al., 1984; Morton et al., 1986)

The ratios of the peak height of norfloxacin to internal standard obtained versus the known norfloxacin concentrations were fitted to a straight line using linear regression (Appendix D).

7. Pharmacokinetic Analysis

Pharmacokinetics of norfloxacin was assumed to be linear. Individual plasma norfloxacin profile from each treatment was fitted to the appropriate pharmacokinetic model using the CSTRIP computer program (Appendix E). Relevant pharmacokinetic parameters (A, B, C, α, β, and K_a) were directly obtained from the computer output. The other parameters, (AUC and $t_{1/2}$) were calculated based on the two compartment open model equations.

$$AUC = A/\alpha + B/\beta + C/K_a \quad \text{--------(1)}$$

where: A, B and C are the coefficient
α is rate constant for the distribution phase
β is rate constant for the elimination phase
K_a is the apparent first order absorption rate constant

$$t_{1/2} = 0.693/\beta \quad \text{--------(2)}$$

8. Bioavailability and Statistical Analysis

The comparative bioavailabilities of all four brands of norfloxacin tablets were evaluated using the following parameters :

a) The peak plasma concentration, C_{max}

b) The time to peak plasma concentration, T_{max}

c) The area under the plasma concentration time curve (AUC_0^∞)

The relative bioavailability of tested products given at the same dosage level were calculated by an equation

$$F_{rel} = \frac{AUC_{test}}{AUC_{ref}} \qquad \text{----------(3)}$$

where; AUC_{test} was the area under the curve of the tested products

AUC_{ref} was the area under the curve of the referenced products

A oneway analysis of variance (ANOVA) and the student's t-test were utilized as a tool to test for the statistically significant differences among the four treatments at the significant level of $\alpha = 0.05$.

All products were completely bioequivalent if their relevant pharmacokinetic parameters (C_{max}, T_{max} and AUC) were not statistically significant difference at $\alpha = 0.05$ and/or these relevant pharmacokinetic parameters

were not different greater than 20% among and between products.

C. **In Vitro-In Vivo Correlative Study**

The relationship between the in vitro and the in vivo parameters was analyzed using the pearson's correlation coefficient tests. The in vitro parameters to be interested were both the disintegration times and the dissolution rate constants whereas the in vivo parameters were those the pharmacokinetic parameters which related to the absorption rate and the extent of absorption of the drug, i.e., 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_0^∞).