CHAPTER III

MATERIALS AND METHODS

Materials

A. Test Products

Eight commercial brands of diclofenac sodium 25 mg enteric-coated tablets were bought from commercial sources (mainly from different drug stores) without any attempt to procure or select lots. One was the innovator's product which was assigned as the reference standard. The letters (A, B, C, D, E, F, G and H) were given to represent the brand names of each product. Other information of these products were all summarized in Appendix A.

B. Reagents

1. Working standard diclofenac sodium powder, (Siam Pharmaceuticals) potency 99.7% Lot No. 914DEG-34

2. Internal standard, mefenamic acid powder, (Siam Pharmaceuticals) potency 100% Lot No. 37854

3. Acetonitrile HPLC grade, (J.T. Baker Inc.), Lot no. 9017-03

4. Methanol GR. (E. Merck, West Germany), Lot no. 033K 14349909

5. Sodium acetate anhydrous GR. (E. Merck, West Germany),Lot no. 036 TA 461168

6. Sodium hydroxide GR. (E. Merck, West Germany), Lot no. 846 C 700998

7. Glacial acetic acid GR. (E. Merck, West Germany), Lot no.K 11297363

8. n-Hexane GR. (E. Merck, West Germany), Lot no. I 808467

9. 2-Propanol GR. (E. Merck, West Germany), Lot no. 640 K 3033134, UN NR 1219

10. di-Sodium hydrogen phosphate anhydrous AR. (Fluka AG, Switzerland), Lot no. 260632 486

11. ortho-Phosphoric acid 85% GR. (E. Merck, Germany), Lot no. 931 K 12677273

12. Heparin 5,000 iu./ml (Novo Industrials, Copenhagen, Denmark) Lot no. 2700031

C. Apparatus

1. Analytical Balance (Sartorius 1615MP6E, GMBH, Germany)

2. Disintegration Tester (Model 64.700-136, Hanson Research Corp., Northridge, CA., U.S.A.)

3. Dissolution Apparatus (72RL, Hanson Research Corp., Northridge, CA., U.S.A.)

4. Spectrophotometer (Spectronic 2000, Bausch and Lomb, N.Y., U.S.A.)

5. High Performance Liquid Chromatography Apparatus (LC-3A, Shimadzu, Japan)

6. Digital pH meter (Orion, U.S.A.)

7. Vortex mixer (Vortex-Genie, Scientific Industries Inc. Bohemia, N.Y., U.S.A.

8. Refrigerated Centrifuge (Sigma 302K, Sigma Lab Centrifuge Gmbt, Germany)

9. Digital Computer (IBM Compatible 16 Bit, Micro Source)

D. **Method**

In Vitro Studies

Since diclofenac sodium is not described in any of the pharmacopoeias, all of the eight commercial brands of diclofenac sodium, 25 mg enteric-coated tablets were then evaluated using the official and non-official tests of the United States Pharmacopoeia XXII and/or British Pharmacopoeia 1988 for enteric-coated tablets. The tests included :

1. Uniformity of Weight (B.P. 1988)

Twenty tablets of each of the eight commercial brands of diclofenac sodium 25 mg enteric-coated tablets were randomly sampled and accurately weighed tablet by tablet. The average weight and standard deviation were calculated for each brand.

2. Assay for Content of Active Ingredient in Tablets

The amount of diclofenac sodium in tablet was determined according to the following procedure (Somkiat, 1991):

Twenty tablets were weighed and finely powdered. A portion of the powder, equivalent to about 50 mg of diclofenac sodium was accurately weighed, and transferred to a 200 ml-volumetric flask. A 20.0 ml of 0.1 N sodium hydroxide was added and shaken for 20 minutes to dissolve the powder, then methanol was added to make up a 200 ml solution. The solution was then mixed using a mixer and filtered through filter paper. A 5.0 ml of clear solution was withdrawn and dilute with 0.01N methanolic sodium hydroxide to 100 ml. The dilute solution was concomitantly determined for its absorbance at the wavelength of maximum absorbance about 280 nm, using a spectrophotometer having 0.01 N methanolic sodium hydroxide as the blank. The amount of diclofenac sodium in the samples were calculated according to the provided Calibration curve (Appendix B).

3. Disintegration test

The disintegration test for all eight brands of diclofenac sodium 25 mg enteric-coated tablets were conducted according to the United States Pharmacopoeia XXII method for enteric-coated tablets (United States Pharmacopoeial Convention Inc., 1990).

Procedure : Placed 1 tablet in each of the six tubes of the basket in water at room temperature for 5 minutes. The apparatus was then operated without adding the disk and using simulated gastric fluid TS maintained at 37 $\pm$ 2° C as the immersion fluid. After

1 hour of operation in simulated gastric fluid TS, show no evidence of disintegration, cracking or softening of the tablet was observed. Then add a disk to each tube, and operate the apparatus, using simulated intestinal fluid TS, maintained at 37 ± 2° C as the immersion fluid. The average disintegration time and standard deviation were then calculated.

4. Dissolution test

The dissolution test of each brand of diclofenac sodium 25 mg enteric-coated tablets were operated using the paddle method of the United States Pharmacopoeia XXII (United States Pharmacopoeial Convention Inc., 1990) and simulated intestinal fluid TS without pancreatin (pH 7.5 ± 0.1) as dissolution medium (Appendix C).

Procedure : Nine hundred millilitres of dissolution medium was placed in each of the six vessels and equilibrated at 37 ± 0.5°C. A tablet was placed in each vessel. Then the apparatus was immediately operated and maintained stirring speed at 100 ± 2 rpm. Five millilitres of samples were withdrawn from each vessel just prior to introduce the tablets and at 5, 10, 15, 20, 25, 30, 40, 50, 60, 75, 90, 105 and 120 minutes after the tablets were already placed in the vessels and replaced at once with the corresponding volumes of the temperature equilibrated dissolution medium. The amount of diclofenac sodium dissolved was determined using a UV

spectrophotometer at maximum wavelength of 280 nm, in comparison with a calibration curve. The dissolution rate of the drug was calculated by sigma-minus method.

Calibration Curve

Standard solutions of diclofenac sodium with concentration of 2.5, 5, 10, 15, 20, 25 and 30 mcg/ml in simulated intestinal fluid TS without pancreatin (pH 7.5 $\pm$ 0.1) were prepared and analyzed using a UV spectrophotometer at the wavelength of 280 nm. Absorbances obtained versus known concentration were fitted to a straight line using linear regression (Appendix B).

In Vitro Evaluation

The characteristics of all eight commerical brands of diclofenac sodium 25 mg enteric-coated tablets were examined and evaluated, using general standard of the United States Pharmacopoeia XXII and/or the British Pharmacopoeia 1988 to determine whether which brand passed the requirements. The differences in disintegration times and dissolution rates among the eight brands were determined by one way analysis of variance (ANOVA) at the significant level of α=0.05. If the results showed statistically significant difference, the difference of these values between the innovator's product and each brand was examined using t-test. The correlation between the disintegration times and the

dissolution rates were determined by correlation coefficient test.

In Vivo Studies

1. Test Products.

Four commercial brands of 25 mg diclofenac sodium enteric-coated tablets with differences in their dissolution properties were selected. One was the innovator's product which was assigned as the reference product. Others were those with maximum, moderate and minimum dissolution rates in simulated intestinal fluid TS without pancreatin, respectively.

2. Subjects.

Twelve healthy male volunteers participated in this study. All of them signed their informed written consents. They were 19 to 41 years old (mean age 31.58 $\pm$ 7.46 years). They had normal body builds with mean weight of 59.33 $\pm$ 7.40 kg. (range 47 to 70 kg) and mean height of 166.67 $\pm$ 5.55 cm (range 157 to 177 cm) (Appendix D). None of the volunteers had a history or evidence of gastrointestinal, cardiac, renal and hepatic diseases. They were assessed by undergoing physical examination and clinical laboratory testing. None was allergic to diclofenac sodium and related compounds. All subject abstained from other drugs intake and alcoholic preparations two weeks prior to the experiment and throughout the study period. The method and conditions of the study were clearly explained to all subjects.

3. Drug Administration.

Two 25 mg of diclofenac sodium enteric-coated tablets were given orally in a single dose. The doses were administered with 200 ml of water after an overnight fast. The subjects were allowed to have breakfast 2 hours after dosing.

4. Experimental Design.

The study was conducted in a crossover design. Each subject received the drug in a randomized order with one week washout period between each administration as shown in Table 3.

Table 3 Dosing Schedule :

Subject	Week			
	1	2	3	4
1 - 3	A*	B*	D*	H*
4 - 6	B	D	H	A
7 - 9	D	H	A	B
10 - 12	H	A	B	D

*Each A, B, D and H represented the brand name of 25 mg diclofenac sodium enteric-coated tablets.

5. Sample Collection

Blood samples were drawn from a forearm vein of each subject. The vein was kept patent by small flushing doses of heparinized saline (100 i.u./ml). Five millilitres of blood samples were collected prior to dosing and at 0.5, 1, 1.5, 2.0, 2.5, 3, 4, 6, 8 and 10 hours after drug administration. The samples were kept in heparinized tube (three drops of 5,000 i.u./ml of heparin solution in the test tube). After centrifugation at 3,000 rpm. for 10 minutes, the plasma samples were separated and stored at -20°C for subsequent analysis.

6. Determination of Diclofenac Sodium in Plasma

Plasma diclofenac sodium concentration were determined by high performance liquid chromatography. The method used was modified from the method as described by Chan *et al.* (1982). The procedure was described as follow.

To 1 ml of plasma in a glass tube was added 200 mcl.of the solution of internal standard (added amount 200 ng) and 2 ml of H_3PO_4 (2.5 N), and the tube was then vortexed for 10 seconds.After agitation, 5 ml of a hexane : isopropanol mixture (90:10) were added to the sample. The tube was vortexed for 3 minutes and centrifuged for 10 minutes at 2500 rpm. The organic layer was transferred to a glass tube and evaporated to dryness under a stream of nitrogen gas. The residue was

reconstituted in 150 mcl. of mobile phase. An aliquot of 50 mcl. was injected into the chromatograph.

HPLC Conditions

Apparatus : LC-3A, Shimadzu, Japan

Column : Analytical column : LiChroCART ^{R} C_{18} 125 x 4.0 mm. encapped

Precolumn : LiChrosphir ^{R} 100 RP-18 5 cm x 2.0 mm. encapped

Mobile phase : 21:17:62 v/v of methanol, acetonitrile and 0.02 M acetate buffer (pH 7) (Appendix C). The mobile phase was mixed, filtered and degassed before use.

Internal standard : Mefenamic acid (concentration 1 mcg./ml. in methanol).

UV detector : 225 nm.

Flow rate : 1.6 ml./min.

Attennuation : 2^{1} mv/full scale

Pressure : 250 kg./cm^{2}

Chart speed : 2 mm./min.

Operating temperature : ambient

Injection volume : 50 mcl.

The concentrations of diclofenac sodium in plasma samples were quantified from the calibration curve (Appendix B).

Calibration Curve

Diclofenac sodium working solution (1, 2, 4, 8 mcg./ml.)and internal standard (mefenamic acid) working solution (1 mcg./ml.) were prepared in methanol. Working standard solution containing 100 to 2,400 ng./ml. of diclofenac sodium and 200 mcl. of mefenamic acid solution were prepared in 1 ml. of pooled drug-free plasma. All samples were analyzed following the same procedure as previously described.

Calibration curves were generated using the least square regression of the peak height ratio of the drug to the internal standard against the known standard plasma diclofenac sodium concentration.

Assay Validation

Within-run precision was determined by analyzing the three sets of the calibration curves at the same day. Peak height ratio of diclofenac sodium to mefenamic acid 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 of three standrad curves injected on three different days, the percent coefficient of variation (%CV) of each concentration was determined.

To assess the recovery of diclofenac sodium and mefenamic acid, peak heights of either diclofenac sodium and internal standrad in plasma obtained from hexane-isopropanol extraction was compared with those in mobile phase solution.

7. Pharmacokinetic Analysis

The pharmacokinetic analysis of individual plasma diclofenac sodium concentrations from each treatment was established using the conventional method and the CSTRIP.

In case of the conventional method the peak plasma concentration and time to peak plasma concentration were directly observed from the data, the area under the plasma concentration-time curve was calculated using the trapezoidal rule. Other pharmacokinetic parameters were obtained using the residual method, and the biological half-life was calculated using an equation.

$$t_{1/2} = 0.693/K_{el} \qquad \text{Eq.1}$$

In case of analysis using the CSTRIP computer program, the data indicated that a biexponential could be best described the concentration time-curve of the diclofenac sodium as shown in equation 2.

$$C_t = A_1 e^{-K_{el}(t-t_{lag})} - A_2 e^{-K_a(t-t_{lag})} \qquad \text{Eq.2}$$

A_1, A_2, K_a, K_{el} and t_{lag} are the parameters estimates obtained directly from the computer output where :

C_t = the plasma diclofenac sodium concentration at any time, t

A_1, A_2 = the ordinate intercept constants

K_a = the absorption rate constant

K_{el} = the elimination rate constant

t_{lag} = the lag time

The peak plasma concentration (C_{max}), the time to peak plasma concentration (t_{max}), the area under the concentration-time curve (AUC) and the biological half-life ($t_{1/2}$) of diclofenac sodium were calculated by the following equations.

$$C_{max} = A_1 e^{-K_{el} t_{max}} - A_2 e^{-K_a t_{max}} \quad \text{Eq.3}$$

$$t_{max} = \ln (K_a / K_{el}) / (K_a - K_{el}) + t_{lag} \quad \text{Eq.4}$$

$$AUC_{0 \to 10} = A_1 / K_{el} - A_2 / K_a \quad \text{Eq.5}$$

Evaluation of Bioequivalence

The bioavailability of 25 mg diclofenac sodium enteric coated tablets in this study relative to the reference were assessed using the three relevant pharmacokinetic parameters, C_{max}, t_{max} and AUC values.

The differences in C_{max}, t_{max} and AUC values among the four selected brands were determined by one way analysis of variance (ANOVA) at the significant level of α=0.05. If the results showed statistically significant difference, the difference of these values between the innovator's product and each selected brand were examined by means of t-test. In the same way, if the result showed no statistically significant differences from those of the innovator's product, the tested brands were bioequivalent to the innovator's product.

9. In Vitro-In Vivo Correlation Study

Correlation coefficient test was used to test the relationship between the in vitro parameters, the disintegration times and dissolution rate constants, and the in vivo parameters, including C_{max}, t_{max} and AUC of all brands.

10. Assumption

Pharmacokinetic of diclofenac sodium was linear. This refered that the rate of elimination of the drug and/or processes occured in the body followed first order kinetic.