CHAPTER III

MATERIALS AND METHODS

Materials

A. Test Products

Four commercial brands of 300 mg gemfibrozil capsules were in vitro and in vivo evaluated. One was the innovator's product which was assigned as the reference standard against the other three locally manufactured brands.

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

B. Reagents

1. Standard gemfibrozil powder (Siam Pharmaceutical)
   Lot No. 90078
2. Standard ibuprofen powder (Siam Pharmaceutical)
   Lot No. 12475
3. Acetonitrile HPLC grade (Baker Analyzed)
   Lot No. H 4799017032 E
4. Monobasic potassium phosphate AR (Merck)
   Lot No. G 715085

5. Methanol HPLC grade (Merck)
   Lot No. 165107
6. Hydrochloric acid AR (Merck)
   Lot No. 13065917
7. Cyclohexane (Baker Analyzed)
   Lot No. 2491D200
8. Sodium hydroxide AR (Merck)
   Lot No. C 788298
9. Orthophosphoric acid 85% GR (Merck)
   Lot No. K 12677273
10. Heparin 5,000 i.u./ml (Leo Pharmaceutical)
    Lot No. B 05 A

C. Apparatus

1. Analytical Balance (Mettler H 51 AR)
2. Disintegration Tester (Manesty machines Ltd., England)
3. Dissolution Appparatus (72 RL, Hanson Research Corp., USA)
4. Spectrophotometer (Spectronic 2000, Bausch & Lomb, USA)
5. Digital pH Meter (Orion, USA)
6. Vortex Mixer (Vortex Genic, Scientific Industries Inc., USA)
7. Refrigerated Centrifuge (Sigma 302 K, Sigma Lab Centrifuge Gmbt, Germany)
8. Shaker (Edmund Buhler, Germany)

9. Water Bath (Mammert, Edelstaph Rost Frei, Germany)
10. High Performance Liquid Chromatography (LC-3A, Shimadzu, Japan)
11. Computer (IBM Compatible, 16 Bit)

Methods

A. In Vitro Studies

All four commercial brands of gemfibrozil capsules were evaluated following the official tests as stated in the monograph of gemfibrozil capsule in the United States Pharmacopoeia XXII (United States Pharmacopoeial Convention Inc., 1990)

1. Weight Variation

Weigh accurately 10 capsules individually, taking care to preserve the identity of each capsule. Remove the contents of each capsule by a suitable means. Weigh accurately the emptied shells individually, and calculate for each capsule the net weight of its contents by subtracting the weight of the shell from the respective gross weight.

2. Assay for Content of Active Ingredient

The method was described as follows :

Mobile Phase : Add 10 ml of glacial acetic acid

to 800 ml of methanol in a 1000-ml volumetric flask, dilute with water to volume, mix, and filter through a membrane filter.

Standard Preparation : Dissolve a suitable quantity of standard gemfibrozil, accurately weighed, in methanol to obtain a solution having a known concentration of about 1 mg per ml. Transfer 5.0 ml of this solution to a 25-ml volumetric flask, dilute with Mobile Phase to volume, and mix.

Assay Preparation : Remove, as completely as possible, the content of not less than 20 gemfibrozil capsules, weigh, and mix. Transfer an accurately weighed portion of the powder, equivalent to about 100 mg of gemfibrozil, to a 100-ml volumetric flask, add about 80 ml of methanol, and shake to dissolve. Dilute with methanol to volume, mix and filter. Transfer 5.0 ml of this clear solution to a 25-ml volumetric flask, dilute with Mobile Phase to volume, and mix.

Chromatographic System : The High Performance Liquid Chromatography is equipped with a 276-nm UV-detector and a 3.9-mm x 30-cm column that contains packing L 1. The flow rate is about 0.8 ml per minute. Chromatograph the standard preparation, and record the peak responses, the relative standard deviation for replicate injections is not more than 2.0%.

Procedure : Separately inject equal volumes (10 mcl) of the Standard Preparation and the Assay Preparation into the chromatograph by means of a suitable microsyringe, record the chromatograms, and measure the response for the major peaks. Calculate the quantity, in mg, of $C_{15}H_{22}O_3$ in the portion of capsules taken by the formula :

$$500\ C\ (r_u/r_s)$$

in which C = Concentration, in mg per ml, of standard gemfibrozil in the Standard Preparation

r_u = Peak responses obtained from the Assay Preparation

r_s = Peak responses obtained from the Standard Preparation

3. Disintegration Test

The disintegration tests for four brands of gemfibrozil capsules were conducted according to the United States Pharmacopoeia XXII (United States Pharmacopoeial Convention Inc., 1990) method for capsules.

Procedure : 1 capsule of the drug was placed in each of the six tubes of the basket, then a disc was added to each tube. The apparatus was operated using water maintained at 37 $\pm$ 1°C as the immersion fluid. The capsules passed the test if all six had disintegrated completely within 15 minutes.

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

4. Dissolution Test

The dissolution test for four brands of gemfibrozil capsules were determined by the paddle method of the United States Pharmacopoeia XXII (United States Pharmacopoeial Convention Inc., 1990) and 0.2 M phosphate buffer (pH 7.5±0.1) was the dissolution medium (Appendix B).

Procedure : Nine hundred millilitres of dissolution medium was placed in each of the six vessels and equilibrated at 37 ± 0.5°C. A capsule was placed in each vessel. The apparatus was immediately operated and maintained stirring at 50 ± 2 rpm. Five millilitres of sample were taken at 5, 10, 15, 20, 30, 45, 60, 90, 120, 150 and 180 minutes after the capsules were already placed in the vessels and replaced by the corresponding volumes of the temperature equilibrated dissolution medium. The amount of drug dissolved was determined by a UV spectrophotometer at 276 nm, in comparison with a calibration curve. The dissolution rate of the drug was calculated by sigma-minus method.

Calibration Curve : Standard solution of gemfibrozil with concentration of 4, 20, 40, 60, 80, 100, 120 and 150 mcg per ml in 0.2 M phosphate buffer (pH 7.5 ± 0.1) were prepared and determined by a UV spectrophotometer at 276 nm.

Absorbance obtained versus known concentrations were fitted to a straight line using linear regression (Appendix C).

5. Evaluation of the In Vitro Studies

The characteristics of all four brands of gemfibrozil capsules were examined and evaluated whether they met the requirements stated in the United States Pharmacopoeia XXII (United States Pharmacopoeial Convention Inc., 1990).

The differences in disintegration times and dissolution rate constants among the four brands were determined by one way analysis of variance (ANOVA) at the significant level of $\alpha = 0.05$. If the results were statistically significant different, the difference of these values between the innovator's brand and each brand was examined by means of t-test. The correlation between the disintegration time and the dissolution rate constant was determined by correlation coefficient test.

B. In Vivo Studies

1. Test Products

All four brands of gemfibrozil capsules commercially available in Thailand were used in this study.

2. Subjects

Twelve male volunteers with a mean age of 31.17 ± 6.85 years (range 20 to 40) participated in this study. They had normal body builds with mean weight of 59.00 ± 6.70 kg (range 47 to 67) and mean height of 168.50 ± 6.53 cm (range 157 to 178). Demographic data are presented in Appendix D. All subjects were healthy based on history, clinical examination and pre-entry hematologic and biochemical tests. None was allergic to gemfibrozil. All subjects abstrainded from other drugs intake and alcoholic preparations two weeks prior to and throughout the study. The method and conditions of the study were clearly explained to all subjects. Informed consent was signed and obtained from each subject prior to entering the experiment.

3. Drug Administration

Two capsules of gemfibrozil were administered to all subjects with water (200 ml) orally 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 two hours after dosing.

4. Experimental Design

The study was conducted in a crossover design. Each subject received the drug in a randomized order (Table 1). The wash-out period was one week.

5. Sample Collection

Blood samples (5 ml) were drawn from the antecubital vein before and at 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 5.0, 7.0 and 10.0 hours post dose. All blood samples were collected in heparinized tubes. After centrifugation at 3000 rpm. for 10 minutes, the plasma was separated and stored at -20°C until subsequent assay.

6. Determination of Gemfibrozil in Plasma

Plasma gemfibrozil concentrations were determined by high performance liquid chromatography using a modification of the method described by Hengy and Kolle (1985). The procedure was developed as follows :

Table 1 Dosing Schedule

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

* A, B, C and D represent the brand name of gemfibrozil capsules

Plasma sample 0.5 ml

- added 2 mcg of ibuprofen (dissolved in 20 mcl of acetonitrile/water 1:1) as internal standard
- added 3 drops of 1 N hydrochloric acid
- mixed 30 seconds
- extracted with 5 ml of cyclohexane for 1 hr. on an automatic shaker
- centrifuged at 3000 rpm for 10 min

↓

transferred the organic top layer to a second tube

- evaporated under a gentle stream of nitrogen

↓

residue

- reconstituted in 100 mcl of the mobile phase

↓

injected 15 mcl of reconstituted sample into the HPLC

HPLC Condition for Gemfibrozil Analysis in Plasma

Apparatus	: HPLC LC-3A; Shimadzu, Japan
Column	: Stainless steel column containing C-18 bonded 5 um silica (Spherisorb ODS II)
Precolumn	: RP-18 encapped (5 um)
Analytical column	: 125 x 4.6 mm, i.d.
Mobile phase	: 1:1 v/v of acetonitrile and 0.2% phosphoric acid
UV detector	: 225 nm
Flow rate	: 2 ml/min
Chart speed	: 2 mm/min
Operating temperature	: Ambient
Pressure	: 180 kg/cm^2
Retention time	: Gemfibrozil ~ 5.10 min Ibuprofen ~ 3.21 min

The gemfibrozil concentration in plasma samples were calculated from the calibration curve (Appendix C).

Calibration Curve : Certain amount (1.0, 2.0, 6.0, 10.0, 14.0, 20.0, 30.0 and 60.0 mcg) of standard gemfibrozil were added to 1.0 ml of pooled drug free plasma. These samples were analyzed following the same procedure as described previously. The ratio of the peak height of gemfibrozil to ibuprofen (internal standard) obtained versus known gemfibrozil concentrations were fitted to a straight line using linear regression (Appendix C).

Assay Validation : The modified Hengy and Kolle's method was validated under the following conditions.

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

To assess the recovery of gemfibrozil, peak height of gemfibrozil and ibuprofen (internal standard) obtained from cyclohexane extraction were compared with those of aqueous solution.

7. Pharmacokinetic Analysis

The pharmacokinetic analysis of individual plasma gemfibrozil levels from each treatment was established using various means such as; curve fitting and/or the CSTRIP, a Fortran IV computer program, for obtaining the polyexponential estimates (Sedmen and Wagner, 1976). The analysis indicated that a biexponential equation could be

best described the concentration-time curve of gemfibrozil as shown in equation 1.

$$C_t = A_1 e^{-K_{el}(t-t_{lag})} - A_2 e^{-K_a(t-t_{lag})} \qquad \text{Eq. 1}$$

t_{lag}, A_1, A_2, K_a and K_{el} are the parameters estimates obtained from curve fitting the data.

where; C_t = the plasma gemfibrozil concentration at any time, t

t_{lag} = the lag time

A_1, A_2 = the ordinate intercept constants

K_a = the absorption rate constant

K_{el} = the elimination rate constant

The peak plasma drug concentration (C_{max}), the time to peak plasma drug concentration (t_{max}) were observed from the plots of the concentrations versus time. The area under the plasma drug concentration versus time curve (AUC) was calculated using trapezoidal rule meanwhile the biological half-life of gemfibrozil and the relative bioavailability of gemfibrozil capsule brands B, C and D with respect to brand A were calculated by the following equations.

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

$$F_{rel} = \frac{AUC_{test}}{AUC_{ref}} \times 100 \qquad \text{Eq. 3}$$

where; F_{rel} = the relative bioavailability

AUC_{test} and AUC_{ref} = the area under the plasma drug concentration versus time curve of test and reference product, respectively.

8. Evaluation of Bioequivalence

The comparative bioavailability of gemfibrozil capsules in the present study relatively to the innovator's product were assessed using the three relevant pharmacokinetic parameters, C_{max}, t_{max} and AUC (Fluehler, Hirtz and Moser, 1981).

The difference in C_{max}, t_{max} and AUC among the four commercial brands were determined by one way analysis of variance (ANOVA) at the significant level of 0.05 (Rodda and Davis, 1980; Westlake, 1972). If the results showed statistically significant differences, the difference of these values between those of the innovator's product and those of each brand were examined by means of t-test. The locally manufactured brands were considered to be bioequivalent to the innovator's product when their C_{max}, t_{max} and AUC values showed no statistically significant difference from those of the innovator's product (Wagner, 1971).

9. In Vitro-In Vivo Correlation Study

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

10. Assumption

Pharmacokinetic of gemfibrozil capsules followed first-order kinetics.