CHAPTER III

MATERIALS AND METHODS

Materials

A. Test Products

Seven commercial brands of glibenclamide tablets were randomly purchased from drugstores. Each tablet contains 5 mg. of glibenclamide. One was the innovator's product which was assigned as the reference standard.

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

B. Reagents

1. Glibenclamide powder (G.P.O.)
   potency : 99.43%
2. Glipizide (F.E. Zuellig)
   potency : 98.90%
3. Acetonitrile HPLC grade (Merck)
   Lot No. I 143930
4. Orthophosphoric acid 85% GR. (Merck)
   Lot No. K 12677273
5. Monobasic potassium phosphate AR.(Merck)

Lot No. A 459773

6. Methanol HPLC grade (Merck)
   Lot No. 165107
7. Methanol AR. (Merck) Lot No. K 13806209
8. Sodium hydroxide AR. (Merck)
   Lot No. C 788298
9. Hydrochloric acid AR. (Merck)
   Lot No. K 13065917
10. Benzene AR. (Merck) Lot No. K 14279883
11. Dichloromethane AR. (Merck)
    Lot No. K 11456150
12. Tetrahydrofuran AR. (Merck)
    Lot No. K 13550231
13. Heparin 5000 i.u./ml. (Leo) Lot No. B01A
    Mfg.date 02/90 Exp.date 02/93

C. <u>Apparatus</u>

1. Analytical Balance (Mettler H51 AR)
2. Disintegration Tester (Manesty machines Ltd., England)
3. Dissolution Apparatus (72 RL, Hanson Research Corp., USA)
4. Spectrophotometer (Spectronic 2000, Bausch & Lomb, USA)
5. Digital pH Meter (Orion, USA)
6. Vortex Mixer (Vortex-Genie, Scientific Industries Inc., USA)
7. Refrigerated Centrifuge (Sigma 302 K,

Sigma Lab Centrifuge Gmbt, Germany)

8. Shaker (Edmund Buhler, Germany)
9. Ultrasonic Bath (Bransonic 221, USA)
10. Water Bath (Memmert, Edelstaph Rost Frei)
11. High Performance Liquid Chromatography (Waters 510 HPLC Pump, equipped with Waters 484 Tunable Absorbance Detector and Waters 745 B Data Module)
12. Personal Computer (IBM Compatible, 16 Bit)

Method

A. In Vitro Studies

All of the seven commercial brands of glibenclamide tablets were evaluated according to the official tests stated in the monograph of glibenclamide tablet in the British Pharmacopoeia 1988 (London Her Majesty's Stationary Office, 1988).

1. Assay for Content of Active Ingredient

The amount of glibenclamide in tablet was determined according to the modified method from the British Pharmacopoeia 1988. The method was described as follows :

Weigh and finely powder 20 glibenclamide tablets. Transfer an accurately weighed portion of the powder equivalent to about 20 mg. of glibenclamide to a 200 ml. volumetric flask. Add 100 ml. of 0.1 M methanolic

hydrochloric acid (Appendix B), shake for about 30 minutes. Adjust to volume with 0.1 M methanolic hydrochloric acid, mix and filter. Collect the filtrate by discarding the first 20 ml. of the filtrate. Measure the absorbance of the resulting solution by a UV spectrophotometer at maximum wavelength 300 nm. Calculate the content of glibenclamide as percent labeled amount, taking 62 as the value of A (1%, 1 cm.) at the maximum 300 nm.

$$\% \text{ labeled amount} = \frac{A_u \times 1000 \times 200 \times \text{mean wt.} \times 100}{62 \times 100 \times \text{wt.of powder taken} \times 5}$$

where A_u = Absorbance of the test solution

mean wt. = Average weight of the tablets

2. <u>Uniformity of Content</u>

The content of glibenclamide of each of ten glibenlamide tablets taken at a random was determined by using the following method modified from the British Pharmacopoeia 1988.

Powder 1 tablet and transfer to a 50 ml. volumetric flask. Add 30 ml. of 0.1 M methanolic hydrochloric acid, shake for about 30 minutes. Adjust to volume with 0.1 M methanolic hydrochloric acid, mix and filter. Collect the filtrate by discarding the first 20 ml. of the filtrate. Measure the absorbance of the resulting solution by a UV spectrophotometer at maximum wavelength 300 nm. Calculate the content of glibenclamide

in each tablet as percent labeled amount, taking 62 as the value of A (1%, 1 cm.) at the maximum at 300 nm. The average percent labeled amount and standard deviation were calculted.

$$\% \text{ labeled amount} = \frac{A_u \times 1000 \times 50 \times 100}{62 \times 100 \times 5}$$

where A_u = Absorbance of the resulting solution

3. Weight Variation

Accurately weigh tablet by tablet of twenty tablets from each brand of glibenclamide tablets which were randomly sampled was performed. The average weight and standard deviation were calculated.

4. Disintegration Test

The disintegration tests for all seven brands of glibenclamide tablets were conducted according to the British Pharmacopoeia 1988.

A tablet was placed in each of the six tubes of the basket, then a disc was added in each tube. The apparatus was operated using water maintained at $37 \pm 1\,^{\circ}C$ as the immersion fluid. The tablets passed the test if all six tablets disintegrate within 15 minutes. The average disintegration time and standard deviation were then calculated.

5. Dissolution Test

The dissolution test of each brand of glibenclamide 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 B) (Jayaswal and Srivastava, 1987).

Procedure : Nine hundred millitres 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 150 + 2 rpm. Five millitres of samples were taken from each vessel just prior to introducing the tablets and at 5, 10, 15, 20, 25, 30, 45, 60, 90, 120, 150, 180, 210, 240, 270, 300 and 360 minutes after the tablets were already placed in the vessels and replaced by the corresponding volumes of the temperature equilibrated dissolution medium. The amount of glibenclamide dissolved was determined using a UV spectrophotometer at maximum wavelength of 207 nm. and a calibration curve. The dissolution rate of the drug was calculated by sigma-minus method.

Calibration Curve

Standard solutions of glibenclamide with concentration of 0.5, 1, 1.5, 2, 4, 6, 8, and 10 mcg./ml. in simulated intestinal fluid TS without pancreatin (pH 7.5 ± 0.1) were prepared. The maximum UV absorption of glibenclamide solution was shown at the wavelength of 207 nm. Absorbances obtained versus known concentrations of glibenclamide obeyed Beer's law (Appendix D).

6. Evaluation of the In Vitro Studies

The physical and chemical characteristics of all seven brands of glibenclamide tablets were examined and evaluated whether they met the requirements stated in the British Pharmacopoeia 1988 and/or the United States Pharmacopoeia XXII.

The differences in disintegration times and dissolution rates among the seven brands were determined by one way analysis of variance (ANOVA) at the significant level of 0.05. If the results showed the 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 time and the dissolution rate was determined by correlation coefficient test.

B. In Vivo Studies

1. Test Products

Four commercial brands of glibenclamide 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 rate in simulated intestinal fluid TS without pancreatin, respectively.

2. Subjects

Twelve healthy male volunteers participated in the study. They were 24 to 41 years old (mean age 30.92 $\pm$ 5.96 years). They had normal body builds with mean weight of 59.42 $\pm$ 7.82 kg. (range 50 to 76 kg.) and mean height of 167.33 $\pm$ 7.50 cm. (range 153 to 178 cm.) (Appendix C). None of the volunteers had a history or evidence of diabetes, gastrointestinal, cardiac, renal and hepatic diseases. This was assessed by undergoing physical examination and clinical laboratory testing. None was allergic to glibenclamide. All subjects 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. Informed consent was signed and obtained from each subject prior to entering the experiment.

3. Drug Administration

Prior to receiving a single oral dose of 5 mg. glibenclamide tablet, all subjects were given a 200 ml. cup of glucose solution (Glucolin^{R} 1 tablespoonful dissolved in 200 ml. of water) in the morning follwing an overnight fast. Fifteen minutes later, the assigned glibenclamide tablets were administered to all subjects with 200 ml. of water. No food and/or soft drinks was allowed until an hour and à half 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 1

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.). Five millilitres of blood samples were collected prior to drug ingestion and at 0.5, 1, 1.5, 2.0, 2.5, 3, 4, 6, 8 and 10 hours post dose. The samples were kept in heparinized tubes (one drops of 5000 i.u./ml. of heparin solution in the test tube). After centrifugation at 3000 rpm. for 10 minutes, the plasma samples were separated and stored at -20°C for subsequent analysis.

Table 1 Treatment Schedule

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

* Each A, C, D and F represented the brand name of glibenclamide tablets

6. Determination of Glibenclamide in Plasma

Plasma glibenclamide concentrations were determined by high performance liquid chromatography. The method was modified from that described by Emilsson et al. (1986). The procedure was described as follows :

A 0.5 ml. of plasma, 1 ml. of 0.05 M hydrochloric acid and 25 mcl. of glipizide internal standard solution (concentration 10 mcg./ml.in methanol) were mixed with 4 ml. of benzene-dichloromethane mixture (3:1) in a test tube. The mixture was gently shaken for 15 minutes using shaker. After centrifugation at 4000 rpm. for 10 minutes, 3.2 ml. of the organic phase was transferred to a test tube and evaporated to dryness under a stream of nitrogen. The residue was reconstitued in 100 mcl. of mobile phase. An aliquot of 20 mcl. was injected into the chromatograph.

Chromatographic Condition

Apparatus : Waters 510 HPLC Pump, equipped with Waters 484 variable wavelength UV detector and Waters 745B Data Module integrator

Column : Spherisorb ODS 2 (C_{18}), Stainless steel column, 10 micron particle size

Analytical column : 250 x 4.6 mm., i.d.

(Phenomenex^{R}, California, USA)

Pre-column : 50 x 2.0 mm., i.d. (C_{18})

Mobile phase : 46 : 54 V/V of acetonitrile and 0.01 M phosphate buffer (pH 3.5) (Appendix B), mixed with 0.2 ml. of tetrahydrofuran. The mobile phase was filtered and degassed before use.

Internal standard : Glipizide (concentration 10 mcg./ml. in methanol)

UV detector : 230 nm.

Flow rate : 1.6 ml./min.

Chart speed : 0.5 cm./min.

Operating temperature : ambient

Injection volume : 20 mcl.

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

Calibration Curve

Ten microlitres of each of standard glibenclamide solutions with concentration of 1, 2, 4, 6, 10, 12, 14 and 16 mcg./ml. in methanol were spiked into 0.5 ml. of pooled drug-free plasma to produce the concentrations of 20, 40, 80, 120, 200, 240, 280 and 320 ng./ml. in plasma, respectively. 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 glibenclamide concentrations.

Assay Validation

The modified Emilsson et al.'s method was validated under the following conditions.

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

To assess the recovery of glibenclamide and internal standard, peak heights of either glibenclamide and internal standard obtained from benzene-dichloromethane extraction was compared with those of aqueous solution directly injected to HPLC.

7. <u>Pharmacokinetic Analysis</u>

The pharmacokinetic analysis of individual plasma glibenclamide levels from each treatment was established using 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 glibenclamide as shown in equation 1.

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

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

where : C_t = the plasma glibenclamide 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 concentration (C_{max}), the time to reach the peak plasma concentration (t_{max}), the area under the concentration-time curve (AUC) and the biological half-life ($t_{1/2}$) of glibenclamide were calculated by the following equations.

$$C_{max} = A_1 e^{-K_{el} t_{max}} - A_2 e^{-K_a t_{max}} \quad \text{Eq. 2}$$

$$t_{max} = \ln (K_a/K_{el})/(K_a - K_{el}) + t_{lag} \quad \text{Eq. 3}$$

$$AUC = A_1/K_{el} - A_2/K_a \quad \text{Eq. 4}$$

$$t_{1/2} = 0.693/K_{el} \quad \text{Eq. 5}$$

8. <u>Evaluation of Bioequivalence</u>

The bioavailability of glibenclamide tablets in the present study relative to the reference were assessed using the three relevant pharmacokinetic parameters, C_{max}, t_{max} and AUC .

The differences in C_{max}, t_{max} and AUC among the four selected brands were determined by one way analysis of variance (ANOVA) at the significant level of 0.05. If the results showed the statistically significant difference, the difference of these values between the innovator's product and each brand were examined by means of t-test. The selected test brands were considered to be bioequivalent to the innovator's product when their C_{max}, t_{max} and AUC values showed no statistically significant differences from those of 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 time and dissolution rate constant, and the in vivo parameters, including C_{max}, t_{max} and AUC of all brands.

10. Assumption

Pharmacokinetics of glibenclamide tablets followed first-order process. The data with too high and/or too low values will be excluded in order to avoid wrong interpretation.