CHAPTER III

MATERIALS AND METHODS

Materials

A. Drug-products

Two drug-products were studied. One was a generic (test) product manufactured by Siam Bheasach Co., Ltd (Diprazide®, Batch no. H42GLA02/1, Mfd. 09/08/2004, Exp. 09/08/2008), and another was an innovator's product manufactured by Pharmacia Italia S.p.A., Ascoli Piceno, Italy (Minidiab®, Batch no. 4ERG81, Mfd. 01/2004, Exp. 01/2009) which was assigned as a reference product.

B. Reagents

1. Working standard Glipizide powder (Siam Bheasach Co.,Ltd., Thailand) potency : 98.94% , Lot No.3404105
2. Working standard Gliclazide powder (Siam Bheasach Co.,Ltd., Thailand) potency : 100.06% , Lot No.03030501
3. Acetonitrile HPLC grade (Lab Scan, Thailand, Ireland), Lot No.03 12 0085
4. Methanol HPLC grade (Lab Scan, Thailand, Ireland), Lot No. 04 07 0068
5. Methanol AR grade (Lab Scan, Thailand, Ireland), Lot No. 04 08 1129
6. ortho-Phosphoric acid 85% (Carlo Erba, Italy), Lot No.406005
7. Potassium dihydrogen phosphate AR (Merck, Germany), Lot No. A397373 247
8. Sodium dihydrogen phosphate anhydrous AR (Merck, Germany), Lot No. K22051045 530
9. Pooled drug free plasma (Thai Red Cross Society, Thailand), Lot No. 100.47.2.00001
10. Glucose anhydrous (Hebei Shegxue Glucose Co., Ltd, China), Lot No. 200405151

C. Apparatus

1. Analytical balance (AG245, Mettler Toledo, Switzerland)
2. Digital pH meter (Backman 040 pH meter, Germany)
3. Vortex mixer (Vortex-Genie, Scientific Industries, Inc., USA)
4. Micropipette 100 µL (Gilson Medical Electronics S.A., France)
5. Micropipette 1000 µL (Socorex, Switzerland)
6. Sonicator (Branson 2200, USA)
7. Glassware (Pyrex, USA)
8. Freezer (FC-27, Sharp, Japan)
9. Centrifuge (Ljungberg, Sweden)
10. Sodium heparin tube 10 mL (BD Vacutainer® Brand, USA)
11. AccuBOND II PHENYL Cartridges 200 mg 3 mL(Agilent Technologies, UK)
12. Vacuum manifold (Agilent Technologies, UK)
13. Pump (Benton Harbor,MI, USA)
14. High performance liquid chromatography
    - HPLC pump (Waters 600 Controller, USA)
    - Autosampler (Waters 717 plus Autosample, USA)
    - Column (µ Bondapak C_{18} column 3.9 x 300 mm 10 µm, USA)
    - Oven (Waters, USA)
    - UV detector (Waters 2487 Dual λ Absorbance Detector, USA)
    - Integrator (Empower Software, Water, Ireland)
15. Water bath (Memmert, Germany)
16. Nitrogen gas (Thai Industrial Gas, Thailand)
17. Spectrophotometer (UV-1601, Shimadzu, Japan)
18. Nylon syringe filters, 17 mm, 0.45 µm (National Scientific Company, USA)
19. Polyamide membrane filter 47 mm, 0.45 µm (Sartorius AG, Germany)
20. Dissolution apparatus
    - Dissolution (VK 7000, Vankel, USA)
    - Heater (VK 650, Vankel, USA)
    - System monitor (VK 8000, Vankel, USA)

Methods

A. Pharmaceutical Equivalence and *In Vitro* Dissolution Testing

In order to confirm the quality of glipizide tablet, pharmaceutical equivalence study of test product versus innovator's product were conducted by following the recommended procedures of United States Pharmacopoeia (USP) 27. These include identification, uniformity of dosage units, assay and dissolution test.

1. Identification

Concentration of 0.05 mg of glipizide per mL of the assay preparation and standard preparation were separately injected with equal volume into HPLC, and the retention times were used for identification. For conclusion, the retention time of the major peak in the chromatogram of the assay preparation corresponds to that of the standard preparation.

2. Uniformity of Dosage Units

2.1 Solution Preparations

Buffer – Dissolve 13.8 g of monobasic sodium phosphate in water, and dilute with water to 1000 mL. Adjust with 2.0 N sodium hydroxide to a pH of 6.00 $\pm$ 0.05.

Mobile phase - Prepare a filtered and degassed mixture of buffer and methanol (45:55).

Standard preparation – Dissolve an accurately weighed 20 mg of glipizide WS in methanol, and dilute to 200 mL with methanol in a 200-mL volumetric flask to obtain a solution having a known concentration of 0.1 mg per mL. Transfer 25.0 mL of this solution into a 50-mL volumetric flask, dilute with buffer to volume, and mix to obtain a solution having a known concentration 0.05 mg per mL.

Test preparation – Transfer 1 tablet to a 100-mL volumetric flask, add a volume of buffer equal to one-half of the total flask volume, and shake by mechanical means for 10 minutes to allow the tablet to disintegrate completely. Dilute with methanol to volume, and sonicate for 15 minutes to obtain a solution having a concentration of about 0.05 mg of glipizide per mL. Filter through a solvent-resistant filter. Ten replicate test preparations have been analyzed.

2.2 Chromatographic System

The liquid chromatograph is equipped with a 225-nm detector and a 30 cm x 3.9-mm column that contains 10 µm packing C_{18}. The flow rate is 1.0 mL per minute. Chromatograph the standard preparation, and record the peak responses as directed under procedure.

2.3 Procedure

Separately inject equal volumes (20 µL) of the standard preparation and the test preparation into the chromatograph, record the chromatograms, and measure the responses for the major peaks. Calculate the quantity, in mg, of glipizide in the tablet taken by the formula:

$$CV(r_u/r_s)$$

in which

C is the concentration, in mg/mL, of glipizide WS in the standard preparation.

V is the volume, in mL, of the test preparation taken.

r_u is the peak responses obtained from the test preparation.

r_s is the peak responses obtained from the standard preparation.

Each of individual content in tablet is between 85% and 115% of the labeled amount of glipizide and relative standard deviation is equal to or not more than 6%.

3. Assay for Content of Active Ingredient

Assay preparation – Weigh and finely powder 20 glipizide tablets. Transfer an accurately weighed portion of the powder, equivalent to about 5 mg of glipizide, to a 100-mL volumetric flask. Add 50 mL of methanol, and place in an ultrasonic bath for 15 minutes. Dilute with buffer to volume, and place in the ultrasonic bath for an additional 15 minutes. Filter through a solvent-resistant filter.

Analytical procedure for the assay preparation is similar to the test preparation as described above. Glipizide tablets contain not less than 90.0% and not more than 110.0% of the labeled amount of glipizide (%L.A). The difference of percent labeled amount of the active ingredient in test and innovator's product should not be more than 5%.

4. *In Vitro* Dissolution Testing

Dissolution test was performed by using the USP apparatus II operated at 50 rpm. Twelve tablets of both test and innovator's products were tested by separating them into four experiments. Each experiment, three tablets of both products were placed in vessels (each tablet of glipizide per vessel) containing 900 mL of phosphate buffer pH 6.8 $\pm$ 0.05 equilibrated at 37 $\pm$ 0.5^{0}C as dissolution medium. Ten mL of samples in each vessel were collected a 5, 10, 15, 20, 30, 45, 60 and 90 minutes, respectively. The same volume of an equilibrated medium was immediately replaced in order to maintain a constant volume. The amount of drug dissolved was measured by spectrophotometer at wavelength of 276 nm and was calculated using the calibration curve.

For constructing the standard calibration curve, an accurately weighed of glipizide WS was dissolved in sodium phosphate buffer pH 6.8 $\pm$ 0.05 to provide the standard solutions of 1, 2, 3, 4, 5, 6, 7 and 8 µg/mL, respectively. All these solutions were analyzed by spectrophotometer. The absorbances of glipizide versus glipizide concentrations were fitted to a straight line using linear regression analysis.

The dissolution profile of test and innovator's products were created by plotting % glipizide dissolved versus time and they were compared using difference factor (f_1) and similarity factor (f_2) (Thai FDA, 2000). The two factors were calculated by the following equations:

$$f_1 = \left[\left\{ \sum_{t=1}^{n} |R_t - T_t| \right\} / \sum_{t=1}^{n} R_t \right] \times 100$$

$$f_2 = 50 \times \log\left[\left\{1 + (1/n)\sum_{t=1}^{n}(R_t - T_t)^2\right\}^{-0.5} \times 100\right]$$

in which;

n	=	Number of sampling points
R_t	=	Mean percent dissolved of innovator's product at time t
T_t	=	Mean percent dissolved of test product at time t

Criteria of dissolution testing, for a single point dissolution testing; the %dissolution of glipizide in 45 minutes is not less than 85% of the labeled amount and for dissolution profiles comparison, the difference factor and similarity factor should be between 0 to 15 and 50 to 100, respectively. The similarity factor is the principal factor for considering the equivalence of dissolution profile.

B. Bioanalytical Method for Determining Glipizide in Plasma

The bioanalytical method used for determining glipizide in plasma was that modified from the article of Kobylinska et al. (2000).

1. Sample Preparations

Ten solid phase extraction (SPE), phenyl columns, were placed on SPE-10 vacuum extraction manifold and were conditioned with 3 mL of methanol, 3 mL of water and 1 mL of 0.1 M H_3PO_4, respectively. Then, 0.5 mL of plasma samples which each sample was mixed with 50 µL of internal standard (gliclazide 12 µg/mL) and 1 mL of 0.1 M H_3PO_4, were loaded into the SPE column. The SPE columns were washed with 2 mL of water two times and 1 mL of 55% methanol in water. Finally, glipizide and internal standard were eluted with 1 mL of methanol.

Because of the low concentration of glipizide in plasma (ng/mL), the eluent was necessary to concentrate by evaporating to dyness in a water-bath at 40^0C under a nitrogen stream. The dried extract was reconstituted in 100 µL of mobile phase. An aliquot of 40 µL of this solution was injected into the HPLC.

2. HPLC System

Column : μ-Bondapak C_{18}, 10 μm, 30 cm x 3.9 mm i.d.

Mobile phase : 0.01 M potassium phosphate buffer pH 3.5: ACN (62:38)

Flow rate : 1.4 mL/min

Oven temperature : 50°C

Autosampler : 8°C

Detector : UV, λ = 225 nm

Retention time : Glipizide ~ 8-10 min, Gliclazide ~ 12-14 min

Response : Peak area ratio (PAR)

Gradient flow rate :

Time	Flow	%Mobile Phase	%ACN
-	1.4	100	0
11	1.4	100	0
12	1.4	60	40
16	1.4	60	40
17	1.4	100	0
22	1.4	100	0

The run time per injection was 24 minutes. The gradient flow rate was used in order to eliminate the interference peaks of the trace detergent deposited at the inner surface of the test tubes with retention time of about 17-24 minutes, which are not shown in chromatogram.

3. Method Validations

For performance characteristics of suitable and reliable method for the intended analytical application, method validations are performed in accordance with the specification given in the Guidance for Industry: Bioanalytical Method Validation of Center for Drug Evaluation and Research (CDER) and Center for Veterinary Medicine (CVM), U.S. Department of Health and Human Services, Food and Drug Administration, 2001. Details of validation were described as follows:

3.1 Selectivity

Six sources of blank plasma and spiked samples with glipizide and internal standard were analyzed in order to ensure that there are no interferences according to the contents in blank plasma and there were selectivity of glipizide and internal standard.

3.2 Linearity and Standard/Calibration Curve

Stock standard solution of glipizide and internal standard (0.1 mg/mL) were prepared in methanol. Working standard glipizide and gliclazide solutions of appropriate concentration were made by dilution the stock solution with 50% methanol/water. The calibration curve were prepared by adding working solution of glipizide in blank plasma to provide the standard solutions of 20, 25, 50, 100, 200, 400, 600 and 1,000 ng/mL. A 50 µL of gliclazide working solution (12 µg/mL in 50%methanol/water) was added to these standard glipizide solutions. All of standard solutions were analyzed by HPLC. The peak area ratios of glipizide to that of internal standard were fitted to straight line using linear regression analysis.

The coefficient of determination should be more than 0.99. The percent recovery for accuracy and percent coefficient of variation for precision of the LLOQ, and standards other than LLOQ should not more than 20% and 15% respectively.

3.3 Lower Limit of Quantification (LLOQ)

Five determinations of lowest glipizide concentration in plasma were analyzed so as to assure that analyte peak (response) was identifiable, discrete, and reproducible with a precision of not exceed 20% and accuracy of 80-120%, and it would serve as the first point of a standard/calibration curve.

3.4 Accuracy

Quality control concentration samples (QC samples) were prepared (Appendix A). Five determinations of QC samples which include three standard concentrations of glipizide in plasma (60, 500 and 900 ng/mL) were analyzed. Accuracy of the analytical method was estimated by the percent recovery of each concentration level using the following equation.

$$\% \text{ Recovery} = \frac{\text{Estimated concentration}}{\text{Know concentration}} \times 100$$

Mean value of the percent recovery of each concentration level should be within 15% of nominal concentration.

3.5 Precisions

3.5.1 Within-run Precision

Five determinations of QC samples were prepared and analyzed in the same day. Precision of the determination is estimated by calculating percent coefficient of variation (%CV) of each concentration level using the following equation.

$$\% \text{ CV} = \frac{SD}{\overline{X}} \times 100$$

The percent coefficient of variation of each concentration level should be less than 15%.

3.5.2 Between-run Precision

Analyzing samples are similar to those stated above (in 3.5.1), but the five determinations of three concentrations of glipizide in plasma were analyzed on five different days. The percent coefficient of variation of each concentration level should not exceed 15%.

3.6 Recovery of Extraction

Five determinations of QC samples and one concentration of gliclazide (as internal standard) in plasma and mobile phase were prepared and analyzed. The percent recovery of extraction was computed by an equation:

$$\% \text{ Recovery of extraction} = \frac{\text{PA of glipizide or int. std. of extracted samples from plasma}}{\text{PA of glipizide or int. std. of unextracted samples from mobile phase}} \times 100$$

PA is peak area. The percent recovery of each concentration level need not be 100%, but the extent of recovery of an analyte and of internal standard should be consistent, precise, and reproducible.

3.7 Stability

3.7.1 Freeze-thaw Stability

Three aliquots of two concentrations of standard glipizide in plasma (60 and 900 ng/mL) were analyzed and stored at -20°C for 24 hours and thawed unassisted at room temperature. This was one freeze-thaw cycle. After complete thaw, the freeze-thaw cycles were repeated two more times under the same conditions, samples were then prepared and analyzed on the third cycle.

3.7.2 Long-term Stability

Three aliquots of two concentrations of standard glipizide in plasma (60 and 900 ng/mL) were analyzed and stored at -20°C for 6 weeks, each standard sample was prepared and analyzed every 2 weeks.

3.7.3 Short-term Room Temperature Stability

Three aliquots of two concentrations of standard glipizide in plasma (60 and 900 ng/mL) were analyzed and stored at -20°C for 24 hours. Afterward, they were thawed at room temperature and analyzed after being kept at this temperature for 4, 8 and 12 hours.

3.7.4 Post-preparative Stability

Three aliquots of two concentration of standard glipizide in plasma (60 and 900 ng/mL) were prepared and analyzed. The prepared samples were analyzed after being kept in autosampler for 6, 12 and 24 hours.

3.7.5 Stock Solution Stability

Standard stock solution of glipizide (0.1 mg/mL) in methanol and gliclazide (0.1 mg/mL) in methanol were prepared and refrigerated. They were then analyzed after being kept for 2, 4 and 8 weeks.

Stability Evaluation

For freeze-thaw, long-term, short-term (room temperature) and, post-preparative stability of glipizide, evaluation is made by comparing analytical results of treated samples with those of freshly prepared samples through an equation:

$$\% \text{ Deviation} = \frac{\text{Est.conc.}_{tn} - \text{Est.init.conc.}_{t0}}{\text{Est.init.conc.}_{t0}} \times 100$$

where;

$\text{Est.init.conc.}_{t0}$ = Estimated initial concentration of freshly prepared sample

Est.conc._{tn} = Estimated concentration of processed sample

Percent deviation of the mean estimated concentrations from that at zero time (initial concentration) should be within $\pm$ 15%.

In order to assess stock solution stability, the estimated concentration were displaced by peak area of glipizide and gliclazide.

C. *In Vivo* Bioequivalence Studies

The methods used for this study were those as stated in USP 27 and the Criteria and Guideline for the Bioequivalence Study of Generic Drugs of Drug Control Department, Office of Food and Drug Administration, Thailand, 2000.

1. Drug-products

Two brands of 5 mg glipizide tablets (Diprazide® and Minidiab®) were tested. Diprazide® was a test product whereas Minidiab® was an innovator's product.

2. Subjects

All volunteers were selected on the basis of medical history, physical examination and clinical laboratory record such as complete blood count, blood urea nitrogen, serum creatinine, AST/ALT, total bilirubin, alkaline phosphatase, total protein, albumin, hepatitis B virus surface antigen, hepatitis C virus antibody, and human immuno-deficiency virus antibody tests. No volunteers had a history or

evidence of hepatic, renal, gastrointestinal or hematologic disease, and of drug allergy to sulfonylurea, sulfonamide and thiazide.

2.1 Inclusion Criteria

Twelve healthy Thai male volunteers, aged between 18 to 45 years with a body mass index ranging from 18 to 24 kg/m^2 precipitated in this study.

All selected volunteers received patient's information sheet and they were explained about the purposes, the benefits and the risks of the experiment prior to entering the test. The study protocol was approved by the Ethics Committee of the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand. Informed consents were obtained from the volunteers before starting the study (Appendix B).

To standardize experiment condition and avoidance the effect of inducing or inhibiting hepatic metabolizing enzyme and the risk of drug interactions, the volunteers were asked not to consume any other drugs, cigarette, alcohol and beverage or food, containing xanthines such as coffee, tea and chocolate for at least two weeks before and during the study period.

No volunteers participated in other clinical trial at least 1 month before the study.

2.2 Exclusion Criteria

The volunteers who did not have above qualifications, or were allergic to glipizide, or showed serious side effect, or consumed drug affecting pharmacokinetic parameters of glipizide and wanted to withdraw from the study, they would be excluded.

3. Experimental Design

The experiment was conducted in a single dose, randomized replicated crossover design with 2-treament, 2-sequence, 4-period and 1-week washout period between treatments, as shown in Table 2.

Table 2 Randomization Schedule

Sequence	Subject no.	Period 1	Period 2	Period 3	Period 4
1	1 3 5 7 9 11	R_1	T_1	R_2	T_2
2	2 4 6 8 10 12	T_1	R_1	T_2	R_2

R is the innovator's product of 5 mg glipizide tablet. T is the test product of 5 mg glipizide tablet. 1 is the first time of drug administration. 2 is the second time of drug administration.

4. Dose and Drug Administration

After an overnight fasted period of at least 8 hours, subjects were admitted to the Faculty of Pharmaceutical Sciences, Chulalongkorn University, at approximately 6.00 a.m. An indwelling catheter was inserted into an antecubital vein to collect the blood samples at specified time point.

Subjects were given a single dose of either formulation (test or innovator's products) of glipizide tablet with 250 mL of 20% glucose solution in water. Following drug administration, 60 mL of 20% glucose solution in water was administered every 15 minutes for 4 hours. The identical lunch and dinner were served at 4 and 10 hours after drug administration in each period.

5. Subject Monitoring

During the first 4 hours of the study, blood pressure and pulse rate were monitored. All monitoring data about blood pressure, pulse rate, unusual symptoms, diagnosis and remedy (if any) were recorded in case record forms by a physician (Appendix B).

6. Sample Collection

Blood samples (5 mL) were collected in sodium heparinized tubes immediately prior to dose administration (time zero) and at 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 6, 8, 10 and 12 hours post dose. They were kept in ice bath until centrifugation. Within 30 minutes after collection, plasma was separated by centrifugation at 3,000 rpm for 15 minutes and stored at -20°C until subsequent assay.

7. Analysis of Glipizide in Plasma Samples

Plasma samples of all subjects were analyzed for glipizide concentrations using the method as described earlier. QC samples in duplicate were also incorporated for accepting or rejecting each run. At least 67% of QC samples must be within 15% of their nominal values (Shah et al., 2000).

8. Evaluation

8.1 Pharmacokinetic Parameters

After plasma samples analysis were complete, plasma glipizide concentration versus time curves were ploted. The relevant pharmacokinetic parameters for bioequivalence evaluation were determined for each subject who completed the study for each treatment using standard non-compartmental methods. They are; the maximum plasma glipizide concentration (C_{max}), the time to peak plasma glipizide concentration (t_{max}) and the area under the plasma glipizide concentration-time curve (AUC).

The maximum plasma glipizide concentrations (C_{max}) and the time to peak plasma glipizide concentration (t_{max}) were obtained directly from the plot of individual plasma glipizide concentration-time profile. The area under the plasma glipizide concentration-time curve to the last measurable concentration (AUC_{0-t}) was

calculated by linear trapezoidal rule. The area under the plasma glipizide concentration-time curve extrapolated to infinite time ($AUC_{0-\infty}$) was calculated as $AUC_{0-t} + \hat{C}/K_e$ where $\hat{C}$ is the last measurable concentration, K_e is the elimination rate constant obtained from least-square analysis of terminal log-linear portion of plasma glipizide concentration-time profile. In addition, other pharmacokinetic parameters; elimination half-life ($t_{1/2}$), clearance/fraction of dose to be absorbed (CL/F), apparent volume of distribution/fraction of dose to be absorbed (V_d/F) and the mean residence time (MRT) were also calculated using the following equations:

$$t_{1/2} = 0.693/K_e$$

$$CL/F = Dose/AUC_{0-\infty}$$

$$V_d/F = Dose/\ AUC_{0-\infty} \times K_e$$

$$MRT = AUMC_{0-\infty}/\ AUC_{0-\infty}$$

Where;

$AUMC_{0-\infty}$ is the area under the moment curve extrapolated to infinite time which is equal to $AUMC_{0-t} + \hat{C}t/K_e + \hat{C}/K_e^2$; $AUMC_{0-t}$ is obtained using trapezoidal rule from the plot of product of plasma glipizide concentration and time (C_t x t)-time curve.

8.2 Statistical Test

Analysis of variance (ANOVA), performed on ln-transformed data of AUC_{0-t}, $AUC_{0-\infty}$ and C_{max} and on observed data of t_{max}, V_d/F, CL/F , MRT, K_e and $t_{1/2}$ at the significant level of 0.05 was constructed to determine the difference of individual corresponding parameter obtained from test and innovator's products using computer program BIOEQ 2x2 (Wijnand, 2003).

AUC_{0-t}, $AUC_{0-\infty}$ and C_{max} were considered as primary variables for the purpose of bioequivalence analysis. The 90% confidence interval for the ratio of each parameter of test to reference product was also obtained from the output of this program.

The test product was considered to be bioequivalent to the innovator's product when 90% confidence intervals for the ratio of test/reference (T/R) of individual parameter base on ln-transformed data was within 80-125% after transformation back to normal data.

The t_{max} difference of test product relative to innovator's product was calculated as percent deviation.

D. Comparison of Pharmacokinetic Parameters of Glipizide

All relevant pharmacokinetic parameters of glipizide in healthy Thai volunteers obtained from this study will be compared to those previously published reports and discussed.