CHAPTER III

MATERIALS AND METHODS

Materials

The following materials were obtained from commercial sources except pooled plasma, which was separated from the whole blood of the rabbits without drug administration.

1. Formulation development and in vitro evaluation

1.1 Active ingredient

- Gliclazide (Lot. No. R7039502, Medirich Sterilab Pvt. Ltd., India)

1.2 Tablet additives

- Hydroxypropyl methylcellulose (4000 cps)
  (Methocel® K4M, Lot. No. 0A 1412N32, Colorcon Limited, USA)
- Xanthan gum (200 mesh)
  (Lot. No. 6328501, supplied by Rama Production, Thailand)
- Dibasic calcium phosphate dihydrate
  (Emcompress®, Lot. No. 7031X, Penwest Pharmaceuticals, U.K.)
- Silicon dioxide
  (Aerosil®, Lot. No. 03A-1, Degussa Ltd, U.K.)

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- Magnesium stearate

  (Lot. No. MAF28, Degussa Ltd, U.K.)

1.3 Commercial product

- Diamicron® MR

  (Lot. No. 3B 2900, Les Laboratoires Servier, France)

1.4 Reagents

The following AR grade reagents were used.

- Hydrochloric acid

  (Lot No. 03 02 0186, Lab-Scan, Ireland)
- Sodium hydroxide pellets

  (Lot No. B 131198 241, Merck, Germany)
- Tribasic sodium phosphate

  (Lot No. 480 277, Carlo Erba, France)
- Potassium dihydrogen orthophosphate

  (Lot No. F2H145, Univar, Australia)
- Methanol, absolute

  (Lot No. 04 03 0188 Lab-Scan, Ireland)

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2. In vivo evaluation

2.1 Pooled plasma

The whole blood of the rabbits was collected in heparinized tube and was

centrifuged at room temperature using a speed of 3000 rpm for 20 minutes. All separated plasma from rabbits was then mixed and stored frozen at –20°C until used.

2.2 Internal standard

- Methyl 4- hydroxybenzoate
  (Lot No. F1E236, Univar, Australia)

2.3 Mobile phase

The following HPLC grade reagents were used.

- Acetonitrile
  (Batch No. 04 01 0109, Lab-Scan, Ireland)
- Methanol
  (Batch No. 04 03 0188, Lab-Scan, Ireland)
- Phosphoric acid
  (Batch No. A3B017, Ajax Finechem, Australia)

2.4 Miscellaneous

- Zinc sulfate anhydrous AR
  (Degussa Ltd, U.K.)
- Citrate phosphate dextrose (CPD)
- Disposable syringe filter (Nylon, pore size 0.45 μm, diameter 13 mm)(Chrom Tech®)

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3. Equipment

- Analytical balance
  (Model A200s, Sartorius GmbH, Germany)
- pH meter
  (Model 210A$^+$, Thermo Orion, Germay)
- Single punch tabletting machine
  (EKO, Viuhang Engineering, Thailand)
- Tablet hardness tester
  (Model 2E/205, Schuleuniger, Switzerland)
- Tablet thickness tester
  (Type SM-112, Teclock, Japan)
- Friabilator
  (Erweka TA-P, Germany)
- Tablet disintegration apparatus
  (Model QC-21, Hanson Research, USA)
- Dissolution apparatus
  (Model DT-6R, Erweka, Germany)
- Ultraviolet / visible recording spectrophotometer
  (Model V-530, Jasco, Japan)
- High performance liquid chromatography system
  (Model SCL-10A VP, Shimadzu, Japan)

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Methods

The procedures in this study can be divided into two main sections with the sequential steps as follows.

1. Formulation development and in vitro evaluation

Gliclazide matrix tablets composed of active ingredient, polymer (HPMC or xanthan gum 200 mesh), diluent (Emcompress ®), anti-adherent (Aerosil ®) and lubricant (magnesium stearate) were prepared using direct compression method. Dissolution of gliclazide tablet formulations was studied in two dissolution media, 0.1 N HCl and phosphate buffer pH 6.8. In addition, the selected formulations were also tested for dissolution using a pH change method (Jonkman, Berg and De Zeeuw, 1983).

1.1 Preparation of gliclazide matrices

The formulation of gliclazide matrices were prepared with varying types and quantities of polymer as shown in Table 1 and Table 2.

1.1.1 Formulation of HPMC matrix system.

The polymer levels were varied in the range of 20% (F1), 40% (F2) and 60% (F3), respectively.

Table 1 Formulations of HPMC containing gliclazide tablets.

Ingredient / Formulation	Quantity		
	F1	F2	F3
HPMC (Methocel® K4M)	4 g (20%)	8 g (40%)	12 g (60%)
Gliclazide	4 g	4 g	4 g
Emcompress®	11.5 g	7.5 g	3.5 g
Aerosil®	0.4 g		
Magnesium stearate	0.1 g		

1.1.2 Formulation of xanthan gum matrix system

The polymer levels were varied in the range of 5% (F4), 7% (F5) and 9% (F6), respectively.

Table 2 Formulations of xanthan gum containing gliclazide tablets.

Ingredient / Formulation	Quantity		
	F4	F5	F6
Xanthan gum(200 mesh)	1 g (5%)	1.4 g (7%)	1.8 g (9%)
Gliclazide	4 g	4 g	4 g
Emcompress®	14.5 g	14.1 g	13.7 g
Aerosil®	0.4 g		
Magnesium stearate	0.1 g		

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1.2 Preparation of gliclazide matrices

Gliclazide tablets were produced by direct compression method. The materials were screened through 30 mesh screens before used. Gliclazide, diluent and polymer (HPMC or xanthan gum) in each formulation were weighed and mixed for 1 minute by geometric dilution method. After this pre-mix step, Aerosil was added and mixed for 1 minute. Finally, magnesium stearate was added and mixed for 1 minute prior to compression. The tablets were compress using a single-punch tabletting machine with an oblong concave faced punch (width 0.3 cm, length 1 cm). The total weight of each tablet was about 150 mg and compressed to have hardness of 5-7 kp.

1.3 Evaluation of physical properties of gliclazide matrix tablet

1.3.1 Determination of weight variation

This test was determined according to BP 2002. The weight of tablet after compression was measured by analytical balance. The mean and standard deviation were calculated from twenty tablets.

1.3.2 Determination of hardness

The hardness of tablet was measured using the hardness tester, the unit of which is expressed in kilopound unit (kp). The mean and standard deviation were calculated from twenty tablets.

1.3.3 Determination of thickness

Thickness of tablet in terms of millimeters was measured using a

micrometer. The mean and standard deviation were calculated from ten tablets.

1.3.4 Determination of friability

The friability of twenty randomly chosen tablets from each variable run was measured by tumbling them for 4 minutes in a Erweka friabilator and then measuring the percent weight loss.

1.3.5 Determination of disintegration time

Disintegration time of tablet was determined according to the method described in USP 25 using the tablet disintegration apparatus. The test was performed without disks in purified water at 37 $\pm$ 2 $^{\circ}$C. Six tablets of each formulation were measured.

1.3.6 Determination of gliclazide content in matrix

Twenty tablets of each formulation were pulverized and three 150 mg powder samples were taken and assayed using UV spectrophotometer at the maximum wavelength of 226 nm. Sample and standard solution were prepared as follows.

1.3.6.1 Sample solution

A quantity of the powdered tablets containing 0.8 g of gliclazide was shaked for 1 hour with 200-ml acetonitrile. Filtered and dilute 10-ml filtrate to 200 ml with a mixture of 2 volumes of acetonitrile and 3 volumes of water.

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1.3.6.2 Standard solution

Forty milligrams of gliclazide was dissolved in 10 ml of acetonitrile and dilute to 200 ml with a mixture of 2 volumes of acetonitrile and 3 volumes of water.

1.4 Determination of drug release from matrices

Dissolution tests were performed by means of USP 24 dissolution apparatus II (paddle type) at 37 °C ± 0.5 °C with a rotation speed at 100 revolutions per minute. The 900 ml of 0.1 N HCl and phosphate buffer pH 6.8 solutions were employed as dissolution media to investigate the influences of pH on drug release from matrix. In order to select the appropriate formulation for investigating the drug release property in pH change medium. The release test of each formulation was done in triplicate.

Sample of 10 ml of the medium was withdrawn at suitable time interval and filtered through a 0.5 μm filter. The same volume of each dissolution medium was immediately added after each sampling to maintain the volume of dissolution medium constant until the end of the experiment.

The absorbance of the filtrate was determined spectrophotometrically in a 1-cm cell at 226 nm for 0.1 N HCl and at 225 nm for phosphate buffer pH 6.8. The model independent approach using mathematical indices to define similarity factor (f_2) and difference factor (f_1), to compare dissolution profile (Moore and Flanner, 1996). A test batch dissolution is considered similar to that of the commercial product if the f_2 value of the two profiles is not less than 50 and the f_1 value is not more than 15. The selected formulation would then be tested in pH-change method.

Dissolution test by pH change method

0.1 N HCl was used as the medium in the first 2 hours and then the pH was adjusted to 6.8 by adding the solution of 19.006 g trisodium phosphate in 220-ml purified water and carried on the test at this pH for 12 hours. The similarity factor (f_2) and difference factor (f_1) were then evaluated as mentioned above. The formulation is considered similar to that of the commercial product, it was then test in the rabbits.

Calibration curve

The standard stock solution was prepared by dissolving 62.0 mg of gliclazide in 20 ml of methanol and adjusted to volume with 0.1 N HCl or phosphate buffer pH 6.8 to 1000 ml. Standard solutions with had known concentrations of about 6, 8, 12, 14 and 18 μg/ml were prepared in duplicate by making dilution of the stock with the medium and analyzed by UV spectrophotometer in a 1-cm cell at 226 nm for 0.1 N HCl and 225 nm for phosphate buffer pH 6.8. The relationship between absorbances and concentration of gliclazide was fitted using linear regression analysis as presented in Table 37, 38 and Figure 24, 25 in the Appendix C.

The photograph of the dissolution study by pH change method

The photographs of test batch matrix (f_2>50, and f_1<15) and commercial product during the course of dissolution test by pH change method. The experiments were performed at the time intervals of 1, 2, 3, 4, 8 and 12 hours.

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Release model analysis in pH change medium

Release data for the first four hours of test batch matrix (f_2>50, **and** f_1<15) and commercial product were plotted based on Higuchi, zero order and- first order equations.

2. In vivo evaluation

2.1 Subjects and drug administration

Twelve male-New Zealand white rabbits, weighing approximately 3.0 kg, were used in this study. All rabbits were acclimatized to the facility unit for 2 weeks before study. The dose of gliclazide (10 mg/kg/day) was chosen because it resulted in a plasma level of gliclazide similar to that achieved in man (Shimizu et al, 1976: Pagano, 1998). All subjects were fasted for at least 10 hours prior to drug administration. Each then received a single dose, followed with 200 ml water. The rabbit abstained from food intake until the 4 hours after drug administration. During drug administration, the plastic tube was inserted into the mouth of rabbit and the matrix tablet was applied through the tube to the throat of animal in order to prevent the tablet chewing. The feces were separated by the tray under the cage.

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2.2 Experimental design

This study was a single-dose, randomized crossover design. All subjects were randomly assigned a number from 1 to 6. Each subject received the drug in a randomized order with two-weeks washout period separated between each dose as shown in Table 3. The subjects number 1 to 3 received a single-dose of HPMC in the first period and they received a single-dose of commercial product in the second

period. For the subjects number 4 to 6, they received a single-dose of commercial product in the first period and they received a single-dose of HPMC in the second period. For XG formulation will be the same as shown in Table 4.

Table 3 Dosing schedule of HPMC and commercial product.

Subject No.	Period	
	1	2
1	HPMC	Commercial product
2	HPMC	Commercial product
3	HPMC	Commercial product
4	Commercial product	HPMC
5	Commercial product	HPMC
6	Commercial product	HPMC

Table 4 Dosing schedule of XG and commercial product.

Subject No.	Period	
	1	2
1	XG	Commercial product
2	XG	Commercial product
3	XG	Commercial product
4	Commercial product	XG
5	Commercial product	XG
6	Commercial product	XG

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2.3 Sample collection

For drug plasma determination, venous blood samples (2.5 ml) were collected from the rabbit's ear vein at 0, 0.5, 1, 2, 3, 4, 6, 8, 12, 18 and 24 hours. Blood sample was collected into heparinized tube. Then, it was immediately centrifuged at a speed of 3,000 rpm for 20 minutes. The separated plasma samples were frozen at –20 °C until analysis.

2.4 Determination of gliclazide in the plasma

Plasma gliclazide concentrations and internal standard (methyl 4-hydroxybenzoate) were analyzed by a high performance liquid chromatography (HPLC) method. The procedure followed a method previously described by Shenfield, Boutagy and Webb (1990).

2.4.1 Preparation of plasma sample

Plasma samples were clarified by deproteinization using zinc sulfate in combination with methanol as follows.

0.3 ml of separated plasma samples, vortex for 5 seconds

↓ add 0.1 ml of 10 % w/v zinc sulfate

vortex for 5 seconds

↓ add 0.8 ml of methanol containing methyl 4- hydroxybenzoate 2 μg/ml (internal standard)

vortex for 5 seconds

centrifuge at 3,000 rpm for 20 minutes

filter and inject supernatant into HPLC column 20 μl

2.4.2 Chromatographic condition

HPLC apparatus (Shimadzu, Japan) comprising :

1. System controller (SCL-10AVP)
2. Auto injector (SIL-10ADVP)
3. UV-detector (SPD-10AVP)
4. Pump (LC-10ADVP)
5. Computing integrator

Column : type C18 Bondclone with particle size 10 μm
300x3.9 mm S/NO. 30038 (Phenomenex, U.S.A.)

Mobile phase : Acetonitrile: 0.01 M Phosphoric acid (1:1)

Flow rate : 1.0 ml/min

Injection volume: 20 μl

Detector : UV 235 nm

Temperature : Ambient

Retention time : internal standard (Methyl 4- hydroxybenzoate) about
4.3 min and gliclazide about 7.2 min

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2.4.3 Calibration curve

Thirty milligrams of gliclazide was accurately weighed into a 100-ml volumetric flask. The drug was dissolved with 30-ml methanol and adjusted to volume with deionized water. This standard stock solution was diluted with mobile phase to obtain the final standard solutions, which had the concentration of 15.0, 30.0, 60.0, 90.0, 120.0,150.0 and 300.0 μg/ml, respectively. Next, exactly 20 μl of each standard solutions was added to 0.3 ml of pooled plasma to make the plasma concentration of 1.0, 2.0, 4.0, 6.0, 8.0,10.0 and 20.0 μg/ml, respectively. Calibration curve was constructed by plotting the ratios of area under the peak of gliclazide to internal standard versus their known gliclazide concentrations. The relationship of these two variables was fitted using linear regression analysis as presented in Table 39 and Figure 26 of the Appendix C.

2.4.4 Assay validation

Method used for analyzing gliclazide in plasma sample was validation under the following conditions:

2.4.4.1 Accuracy

Accuracy in term of percent analytical recovery was done by computing the ratio of inversely estimated concentrations obtained using linear regression equation of standard calibration (low, medium and high) to known concentration of each standard gliclazide concentration in plasma multiplied by one hundred. Each concentration was determined triplicate.

2.4.4.2 Precision

Within run precision

Within run precision was determined by analyzing three sets of standard gliclazide concentrations in plasma (low, medium and high) on the same day. Each estimated concentration was computed and the percent coefficient of variation (%C.V.)for each concentration was calculated. Each concentration was determined triplicate.

Between run precision

This precision was determined by estimating the concentrations of three sets of standard gliclazide concentrations in plasma (low, medium and high) on three different days and the percent coefficient of variation (%C.V.) for each concentration was calculated. Each concentration was determined triplicate.

2.4.4.3 Linearity

Linearity in term of the coefficient of determination (r^2) was calculated from the linear regression of the calibration curve.

2.4.4.4 Acceptance criteria

The percent recovery was within $\pm$ 15%. The percent coefficient of variations were less than 15% and the coefficient of determination was greater than 0.99 (Shah, 1992).

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2.5 Pharmacokinetic analysis

Plasma gliclazide concentrations versus time curves from each subject were plotted and the pharmacokinetic parameters were determined as follows:

2.5.1 Peak plasma concentration (C_{max})

The peak plasma concentrations were obtained directly from the concentration-time curve (maximum concentration point).

2.5.2 Time to peak plasma concentration (t_{max})

The time to peak plasma concentration were obtained directly from the concentration-time curve (time to maximum concentration point).

2.5.3 Area under the plasma concentration-time curve (AUC)

The area under the plasma concentration-time curve was calculated using the trapezoidal rule ($AUC_{gliclazide-24}$). Extrapolated AUC from C_t to infinity (AUC_t^{∞}) was calculated as: $AUC_t^{\infty} = C_t / K_e$. Total AUC ($AUC_0^{\infty}$) was resulted from the summation between $AUC_{gliclazide-24}$ and AUC_t^{∞}.

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2.5.4 Other pharmacokinetic parameters (Ka, Ke and $t_{1/2}$)

2.5.4.1 Absorption rate constant (Ka)

The absorption rate constant was determined by using residual method in semilogarithmic scale, in accordance with the equation; $-Ka/2.303 = (\log C_2 - \log C_1)/(t_2 - t_1)$

2.5.4.2 Elimination rate constant (Ke)

The elimination rate constant was calculated from slope of the plasma drug concentration-time curve in semilogarithmic scale (between elimination point), in accordance with the equation; $-Ke/2.303 = (\log C_2 - \log C_1)/(t_2 - t_1)$

2.5.4.3 Biological half-life ($t_{1/2}$)

The biological half-life was determined by using an equation: $t_{1/2} = 0.693/Ke$.

2.6 Statistical evaluation of pharmacokinetic parameters

The pharmacokinetic parameters of gliclazide matrix tablets were compared statistically as follow:

2.6.1 Mann-Whitney U test

The Mann-Whitney U test is used to compare the pharmacokinetic parameters between HPMC versus XG (at $\alpha = 0.05$). The reason that Mann-Whitney U test was employed because all subjects were in different group.

2.6.2 Wilcoxon Singed Rank test

The Wilcoxon Singed Rank test is used to compare the pharmacokinetic parameters between HPMC versus commercial product and XG versus commercial product (at α =0.05). The reason that Wilcoxon Singed Rank test was employed because all subjects were in same group.

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