# CHAPTER III

# MATERIALS AND METHODS

## Materials

1. Formulation development and *in vitro* study

1.1 Model drug : Diltiazem hydrochloride ( Donated by Siam Bhaesach Co., Ltd) potency: 99.26%

1.2 Additives

- Hydroxypropylmethylcellulose 4000 cps. (Methocel® K4M Premium EP, Lot. No. ND24012N01, Colorcon Limited., USA)
- Xanthan gum 200 mesh (Rheogel® 200 mesh, Lot. No. 57161A, CNI Colloid Natural Internatural, France)
- Lactose monohydrate (Tablettose® 80, Lot. No. L0021 A4003, Meggle GmbH, Germany)
- Dibasic calcium phosphate dihydrate (Emcompress® , Lot. No. X05E, Penwest Pharmaceutical Co., Ltd., UK)
- Magnesium stearate USP (Lot. No. MGS 80014, Carasgo (Genova), Italy)
- Talcum (Lot. No. 450014200006, China)

1.3 Innovator's (Reference) Product : Cardil® 120 mg Tablet Batch No.1C 254/34, (Orion Pharmaceutica, Espo, Finland)

1.4 Reagents

- Hydrochloric acid solution 37% , sp.gr.1.18 , AR grade (Batch No. 01070070, Lab Scan, Thailand)
- Potassium dihydrogen phosphate , AR grade (Lot. No. A262673045, Merck, Germany)

- Sodium hydroxide , AR grade (Lot. No. A002673098, Merck, Germany)
- Methanol , HPLC grade (Lot. No. 01051032, Lab Scan, Thailand)

1.5 Equipment

- Analytical balance (Sartorius,1615MP, Germany)
- Digital pH meter (Orion, Germany)
- Dissolution Apparatus (Sortax AT7, Switzerland)
- Hydraulic equipment (Model C, Carver Laboratory Press, USA)
- Micropipet (Socorex, Switzerland)
- Single punch tabletting machine (Yeawheng, Thailand)
- Sonicator (Bransonic 221, USA)
- Spectrophotometer (Jasco 7800, Jasco Corp., Japan)
- Speed vacuum concentration (Maxi Dry Plus, Heto, Denmark)
- Tablet hardness tester (Schleunginer-2E, Switzerland)
- Tablet friability tester (Erweka, Germany)
- Ultrasonic bath (Transonic Digital, Diethelm & Co.Ltd, Germany)
- Vortex mixer (Sigma 302K, Sigma Lab, Centrifuge Gmbh, Germany)

2. *In vivo* study

2.1 Subjects

Twelve male healthy white New Zealand rabbits weighing between 2.8 and 3.5 kg were obtained from the National Laboratory Animal Center, Mahidol University, Salaya, Nakornpathom, Thailand. Animals were housed individually at the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok and acclimatized for at least 1 week before experiment. All animals were allowed freely to assess food (C.P. Co., Ltd, Thailand) and drinking water.

2.2 Pooled plasma

Whole blood of rabbits without drug were collected in heparinized tube and centrifuged at 5000 rpm for 10 minutes. Plasma was separated and mixed together. It was then kept at –20 °C. This pooled plasma was used for assay validation and standard curve construction.

2.3 Internal standard : ethylparaben (Lot. No. VB19/1, Srichand United Dispensary Co., Ltd.)

2.4 Reagents

- Acetonitrile, HPLC grade (Lot. No. 01120107, Lab Scan, Thailand)
- Disodium phosphate AR grade (Lot. No. A254121457, Merck, Germany)
- Triethylamine (Lot. No. 3312773, Merck, Germany)
- 85% Orthophosphoric acid (Lot. No. 1G 369191L, Carlo Erba, Italy)

2.5 Equipment

- Analytical balance (Sartorius,1615MP; S/N 3209026, Germany)
- Digital pH meter (Orion, Germany)
- High Performance Liquid Chromatography (LC-10AD, Shimadzu, Japan)
  - Communications bus module (CBM-10A, Shimadzu, Japan)
  - Pumps (LD-10A)
  - UV-detector (SPD-10A)
  - Autosampler (SIL-10A)
- Vortex mixer (Sigma 302K, Sigma Lab, Centrifuge GmbH, Germany)
- Microcentrifuge (Z 230 MA, 15000 rpm., Germany)
- Micropipet (Socorex, Switzerland)
- Sonicator (Bransonic 221, USA)

## Methods

1. Formulation development and *in Vitro* evaluation

1.1 Preliminary study

1.1.1 Formulation of diltiazem hydrochloride matrices\

One hundred and twenty milligrams of diltiazem hydrochloride powder was mixed with polymers and fillers as shown in Tables 1 and 2 for 30 minute followed with talcum and magnesium stearate for 5 minute. In preliminary study, tablet was punched using hydraulic press by direct compression. Weight of each tablet was about 375 mg and hardness of tablet was in the range of 6-8 kps.

Table 1. Formulation of diltiazem hydrochloride matrices

| Ingredients | % W/W |
|---|---|
| Diltiazem hydrochloride | 32 (120 mg/tablet) |
| Polymers[a] | * |
| Fillers[b] | qs 375 mg |
| Talcum | 2 |
| Magnesium stearate | 1 |

a) Polymers : Methocel® K4M (HPMC) and/or Rheogel® 200 mesh (XG)

b) Fillers : Tablettose® (lactose) and/or Emcompress® (dibasic calcium phosphate)

* Amount of polymers were shown in Table 2

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Table 2. Compositions of polymers, amounts and types of fillers in each formulation

| No. | Formulations | % of Fillers | | % of Polymers | |
|---|---|---|---|---|---|
| | | Tablettose® | Emcompress® | HPMC | XG |
| 1. | 0% P/T | qs 375 mg | - | - | - |
| 2. | 0% P/E | - | qs 375 mg | - | - |
| 3. | 5% H/T | qs 375 mg | - | 5 | - |
| 4. | 5% H/E | - | qs 375 mg | 5 | - |
| 5. | 5% X/T | qs 375 mg | - | - | 5 |
| 6. | 5% X/E | - | qs 375 mg | - | 5 |
| 7. | 10% P/T | qs 375 mg | - | 5 | 5 |
| 8. | 10% P/E | - | qs 375 mg | 5 | 5 |
| 9. | 20% P/T | qs 375 mg | - | 10 | 10 |
| 10. | 20% P/E | - | qs 375 mg | 10 | 10 |
| 11. | 30% P/T | qs 375 mg | - | 15 | 15 |
| 12. | 30% P/E | - | qs 375 mg | 15 | 15 |

1.1.2 Evaluation of tablets

Tablets of all formulations were evaluated as follows:

1.1.2.1 Tablet hardness

Twenty tablets of 120 mg diltiazem hydrochloride tablet were selected. They were tested using Schleunginer-2E hardness tester. Average, standard deviation of tablet hardness from twenty tablets were calculated.

1.1.2.2 Weight variation

Weight of each tablet after compression was measured using analytical balance. Average and standard deviation of twenty tablets were calculated.

1.1.2.3 Content of active ingredient

1.1.2.3.1 Standard preparation

Standard diltazem hydrochloride solution was prepared at a concentration of 120 mcg/mL in water. One millilitre of this solution was placed into a 10 mL volumetric flask and diluted with water to volume. Absorbance of the final solution was measured at maximum wavelength of 237 nm using spectrophotometer.

1.1.2.3.2 Assay preparation

Twenty tablets of 120 mg diltiazem hydrochloride tablet from each formulation were ground and transferred an accurately weighed portion, equivalent to about 120 mg diltiazem hydrochloride to 1000 mL volumetric flask. Water was added and shook until complete extraction. One millilitre of the supernatant was transferred to 10 mL volumetric flask and diluted to volume with water. The absorbance of final solution was measured as standard solution. The content of diltiazem hydrochloride was calculated as follow :

$$C = 10000C(A_u/A_s)$$

in which

C = Concentration in mcg/mL of diltiazem hydrochloride in the standard preparation

$A_u$, $A_s$ = Absorbance from the assay and standard preparation, respectively

1.1.2.4 Dissolution test

The USP dissolution apparatus I was used for drug release testing of diltiazem hydrochloride tablets. Three dissolution media (deionized water, 0.1N hydrochloric acid pH 1.2 and phosphate buffer pH 7.2) were employed. One tablet of diltiazem hydrochloride was placed in a glass vessel containing 900 mL of dissolution medium at 37 $\pm$ 0.5°C. According to the USP 24, the apparatus was operated at the speed of 50

rpm in phosphate buffer pH 7.2, 100 rpm in water and 0.1N hydrochloric acid pH 1.2. Five milliliters of sample solution were withdrawn at 0.25, 0.5, 0.75, 1, 2, 3, 4, 5, 6, 7, 8, 10 and 12 hours, respectively. The same quantity of equilibrated 37°C medium was added immediately to maintain the volume of dissolution medium. Six tablets of each formulation were tested. The sampling solution was quantitated using spectrophotometer by measuring its absorbance at the maximum wavelength of 237 nm and calculated amount of diltiazem hydrochloride using the calibration curve. The release profiles of diltiazem hydrochloride were plotted between the percent drug released versus time.

Calibration curve

Stock standard solutions of diltiazem hydrochloride (10 mg/mL in water or in 0.1N hydrochloric acid and 1 mg/mL in phosphate buffer pH 7.2) were prepared. Then 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6 and 1.8 mL of each stock standard solution were transferred into 10 mL volumetric flask and diluted with each medium. The final concentrations of diltiazem hydrochloride in standard solution were 2, 4, 6, 8, 10, 12, 14, 16 and 18 mcg/mL. They were analyzed using spectrophotometer at the maximum wavelength of 237 nm. The absorbance of diltiazem hydrochloride versus known diltiazem hydrochloride concentrations were fitted to straight line using linear regression.

1.1.2.5 *In vitro* evaluation

In order to select the formulations for scale up study, the release profile of the drug from each formulation was compared to that of the reference product using the difference factor ($f_1$) and the similarity factor ($f_2$). Difference factor of selected formulations should less than 15 and similarity factor should more than 50.

1.2 Scale up study

The selected formulae from preliminary study were varied with respect to polymer concentrations. They were prepared using single punch tabetting machine and evaluated as follows

1.2.1 Tablet hardness

Tablet hardness was evaluated as described in 1.1.2.1.

1.2.2 Weight variation

Weight variation of tablet was evaluated as described in 1.1.2.2.

1.2.3 Content of active ingredient

Content of active ingredient of tablet was evaluated as described in 1.1.2.3.

1.2.4 Dissolution test

Dissolution test of tablet was evaluated as described in 1.1.2.4.

1.2.5 Thickness of tablet

Thickness of tablet was evaluated using Vernia Caliper in term of millimeters.

1.2.6 Tablet friability

Twenty tablets of 120 mg diltiazem hydrochloride tablet were selected and any dust was removed using soft brush. They were accurately weighed and afterward placed in the drum. The drum was rotated according to the procedure specified in the USP. Any dust was removed from the tablets as stated earlier and they were weighed again . The weight of 20 tablets before and after the test were compared (USP 24, 2000).

2. *In Vivo* Evaluation

2.1 Products

Three formulations of controlled release of diltiazem hydrochloride tablets with the most satisfactory dissolution characteristics were selected to be *in vivo* evaluated. All tablets were newly prepared by individual condition and subjects to be investigated for the pattern of *in vitro* drug release. Cardil®, the innovator's product to be used as a reference material was also tested.

2.2 Subjects and drug administration

Twelve male white New Zealand rabbits, weighing between 2.8 and 3.5 kg. were acclimatized to the research facility for two week before study. Two tablets of each formulation containing 120 mg of diltiazem hydrochloride were given orally in a single dose. All of them received each dose in the morning after being fasted overnight with water ad libitum. No food was permitted until 4 hours after dosing.

2.3 Experimental design

The study was conducted in a randomized four way balanced crossover

design. Each subject received the drug in a randomized order with one week washout period separated between each dose as shown in Table 3.

Table 3. Dosing schedule

| Sequence | Subject No. | Period | | | |
|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 |
| I | 1-3 | A | B | C | D |
| II | 4-6 | B | C | D | A |
| III | 7-9 | C | D | A | B |
| IV | 10-12 | D | A | B | C |

where A = 7% mixed polymers in Emcompress® (7% P/E)

B = 10% mixed polymers in Emcompress® and Tablettose® (2:1) (10% P/E+T)

C = 15% mixed polymers in Tablettose® (15% P/T)

and D = Cardil®

2.4 Sample collection

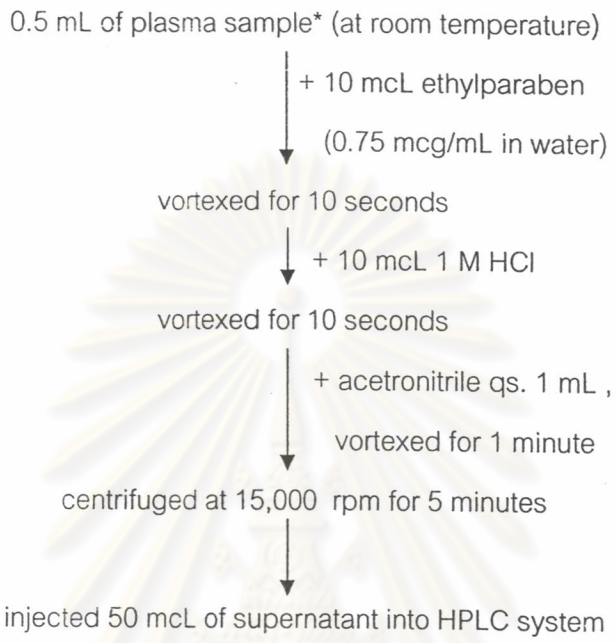
Three milliliters of blood sample was collected from a marginal ear vein using a disposible needle No. 22 at 0, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 15 and 18 hours after each dosing. Blood sample was transferred into a heparinized tube. Then, it was immediately centrifuged at speed of 5000 rpm for 10 minutes. All plasma samples were separated and kept at –20° C until subsequent analysis.

2.5 Determination of plasma diltiazem hydrochloride concentration

2.5.1 Sample preparation

The modified Chaudhary et al.'s (1993) method used for analyzing diltiazem

hydrochloride in plasma sample. After thawing, plasma sample was mixed with internal standard (0.75 mcg/mL of ethylparaben in water) and extracted with acetonitrile as follows :




* = 0.49 mL of blank plasma + 0.01 mL of diltiazem hydrochloride in calibration curve

2.5.2 Chromatographic system

| | | |
|---|---|---|
| Apparatus | : | Shimadzu® LC 10 AD. |
| Column | : | Inersil® ($C_8$), stainless steel column, GC Science Inc., Japan |
| UV detector | : | 237 nm. |
| Flow rate | : | 1.2 mL/min |
| Pressure | : | 56 $kg/cm^2$ |
| Injection volume | : | 50 mcL |
| Mobile phase | : | 28% acetonitrile in 0.01M disodium phosphate pH 5.0 |
| Retention time | : | diltiazem hydrochloride ≈ 8.8 min. |
| | : | ethylparaben ≈ 10.9 min. |

The concentrations of diltiazem hydrochloride in plasma samples were computed using a standard calibration curve.

Calibration curve

Seven standard concentrations of diltiazem hydrochloride in pooled plasma were prepared. They were 70, 100, 200, 400, 1000, 1500 and 2000 ng/mL, respectively. The plasma standards were finally clarified and analyzed following the same procedure as mentioned earlier. Calibration curve was constructed by plotting the ratios of area under the peak of diltiazem hydrochloride to that of ethylparaben versus standard concentrations of diltiazem hydrochloride using linear regression .

2.6 Assay validation

Method used for analyzing diltiazem hydrochloride in plasma sample was validated under the following conditions :

2.6.1 Accuracy

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

2.6.2 Precision

2.6.2.1 Within-run precision

This precision was determined by analyzing three sets of standard diltiazem hydrochloride concentrations in plasma (low, medium, 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 triplicately.

2.6.2.2 Between-run precision

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

2.6.3 Linearity

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

2.6.4 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 et al.,1991).

2.7 Pharmacokinetic analysis

Plasma diltiazem hydrochloride concentration (C) versus time (t) curves from each treatment were plotted and the pharmacokinetic parameters were determined. The peak plasma concentration ($C_{max}$) and the time to reach the peak plasma concentration ($t_{max}$) of diltiazem hydrochloride were directly inspected from the data. The area under the plasma concentration-time curve ($AUC_0^{\infty}$) was calculated using trapezoidal rule and extended to infinity by adding with $\hat{C}/K$ term, where $\hat{C}$ was the last measurable plasma diltiazem hydrochloride concentration and K was the terminal

elimination rate constant. The elimination half-life ($t_{1/2}$) was calculated using an equation : $t_{1/2} = 0.693/K$.

2.8 Evaluation of bioequivalence

The bioequivalence of three formulations of diltiazem hydrochloride controlled release tablets relative to Cardil® was assessed using comparison of corresponding pharmacokinetic parameters ($C_{max}$ and $AUC_0^{\infty}$) values based on logarithmic transformed data.

2.8.1 Statistical test

The differences of these corresponding pharmacokinetic parameters in terms of ln-transformed data among four formulations were determined by analysis of variance for randomized crossover design at $\alpha = 0.05$.

2.8.2 Construction of 90% confidence interval

A 90% confidence interval of individual parameter ratio based on ln-transformed data was constructed using an equation

$$90\%\ CI = (\overline{x}_T - \overline{x}_R) \pm (t_{0.1,df} \times S.E.)$$

where $\overline{x}_T$, $\overline{x}_R$ = Mean ln $C_{max}$ and mean ln AUC values of test and reference products, respectively.

$t_{0.1,df}$ = Tabulated t value at $\alpha = 0.1$, df of MSE

S.E. = $\sqrt{2MSE/n}$

MSE = Mean square error obtained from the ANOVA table

$$\text{Lower limit} = \text{anti ln}\left\{ (\bar{x}_T - \bar{x}_R) - (t_{0.1,df} \times \text{S.E.}) \right\} \times 100$$

$$\text{Upper limit} = \text{anti ln}\left\{ (\bar{x}_T - \bar{x}_R) + (t_{0.1,df} \times \text{S.E.}) \right\} \times 100$$

The test formulation was considered to be bioequivalent to the reference product, when 90% confidence interval of individual parameter of test formulation relative to that of the reference product was within 80-125%.