# Chapter II

## Materials and Methods

### Materials

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

1. Formulation Development and In Vitro Evaluation

1.1 Active Ingredient

- Theophylline anhydrous BP, Batch No. 890314 (Vertex Chemicals Co., Hong Kong)

1.2 Tablet Additives

- Methocel E4M, Batch No. MM90071802 (Colorcon Ltd., England)
- Lactose hydrous (Wyndale, New Zealand)
- Magnesium stearate (supplied by Pharmaceutical Sciences, Thailand)

1.3 Commercial Products

- Theo-Dur® 300 mg, Batch No. SA754
  (Astra, Sweden)
- Nuelin® 250 mg, Lot No. 7646A
  (3M Pharmaceuticals, Australia)

1.4 Reagents

- Potassium dihydrogen phosphate, AR (E. Merck, Darmstadt, Germany)
- Sodium hydroxide, AR (E. Merck, Darmstadt, Germany)
- Hydrochloric acid, AR (E. Merck, Darmstadt, Germany)
- Ethanol absolute, AR (E. Merck, Darmstadt, Germany)

2. In Vivo Evaluation

2.1 Pooled Plasma

After the whole blood of the rabbits without drug given was collected in heparinized tube, it was centrifuged at a

speed of 3000 rpm for 20 minutes. All separated plasma from ≥ three rabbits was then mixed together and kept refrigerated at -10°C until subsequent analysis.

2.2 Internal Standard

- β-Hydroxyethyltheophylline (Sigma Chemical Co., St. Louis, USA)

2.3 Mobile Phase

- Acetonitrile, HPLC grade (May & Baker Ltd., Dagenham, England)
- Acetic acid glacial, AR (E. Merck, Darmstadt, Germany)
- Sodium acetate anhydrous, AR (Farmitalia Carlo Erba, Italy)

2.4 Miscellaneous

- Methanol, HPLC grade (Labscan Ltd, Dublin, Ireland)
- Zinc sulfate anhydrous, AR (E. Merck, Darmstadt, Germany)
- Heparin sodium 1000 iu/ml
- Membrane filter 0.45 μm
- Commercial rabbit food (Gold Coin Mill, Singapore; Distributed by F.E.Zuellig Ltd, Thailand)

Methods

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

1. Formulation Development and In Vitro Evaluation

First of all, the series of tablets composed of active ingredient and retarding agent were prepared by means of conventional wet granulation and studied for their release profiles in two dissolution medium, 0.1 N HCl and pH 6.8 phosphate buffer. The most suitable formulation was then selected for further development by adding various amount of lactose as channeling agent.

1.1 Preparation of Granules

The granules of theophylline with varying quantities of Methocel E4M (Table 2) were prepared by means of wet granulation. Both ingredients were firstly weighed and mixed in a mortar by geometric dilution method. Then absolute ethanol was added gradually as the wet granulation process was carried out until a subjunctive end-point. The wet mass was next pressed through a 16-mesh screen and placed in the air for 15 minutes to evaporate ethanol before drying in a hot air oven at 50°C for one hour. The dried granulation was finally milled by pushing it through a 18-mesh screen.

**Table 2** Formulations of Theophylline Granules with Methocel E4M

| Ingredient | Quantity |
|---|---|
| Theophylline, | 300 mg |
| Methocel E4M | 5, 7, 10 % * |

* represents the formulation 1, 2 and 3 respectively

1.2 Evaluation of Granules

1.2.1 Moisture Content : About 5 g of sample was accurately weighed on a pan of Mettler LP16 moisture determination balance (Mettler, USA) and it was dried by an IR lamp until constant weight was obtained. The moisture content was automatically calculated in percentage and was averaged from three determinations.

1.2.2 Particle Size Distribution : Using the method of sieve analysis, the approximately 30 g of granule was put on the top of a sieve series whose sizes ranging from 850, 425, 250, 180 and 150 μm respectively. The nest of sieve (Endocotts, England) was placed on the sieve shaker ( Josef Deckelman, Germany ) for 20 minutes. The results averaged from two determinations were reported as percentage of weight retained on each sieve and pan.

1.2.3 Angle of Repose : Angle of repose was determined by fixed-bed cone method. An appropriate amount of granule was filled in the funnel of suitable diameter and allowed to flow freely onto the center of a petridish of known radius until its perimeter was touched by the granule heap. Angle of repose was calculated from the following equation :

$$\propto = \tan^{-1} \frac{H}{R} \quad \text{..... Eq. 1}$$

where $\propto$ is the angle of repose, H and R are the height and radius of heap respectively. The results was reported as an average of three determinations.

1.2.4 Bulk, Tap Density and Compressibility : An accurate weight of about 20 g of each granulation was slowly poured into a 100-mL graduated cylinder and the bulk volume was measured. The cylinder was later dropped onto a hard wood surface from a height of 5 cm until a constant volume was obtained. The bulk and tap density were calculated by dividing the weight of sample by its bulk volume and tap volume respectively. Both parameters were averaged from three trials and was employed to calculate the compressibility by using the following equation :

$$\% \text{ compressibility} = \frac{(T-B)}{T} \times 100 \quad \text{..... Eq. 2}$$

where T = Tap density

and B = Bulk density

1.2.5 Content of Active Ingredient : The procedure used to determine theophylline content was modified from the one described by Sa, Bandayopadhyay, and Gupta (1990). An approximately 100 mg of sample was accurately weighed and after that a 150 mL of 0.1 N HCl was added and stirred for an hour using magnetic stirrer. The solution was then adjusted to a volume of 200 mL with 0.1 N HCl in volumetric flask and filtered. After discarding the first 10 mL of filtrate, an aliquot of 10 mL was transferred into 50-mL volumetric flask and adjusted to volume with 0.1 N HCl.The absorbance of final solution was measured at 268.5 nm using double beam spectrophotometer (Spectronic 2000, Bausch & Lomb, USA). The content of theophylline was then calculated from absorbance-concentration calibration curve.

1.3 Preparation of Tablets

The granules of each formulation were mixed with 0.5 % magnesium stearate and then compressed into tablets of about 10-kp hardness by Carver Laboratory Press (Perkin-Elmer, Model C, Fred & Carver Inc., USA) using 3/8-inch round flat-faced punch. The

compression pressure was maintained for 10 seconds and quickly released.

1.4 Evaluation of Tablets

1.4.1 Hardness : The hardness of compressed tablets was measured by Schleuniger-2E hardness tester (Model 2E/205, Dr. K. Schleuniger & Co., Switzerland) and expressed in kilopound unit (kp). The mean and standard deviation were calculated from ten determinations.

1.4.2 Disintegration Time : It was determined according to the method for uncoated tablets specified in USP XXII with the USP disintegration apparatus (Model QC-21, Hanson Research, USA). The test was performed with disk in purified water at 37 ± 2 ° C. The average time was calculated from six determinations.

1.5 Effect of pH on Dissolution Characteristics

Since the different pH values of the medium may affect the dissolution of theophylline from tablets, an in-vitro drug release was therefore studied in two dissolution media i.e. 0.1N HCl and pH 6.8 phosphate buffer. Following the paddle method of USP dissolution test, 900 mL of the dissolution medium was placed in a glass vessel and equilibrated at 37 ± 0.5 ° C. One tablet was immersed in each of the vessel and the dissolution apparatus (Model SR2, Hanson Research,

USA) was then immediately operated with the center-positioned paddle stirring at a speed of 50 rpm. 5 mL of sample solution was taken at appropriate time intervals up to 12 hours and the same quantity of the medium was added at once after each sampling to keep the volume of dissolution medium constant during the whole test period. Each sample was next diluted with the same medium to suitable concentration and the absorbance was measured using spectrophotometer at 268.5 nm for 0.1 N HCl and 270.3 nm for pH 6.8 phosphate buffer. The amount of theophylline dissolved at any time interval was calculated from the calibration curve. A cumulative correction was also made for the previously removed sample to determine the total amount of drug release. Three tablets of each formulation were evaluated.

Calibration Curve : About 200 mg of theophylline was accurately weighed and transferred to a 2000-mL volumetric flask. It was dissolved and adjusted to volume with 0.1 N HCl or pH 6.8 phosphate buffer to produce the stock solution. Standard solutions which had the known concentrations of about 4, 6, 8, 10, 12 and 14 mcg/mL were then prepared in a duplicate by dilution of the stock with the medium and analysed spectrophotometrically in a 1-cm cell at the same wavelength as used in the dissolution study. The absorbance of each concentration and the calibration curve in both media are presented in Tables 34, 35 and Figures 21, 22 of the Appendix B respectively.

1.6 Modification of Drug Release

1.6.1 Addition of Channeling Agent : The formulation which provided the most similar dissolution profiles in both 0.1 N HCl and pH 6.8 phosphate buffer was selected for further study by reason of its constant release over that wide pH range. However, it was found that the release rate of such formulation was rather slow especially in pH 6.8 phosphate buffer. To increase the rate of drug release, lactose as a channeling agent was incorporated into the formulation in various quantities as shown in Table 3. All tablets were evaluated for physical properties by the same method as described in 1.4.

**Table 3** Formulations of Theophylline Sustained-release Tablets with Lactose as Channeling Agent

| Ingredients | Amount per tablet |
|---|---|
| Theophylline, anhydrous | 300 mg |
| Methocel E4M | 22.54 mg |
| Lactose | 20, 30, 40 %* |
| Magnesium stearate | 0.5 % |

* represents the formulation 4, 5, and 6 respectively

1.6.2 Dissolution Test by pH Change Method : As oral sustained-release tablets were supposed to pass the entire upper gastrointestinal tract, it would be ideal when the active drug was released at a constant rate over a wide range of pH values (from 1 to about 7). Therefore, an in-vitro dissolution test should at least cover this pH range to reflect the real condition of drug release in vivo. For this reason, pH change method was used ( Jonkman , Berg and De Zeeuw , 1983 ). 0.1 N HCl was used as the medium in the first two hours and then the pH was increased to 6.8 by adding sufficient volume of 0.1 N HCl containing 4.41 g of sodium hydroxide and 6.12 g of potassium dihydrogen phosphate. Purified water was boiled to deaerate before use. Other procedures were the same as described in 1.5. Six tablets of each formulation were evaluated and compared with the commercial product, Theo-Dur® and Nuelin®.

2. In Vivo Evaluation

2.1 Products

The experimental theophylline sustained-release formulations of theophylline prepared by techniques of spray drying (Vipaluk , 1993), fluidized-bed coating (Sudarat, 1994) and conventional wet granulation which possessed the most satisfactory dissolution characteristics were selected to be evaluated. All tablets were newly prepared by individual condition and investigated for the pattern of in-

vitro drug release. The commercial products such as Theo-Dur® which used as a reference and Nuelin® were also tested.

### 2.2 Subjects and Drug Administration

Twenty male New Zealand White rabbits, weighing between 2.8 and 3.5 kg, were acclimatized to the research facility for one week before the study. Each then received a single oral dose of theophylline as an intact tablet in the morning after being fasted overnight with water *ad libitum*. Food was withheld for two hours after the administration of the various drug formulations.

### 2.3 Experimental Design

The study was conducted in a balanced incomplete block design which had the general rule that every subject received an equal number of formulations and each pair of formulations occurred together in a block (subject) at the same number of times (Westlake, 1988). One tablet of each formulation was given orally to each subject in a crossover manner with a washout period at least one week between two consecutive dosings as shown in Table 4. It should be noted that a second replicate was conducted to minimize any uncontrolled variations.

**Table 4** Dosing Schedule in a Balanced Incomplete Block Design

| Subject number | Week 1 | Week 2 |
|---|---|---|
| 1, 11 | A | B |
| 2, 12 | B | C |
| 3, 13 | C | D |
| 4, 14 | D | E |
| 5, 15 | E | A |
| 6, 16 | A | C |
| 7, 17 | C | E |
| 8, 18 | E | B |
| 9, 19 | B | D |
| 10, 20 | D | A |

Where A = Theo-Dur®; B = Nuelin®; C, D, E = Tablets prepared by spray drying, fluidized-bed coating, and wet granulation respectively

2.4 Sample Collection

3 mL of blood sample was collected from a marginal ear vein using a disposable needle No. 21 at 1, 2, 5, 7, 9, 12, 24, 36, 48 and 72 hours after each dosing. Blood sample was allowed to flow directly through the needle into a heparinized tube. Then, it was immediately centrifuged at a speed of 3000 rpm for 20 minutes. All of the plasma was separated and stored at -10 ° C until subsequent analysis.

2.5 Determination of Plasma Theophylline Concentration

Concentrations of theophylline in plasma samples were determined using high performance liquid chromatography (HPLC) (Milton Roy, LDC Division, USA) by following the procedure modified from that described by Bock , Lam , and Karmen ( 1984 ).

β-Hydroxyethyltheophylline of which chemical structure was similar to theophylline was used as an internal standard.

2.5.1 Preparation of Plasma Sample : Before introducing into HPLC system, each plasma sample had to be clarified by deproteinization using zinc sulfate in combination with methanol as follows.

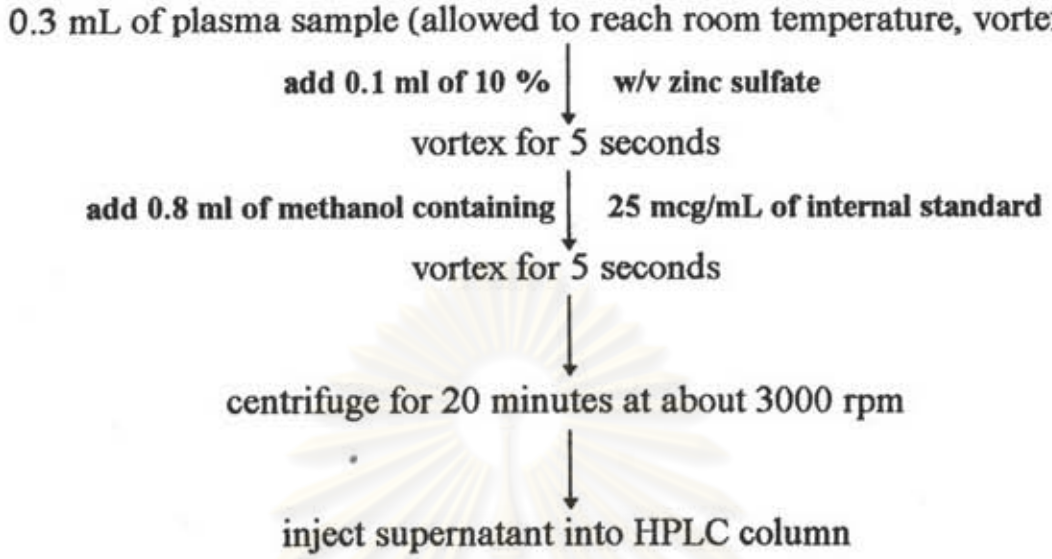


**Figure 1** Schematic Process for Plasma Sample Preparation

2.5.2 Chromatographic Condition :

HPLC apparatus (Milton Roy, USA) consists of : 1. Multiple solvent delivery system, Model CM4000
2. Programmable wavelength detector, Model SM4000
3.Computing integrator, Model CI4000

Injector : Rheodyne 7100 Injection Port (Rheodyne, USA)

| | | |
|---|---|---|
| Column | : | Bondclone C18 with particle size of 10 μm, 300 × 3.9 mm, serial no. 30038 (Phenomenex, USA) |
| Guard column | : | Bondapak C18/Corasil with particle size of 37-50 μm (Millipore Corp., USA) |
| Mobile phase | : | Acetonitrile : 0.01 M sodium acetate buffer, pH 4.0 = 1 : 9 |
| Flow rate | : | 1.5 mL/min |
| Pressure | : | 1400-1900 psi |
| Injection volume | : | 20 μL |
| Detector | : | UV, 280 nm |
| Chart speed | : | 3 mm/min |
| Attenuation | : | $2^3$ |
| Retention time | : | about 5.3 min for theophylline<br>about 6.1 min for internal standard |
| Temperature | : | Ambient |

The area under the peak of theophylline and internal standard were calculated by the integrator and theophylline concentrations in plasma were subsequently quantified from the calibration curve of peak area ratio against known drug concentration.

2.5.3 Calibration Curve : About 300 mg of theophylline was weighed accurately and transferred to 100-mL volumetric flask. Dissolve the drug and adjust to volume with tridistilled water. This was used as stock solution. Standard solutions which had the known concentration of about 30, 180, 360, 480, 600, 900, 1200 and 1500 mcg/mL were then prepared by dilution of the stock with tridistilled water. Next, exactly 20 µL of each standard solution was individually added, using micropipette (Socorex ISBA S.A., Switzerland) , to 0.3 mL of pooled plasma and vortexed for 5 seconds to make the plasma concentrations of 2, 12, 24, 32, 40, 60, 80 and 100 mcg/ml, respectively. These plasma standards were finally clarified and analysed following the same procedure as mentioned previously. Calibration curve was constructed on each day of sample analysis by plotting the ratios of area under the peak of theophylline to that of β-hydroxyethyltheophylline against their known drug concentrations. The relationship of these two variables was fitted to a straight line using linear regression as presented in Table 36 and Figure 23 of the Appendix B .

## 2.6 Pharmacokinetic Analysis

Theophylline concentration (C) versus time (t) curves from each treatment were plotted and the pharmacokinetic parameters were determined using conventional method. The peak plasma concentration ($C_{max}$) and the time to reach the peak ($t_{max}$) were directly inspected from the data. The area under the plasma concentration-time curve ($AUC_0^\infty$) was calculated using the trapezoidal rule, with extrapolation to infinity, by dividing the last experimental point by the linear terminal slope of the logC vs t plot (Gibaldi and Perrier, 1982). The absorption and elimination rate constant ($K_a$ and $K_{el}$) were both obtained by the method of residuals and the biological half-life ($t_{1/2}$) was calculated using the quotient $0.693 / K_{el}$.

## 2.7 Evaluation of Bioequivalence

The assessment of the comparative bioavailability of the five formulations of theophylline sustained-release tablets was established employing the three relevant pharmacokinetic parameters, i.e. $C_{max}$, $t_{max}$, and $AUC_0^\infty$ values. The differences in these parameters among all products were found out by one way analysis of variance at 95 % confidence interval ($\alpha = 0.05$). If the results showed statistically significant difference, the difference of those values between each pair of treatment, especially the comparison with Theo-Dur®, would be examined

by the method of Duncan's new multiple range test. Provided that there was no significant difference, the two formulations would be considered to be bioequivalent.

## 2.8 In Vitro-In Vivo Correlation

The dissolution rate constants, as the in vitro parameter, and the bioavailability parameters $C_{max}$, $t_{max}$ and $AUC_0^\infty$ of all tablets were subjected to linear regression analysis and the correlation coefficients were calculated.