CHAPTER III

MATERIALS AND METHODS

I Materials

Test Products

Four commercial brands of theophylline sustained-release products, were bought from Siriraj Hospital. The letters A, B, C, and D were given to represent the brand names of the products. Information of these products were reported in Appendix A.

Reagents

1. Working standard theophylline powder potency 100.35%(Fluka Chemika, Switzerland) lot no. 278460 688

2. Working standard etofylline powder (Sigma) lot no. 78F0419

3. Working standard caffeine anhydrous powder potency 98.04% (FA trading Co.) lot no. 890022

4. Methanol HPLC grade(J.T. Baker Inc., U.S.A.)lot no.24P0198

5. Acetonitrile HPLC grade (J.T.Baker Inc., U.S.A.) lot no, 24P2300

6. Potassium dihydrogenphosphate GR. (E.Merck, Germany) lot no. 304A707573

7. Sodium acetate trihydrate (May & Baker, England) lot no. MX7009

8. Zinc sulfate heptahydrate GR. (E.Merck, Germany) lot no. 244TA327983

9. ortho-Phosphoric acid 85% GR. (E.Merck, Germany) lot no. 046K15068573

10. Hydrochloric acid (BDH, England) lot no. 6983370J

11. Sodium hydroxide pellets GR. (EKA Nobel, Sweden) lot no. 8070-408

12. Potassium hydroxide pellets GR. (E.Merck, Germany) lot no. 0101195

13. Heparin 5000 IU/ml (NOVO Industrials, Denmark) lot no. 2437612

14. Sodium chloride GR. (E.Merck, Germany) lot no. 009K13577604

15. Glacial acetic acid 100% GR. (E.Merck, Germany) lot no. 0118K14090663

Apparatus

1. Analytical balance (Sartorius, 1615 MP, Germany)

2. Dissolution apparatus (72 RL, Hanson Research Corp., Northridge, Calif., U.S.A.)

3. Spectrophotometer (U-200, Hitachi, Japan)

4. Digital pH meter (SA 520, Orion, U.S.A.)

5. Vortex mixer (Ofnie, K-550-GE, Scientific industries, Inc., Bohemia, N.Y., U.S.A.)

6. Refrigerated centrifuge (Sigma 302 K, Sigma Lab, Centrifuge Gmbtt, Germany)

7. High Performance Liquid Chromatography
   - Pump (Waters 510, Millipore, U.S.A.)
   - Turnable absorbance detector (Waters 484, Millipore, U.S.A.)
   - Data module (Waters 745 B, Millipore, U.S.A.)
   - Automatic injector (Waters 721 Wisp, Millipore, U.S.A.)

8. Waterbath (Hetofrig CB60, DT. Hetotherm, Heto, Denmark)

9. Micropipet (Socorex, Swiss)

10. Sonicator (Bansonic 221, U.S.A.)

11. Glassware

II Methods

A. In Vitro Studies

Four commercial brands of theophylline sustained-release products were bought from Siriraj hospital. The letters A, B, C and D were given to represent each brand names of the products. Information of these products were reported in Appendix A.

All of these brands were evaluated using both official and non-official tests as stated in the pharmacopoeia. The tests were:

1. Uniformity of dosage units (U.S.P.XXII)

Weight variation

Select not less than 30 tablets or capsules of theophylline sustained-release tablets from each brand then weigh accurately 10 tablets individually, and calculate the average weight. (differential weights not more than $\pm$ 5 %)

2. Identification (U.S.P. XXII)

2.1 From each brands, triturate a quantity of finely powdered tablets, equivalents to about 100 mg of anhydrous theophylline, put into a suitable conical flask. Add 150 ml of methanol and sonicate until the insoluble material dispersed into fine particles. Shake by mechanical means for 15 minutes, and filter

into a 250-ml volumetric flask. Dilute with water to volume, and mix. Pipet 5 ml of this solution into a 200-ml volumetric flask, dilute with 0.1 N hydrochloric acid to volume, and mix: the ultraviolet absorption spectrum of the solution so obtained exhibits maxima and minima at the same wavelengths as that of a similar solution of working standard theophylline, concomitant measured.

2.2 The retention time of the major peak in the chromatogram of the assay preparation corresponds to that of the standard preparation, as obtained in the assay.

3. Assay (U.S.P.XXII)

The contents of at least 20 tablets of each brand were carefully grinded and mixed uniformly. An accurately weighed amount of this powder, equivalent to about 100 mg of anhydrous theophylline, was added with 2 ml of methanol, and swirled until the powder is wetted. Then add 80 ml of phosphate buffer (Appendix C), sonicate with frequent swirling until the powder was completely dispersed, and heat on a steam bath for 15 minutes It was left cool. The solution was adjusted to volume (100 ml) with phosphate buffer. After filtering, 10 ml of solution was diluted to 100 ml with the mobile phase (Appendix C) and this sample was assayed with High Pressure Liquid Chromatography compared with standard theophylline which prepared by the same method. From 5 replicate chromatograms, calculate for the quantity in mg of anhydrous theophylline.

High Performance liquid Chromatography Condition

Apparatus : HPLC Waters 510, Waters Ass. (Millipore), U.S.A.

Columnn : μ-Bondapak C18 stainless steel column, 3.9 x 300 mm, 125 ^{0}A 10 mcm of Dimethyloctadecylsilyl bonded amorphous silica, part no. 27324, Waters Associates Pty-Ltd., U.S.A., Pre-column 5 cm x 2.0 nm i.d.

Mobile Phase : Phosphate buffer (Appendix B) : Methanol (7:3)

UV detector : 254 nm

Flow rate : 1 ml/minute

Attnuation : 256 mv/full scale

Chart speed : 0.25 cm/min.

Pressure : 1500-2000 psi.

Temperature : Ambient

Resolution solution : Prepare a solution of anhydrous caffeine in a mixture of methanol and water (9:1) containing about 2 mg/ml. Pipet 6 ml of this solution and 10 ml of the stock standard solution into a 100-ml volumetric flask, dilute with the mobile phase (Appendix C) to volume, and mix.

Retention time : Theophylline, anhydrous 6.4 minutes
Caffeine, anhydrous 8.9 minutes

4. Dissolution test (Al-Angary et al., 1990)

The dissolution test method for theophylline sustained-release tablets was not available in any pharmacopoeias. Dissolution profiles of theophylline sustained-release tablets were determined according to the method which modified from that of Al-Angary et al. It was described as follow.

The dissolution test was carried out using the U.S.P.XXII apparatus type I (Appendix D) at 100 rpm. The dissolution medium, at 37 ± 0.5 °C, consisted of 900 ml of simulated gastric fluid at pH 1.2 (Appendix C) for 1 hr., then substituted by 900 ml of simulated intestinal fluid at pH 7.5 (Appendix C) for 11 hr.(or 23 hr. for once-a-day theophylline sustained-release capsules). Both fluids were without enzymes. At predetermined time intervals 5 ml aliquots were withdrawn and were immediately replaced with 5 ml of fresh dissolution medium equilibrated at 37°C. Theophylline concentration fromthe filtered samples was determined at 270 nm and the results were reported as the mean percent dissolved. Each point represent the average of six determinations.

Calibration curve

Standard solutions with known concentration of theophylline in dissolution medium (2, 4, 6, 8, 10, 12, 16, 18, and 20 mcg/ml) were prepared and they were analysed using spectrophotometer at 270 nm. Absorbance obtained versus known concentrations were fitted to a straight line using linear regression (Appendix B).

5. In Vitro Evaluation

Physical characteristics of all four brands of theophylline sustained-release tablets were examined and evaluated to determine whether each brand passed the general standard of U.S.P.XXII requirements. A one way analysis of variance and Least Significance Difference (LSD) (Appendix E) were performed to assess the difference of the dissolution rate constant between the values of the reference product and those of the other manufactured brand.

B. In Vivo Studies

1. Products

All brands of theophylline sustained-release tablets commercially available in Thailand were used in this study.

2. Subjects

Thirteen volunteers with the ages ranged from 20 to 45 years partiipated in this study. All subjects were healthy based on history, clinical examination and pre-entry hematologic and biochemical tests. Demographic data are presented in Appendix D. The methods of the study were fully explained to all subjects. They were taking no medication, alcohol and cigarette for at least one week prior to and throughout the study.

3. Dose and Drug Administration

One or halved tablet (only in breakable brands) of theophylline sustained-release tablets was given orally with water every 12 hours for one week (or every 24 hours for once-a-day preparation)

4. Experimental Design

The study was conducted in a randomized crossover design. Each subject received the drug in a randomized order as shown in Table 1.

5. Sample Collection

Five millilitres of blood samples were collected from a forearm vein using a heparinized IV cathetor. They were immediately transfered to heparinized tubes. Blood sample was collected before drug administration and at 0.25, 0.5, 1, 2, 3, 4, 6, 8, and 12 hr. after dosing (for once-a-day preparations, the blood sample was further collected at 16 and 24 hr. later). The blood was immediately centrifuged at 3000 rpm for 5 minutes and the plasma was seperated and kept at -20^{0}C until subsequent analysis.

Table 2 Dosing Schedule

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

6. Determination of theophylline in plasma

Concentrations of theophylline in plasma were determined by High Performance Liquid Chromatography using a method modified from those described by Jung,1989. The procedure was shown as follows :

Plasma sample (or standard) 0.5 ml
- add 0.1 ml of zinc sulfate solution (10% w/v) followed by 0.75 ml of methanol containing 10 mcg/ml of the internal standard, etofylline
- mixed by vortexing for 30 seconds then centrifuged for 15 min. at 1200 rpm

↓

25 mcl of supernatant

was injected into the HPLC system

7. Operating Condition

Apparatus : HPLC Waters 510, Waters Ass. (Millipore), U.S.A.

Columnn : μ-Bondapak C18 stainless steel column, 3.9 x 300 mm, 125 ^{0}A 10 mcm of Dimethyloctadecylsilyl bonded amorphous silica, part no. 27324, Waters Associates Pty-Ltd., U.S.A., Pre-column 5 cm x 2.0 nm i.d.

Mobile Phase : 11% acetronitrile in 0.01 sodium acetate buffer pH 4.0 (Appendix C)

UV detector : 280 nm

Flow rate : 1.5 ml/minute

Attenuation : 32 mv/full scale

Chart speed : 1.0 cm/min.

Pressure : 1500-2000 psi.

Temperature : Ambient

Retention time : Theophylline, anhydrous 5.5 minutes
Etofylline 6.5 minutes

The theophylline concentration in plasma samples were quantified from the calibration curve.

8. Calibration Curve

Known amounts of standard theophylline were prepared in distilled water to yield 0.625, 1.25, 2.5, 3.75, 5.0, 7.5 and 10.0 mcg /mcl of theophylline.

Exactly 10 mcl of each theophylline standard solution was added, using a microsyringe, into each of seven test tubes containing 0.5 ml human blank plasma, except the first test tube was added with 10 mcl of distilled water. Finally, the concentrations of plasma standards were 0, 1.25, 2.5, 5.0, 7.5, 10.0, 15.0, and 20.0 mcg/ml, respectively. These plasma standards were then prepared and analyzed following the same procedure as described previously. The ratio of the peak area of theophylline to inter nal standard obtained versus

known theophylline concentrations were fitted to a straight line using linear regression (Appendix B).

9. Pharmacokinetic analysis

Since in each brand had differences in dosage, all data was normalized to be 200 mg dose twice daily (or 400 mg dose once daily for once-a-day preparation) before.

The pharmacokinetic analysis of individual plasma theophylline concentrations from treatment was established using the conventional method.

In case of the conventional method the peak plasma concentration and the time to peak plasma concentration were directly observed from the data, meanwhile, the area under the plasma concentration-time curve was calculated using the trapezoidal rule (all data formulate AUC were normalized to 400 mg per day).

Fluctuation of each drug and each subject was calculated using an equation.

$$\% \text{ fluctuation} = (\text{peak} - \text{trough})/\text{trough} \times 100$$

10. Evaluation of Bioequivalence

The comparative bioavailability of all four brands of theophylline sustained-release tablets in this study were assessed using the four relevant pharmacokinetic parameters, C_{max}, t_{max}, AUC and %fluctuation.

The differences in C_{max}, t_{max}, AUC and %fluctuation among the four brands were determined by one way analysis of variance (ANOVA)

at the significant level of α = 0.05. If the results showed statistically significant difference, the difference of these values between the values of the reference product and those of each brand were examined by LSD. In the same way, if the result showed no statistically significant differences from those of the reference product, the test brands were considered to be bioequivalent to the reference brand.

11. In Vitro-In Vivo Correlation Study

Correlation coefficient test was used to test the relationship between the in vitro parameters, (the dissolution rate constants), and the in vivo parameters (C_{max}, t_{max}, AUC and % fluctuation) of all brands.

12. Assumption

The pharmacokinetics of theophylline following drug administration to human followed the first-order kinetics.