CHAPTER III

MATERIALS AND METHODS

MATERIALS

A. Theophylline Test Product

Theophylline syrup dosage form was used in this study. "Nuelin® syrup" (Riker Laboratories Australia PTY.LTD., Lot no. 4634B) was selected for using throughout this study. It contained 80 mg of theophylline per 15 ml. No other active ingredients or alcohol were present in this dosage form.

B. Reagents

1. Working Standard Theophylline Powder, potency 100.35% (Fluka Chemika, Switzerland) Lot no. 278460 688
2. Internal Standard, 8-Chlorotheophylline Powder (Sigma,U.S.A) Lot no. 27F-0020
3. Acetonitrile HPLC grade (E.Merck, Germany) Lot no. I007930
4. Methanol Absolute HPLC grade (BDH,England) Lot no. 3628620j
5. Acetic Acid, glacial AR (E.Merck, Germany)

Lot no. 542 HI 350663

6. Sodium Acetate AR (Ajax Chemicals LTD., Sydney-Melbourne) Lot no. 41868
7. Zinc Sulfate AR (E.Merck, Germany) Lot no. 7499801

C. Apparatus

1. HPLC Column (μ-Bondapak-C_{18}, Waters Associates, Inc., Milford, MA., U.S.A)
2. HPLC Pump (Model 510, Waters Associates, Inc., Milford, MA., U.S.A)
3. Injector (Model U6K, Waters Associates, Inc., Milford, MA., U.S.A)
4. UV Absorbance Detector (Model 440, Waters Associates, Inc., Milford, MA., U.S.A)
5. Integrator (745 Data Module, Waters Associates, Inc., Milford, MA., U.S.A)
6. Analytical Balance (Sartorius 2442, Sartorius GMBH., Germany)
7. Centrifuge (Model 301, Sigma Laborzentrifugen GMBH, Western Germany)
8. Vortex (Model G-560-E, Scientific Industries, Inc., Bohemia, N.Y., U.S.A)

METHODS

1. Subjects

Forty Thai healthy volunteers participated in this study. The subjects were divided into 4 groups according to sex, smoking status, and age as follows.

Group A. Nonsmoking Males

This group consists of 10 men, who had either never smoked or had not smoked within the past 2 years, ranged in age from 23 to 38 years.

Group B. Nonsmoking Females

Ten women had the same smoking status as nonsmoking males, ranged in age from 22 to 34 years. None of them were pregnant or on contraceptive medications.

Group C. Smoking Males

This group consists of 10 men who smoked at least 15 cigarettes per day during the study and at least 1 year before the study, ranged in age from 21 to 33 years.

Group D. Children

This group consists of 10 boys ranged in age from 7 to 12 years.

Other characteristics of each subject are presented in Appendix A.

In all subjects, a medical history, physical examination and standard laboratory screen (Appendix A) were performed prior to the study, to ensure that the subjects were normal,i.e., healthy and absent from any significant hepatic or renal disturbance.

The purpose and method of the study were fully explained to all subjects and the parents of children subjects in group D.

No subjects had taken any medications within 1 week before the study. All of them were asked to abstain from food or beverages containing xanthines (coffee, tea, chocolate and cola beverages) and alcohol for at least 24 hours prior to and during the study.

2. Drug Administration

Each subject received a single oral dose of 2.4 mg/kg of theophylline in syrup with 100 ml of water. Subjects had fasted 12 hours before and 2 hours after taking the theophylline dose.

3. Sample Collection

Blood samples (5 ml) were drawn from an intravenous cannula in a forearm vein of each subject prior to dosing and at 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10,

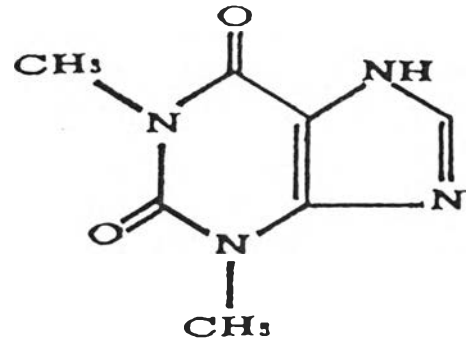
and 12 hours after the dose and immediately collected in 10 ml heparinized tube. Plasma was separated after collection by centrifugation (1200 *g* for 15 minutes at room temperature). All plasma samples were stored at -20 ^{o}C until subsequent analysis.

4. Analytical Method

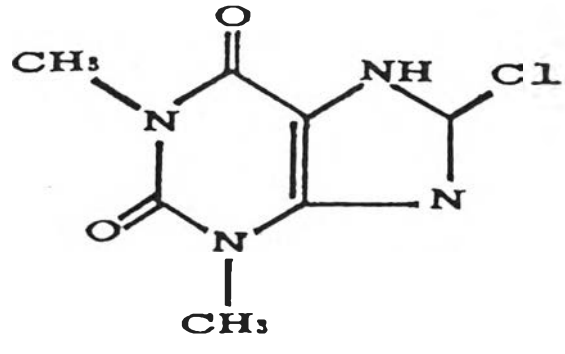
Theophylline concentrations in plasma samples were determined by High Performance Liquid Chromatography (HPLC) method. 8-Chlorotheophylline (8-chloro-3,7-dihydro-1,3-dimethyl-1H-purine-2,6-dione) of which chemical structure was similar to theophylline, was used as the internal standard. Chemical structures of theophylline and 8-chlorotheophylline are shown in Figure 2. The procedure was modified from the method of Bock et al. (52) as follows.

4.1 Sample preparation

Plasma samples were clarified before introduced into HPLC system. The clarification by protein-precipitation with zinc sulfate and methanol was used in this procedure.

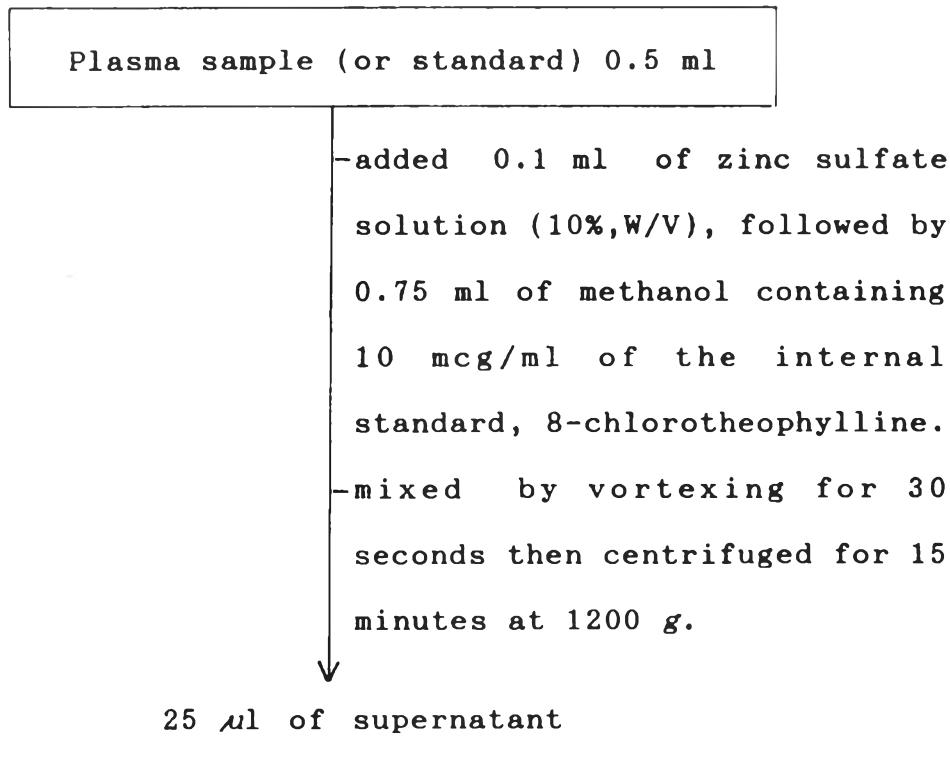


Theophylline



8-Chlorotheophylline

Figure 2. Chemical structures of theophylline and 8-chlorotheophylline



The supernatant was injected into the column and analyzed by HPLC as described under the section of chromatographic conditions.

4.2 Plasma standard preparation

Standard solutions of theophylline were prepared in distilled water to yield 0.625, 1.25, 2.5, 3.75, 5.0, 7.5, and 10.0 mcg per 10 μl of theophylline.

Exactly 10 μl 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 μl 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 along with plasma samples. On each day of sample analysis, the plasma standards were prepared and analyzed concomitantly.

4.3 Liquid chromatographic conditions

Apparatus	:	HPLC Pump, Injector, UV Absorbance Detector, and Integrator
Column	:	μ-Bondapak-C_{18}, 30 cm x 3.9 mm id.
Mobile Phase	:	11% acetonitrile in 0.01 M sodium acetate buffer pH 4.0 (Appendix B)
Flow Rate	:	1.5 ml per minute
Pressure	:	2500-3000 psi
Temperature	:	ambient
Detector	:	UV 280 nm
Integrator	:	attenuation 16 chart speed 1.5 cm per minute
Injection Volume	:	25 μl

A 25-μl volume of supernatant, prepared from plasma samples or standards, as described previously, was injected into the column and analyzed by an HPLC under the above conditions. The absorbance of theophylline and 8-chlorotheophylline were detected by a UV detector at

280 nm wave length and measured by an integrator. The area under the peaks of theophylline and 8-chlorotheophylline were calculated by the integrator.

Theophylline concentration in each plasma sample was determined by comparing the ratio of area under the peak of theophylline to that of 8-chlorotheophylline with the standard curve.

4.4 Standard curve

Standard theophylline concentration curve was constructed by plotting the ratios of area under the peak of plasma-standard theophylline to that of 8-chlorotheophylline against their known theophylline concentrations. The ratios were analyzed by linear regression with respect to their concentrations (Appendix C). The concentrations of theophylline in plasma samples were determined by inverse predication from the linear regression of the standards.

5. Data Analysis

The theophylline plasma concentration-time data obtained was resolved by pharmacokinetic and statistical analysis. The appropriate pharmacokinetic model was used to describe the data obtained from the experiments. The theophylline pharmacokinetic parameters of each group of subjects (i.e.,nonsmoking males,

nonsmoking females, smoking males, and children) were determined and compared.

5.1 Pharmacokinetic analysis

The theophylline plasma concentration versus time from all subjects data were plotted on the semilogarithmic graph paper. The concentration-time profiles obtained from these experimental data could be well described by a one-compartment open model with first-order absorption and first-order elimination (16,18,39,43,45), as shown in Figure 3.

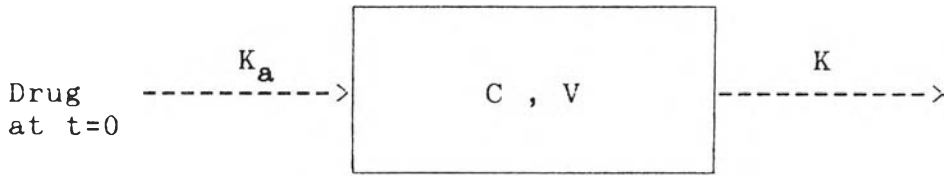


Figure 3. Diagram of one-compartment pharmacokinetic model for first-order drug absorption and first-order elimination. K_a and K are the first-order rate constants for absorption and elimination, respectively. C is the drug concentration in plasma and V is the volume of distribution.

This model is described by the following equation (53).

$$C = \frac{K_a F X_o}{V (K_a - K)} (e^{-Kt} - e^{-K_a t})$$

C - plasma concentration of drug at time t

F - fraction of the administered dose (X_o) that is absorbed

V - apparent volume of distribution of drug in the body

K_a,K - first-order rate constants for absorption and elimination, respectively

To obtain the best estimates of the parameters, this equation was used to fit the data using a nonlinear estimation program, PCNONLIN. The initial estimates (V, K_a and K) were obtained by using graphical method with the method of residuals (53) (Appendix D).

By introducing the obtained initial parameter estimates (V, K_a and K) into PCNONLIN nonlinear estimation program and a using method 2 for analysis could enable the computer program to estimate the best estimates of V, K_a and K. The best estimates of these parameters then were used to calculate the other parameters [i.e., the area under the concentration-time curve from time zero to time infinity AUC, half-lives of K_a and K, time to peak plasma concentration (t_{max}) and the maximum plasma

concentration at this time (C_{max})] by using the equations shown in Appendix D and E.

5.2 Statistical analysis

The results obtained from the pharmacokinetic evaluation were subject to statistical analysis. The Student's t-test was employed, at the 5% significant level, to analyze the differences between the nonsmoking males and the other groups of subjects (i.e., nonsmoking females, smoking males, and children). The SPSS/PC package was used to perform all statistical analysis in this study (Appendix F).