CHAPTER II

MATERIALS AND METHODS

MATERIALS

A. Theophylline Test Product

Theophylline tablet containing 100 mg. per tablet. (Unichem Pharmaceuticals Reg. No. 1A203329, Batch No. 22035) was selected for used throughout this study.

B. Reagent

1. Theophylline ; Aldrich, Milwaukee, WIS, USA.
2. β - Hydroxyethyltheophylline ; Sigma, St.Louis, MO, USA.
3. β - Hydroxypropyltheophylline ; Sigma, St.Louis, MO, USA.
4. 8-Chlorotheophylline ; Sigma, St.Louis, MO, USA.
5. Caffeine ; Sigma, St.Louis, MO, USA.
6. Zinc sulfate, AR grade ; E.Merck, Damstadt, Germany
7. Methanol, HPLC grade ; J.T. Baker Chemical, NJ, USA.
8. Acetonitrile, HPLC grade ; J.T. Baker Chemical, NJ, USA.
9. Citric acid, AR grade ; Farmitalia Carlo Erba, Milano,Italy.

10. Sodium acetate, AR grade ; E.Merck, Damstadt, Germany
11. Glacial acetic acid, AR grade ; E.Merck, Damstadt, Germany
12. Heparin 5000 IU. ; Leo, USA.
13. Normal saline solution, 0.9% ; General Hospital Products, Bangkok, Thailand

C.. Human Plasma and Saliva

Human plasma used in the part of analytical method was generously supplied from the Plasma Division, Thai Red Cross Society. The pooled blank plasma from volunteers was used in analysis of sample. Human saliva own expectorated and the pooled blank saliva from volunteers were ultilized for analytical method part and sample analysis, respectively.

D. Apparatus

1. HPLC ; Millipore Waters Chromatography Division, Milford, Massachusetts, USA.
   compose of :
   1.1 Model 600 E multi solvent delivery system
   1.2 Water 746 data module
   1.3 Water 484 tunable absorbance detector
   1.4 Model 712 Waters Intelligent Sample Processeor (WISP™)

2. Ultraviolet double beam Spectrophotometer ; Shimadzu, Model UV-180 , Shimadzu, Japan
3. Centrifuge ; Labofuge 1 , Heraeus-Christ GMBH, West Germany
4. Analytical balance ; Precisa 300 A, PAG oerikon AG, Switzerland
5. pH meter ; Consort pH meter, P 307, Germany
6. Vortex mixer ; Vortex-Genie, Scientific Industries Inc., NY, USA.
7. Chromatographic column ; 300 x 3.9 mm. I.D. stainless-steel 10 mcm. Bondclone 10 C18 colume Phenomenex(R), CA, USA.)
8. Guard column ; 50 x 2.0 mm. I.D. stainless-steel packed with 37-50 mcm C18 (Coracil(R)_Water Associates, Massachusetts, USA.)
9. Sonicater ; Bransonic 321, Smith Kline, CT, USA.
10. Micropipet : (10-20 mcl.), (100-1000 mcl.) ; Pipetman(R), Gilson, UK.

    : (10-100 mcl.), (100-1000 mcl.) ; Socorex(R) , USA.
11. Screw-capped glass tube, 15 cc. ; Pyrex (R), USA.
12. Disposable Needle No. 21, 23 ; Terumo(R), USA.
13. Disposable Syringe 5 ml. ; Terumo(R) , USA.
14. Heparin Lock No. 21 ; Terumo(R) , USA.

E. Preparation of Chemical Solutions

1. Solutions for Internal Standard Selection

The stock solutions of β - Hydroxyethyltheophylline (IS_1), β -Hydroxypropyltheophylline (IS_2) and 8- Chlorotheophylline (IS_3) in the concentration of 0.35 mg./ml. were each prepared by dissolving 0.035 g. into individual 100.0 ml. volumetric flask and adjusted to volume with methanol. Each working solution was made by diluting one milliliter of stock solution into 1000.0 ml. with methanol.

2. Caffeine Aqueous Solution (0.4 mg./ml.)

A 0.1000 g. of caffeine was accurately weighed, dissolved in distilled water and made up to 50.0 ml. By diluting ten milliliters of this stock solution into 50.0 ml. with distilled water, a final concentration of 0.4 mg./ml. was made.

3. Stock Solution of theophylline

A 0.1000 g. of theophylline was accurately weighed and transferred into a 50.0 ml. volumetric flask, dissolved and adjusted to volume with distilled water, resulting concentration of 2.0 mg./ml. The stock solution was prepared for every two weeks.

4. Stock Solution of β -Hydroxyethyltheophylline (IS_1)

A 1.0 mg/ml of IS_1 aqueous solution was prepared by transferring 0.1000 g. of IS_1 into a 100.0 ml. volumetric flask and made up to volume with distilled water. This stock aqueous solution was reprepared every two weeks.

5. 10 % w/v Zinc Sulfate Aqueous Solution

A 10.0 g. of zinc sulfate was dissolved and made up to 100.0 ml with distilled water.

6. Mobile Phase for HPLC

The mobile phase composes of acetonitrile and 0.01 M sodium acetate buffer (pH 3.8) in the ratio of 1:9.

sodium acetate 0.01 M (pH 3.8)

A 0.8203 g. of sodium acetate was dissolved and adjusted pH to 3.8 with glacial acetic acid. The volume was made up to 1000.0 ml with distilled water.

The mobile phase was freshly prepared, filtered through 0.45 micron membrane filter and degassed before used.

METHODS

This study was divided into 4 parts, they are

Part 1 The Analytical Method for Theophylline

- 1.1 Investigation of Optimum UV Detector Wavelength
- 1.2 Internal Standard Selection
- 1.3 Validation of the Analytical Method

Part 2 Experiment in Volunteers

Part 3 Data Analysis

Part 4 Application to Patients

Part 1 Analytical Method for Theophylline

An isocratic reversed-phase high performance liquid chromatography (HPLC) technique used for determining theophylline concentration in plasma and saliva samples was modified from Orcutt et. al. (1977) and Bock et. al. (1984).

1. The Investigation of Optimum UV Detector Wavelength

A 10 mcg./ml. theophylline in distilled water was filled into a 1 cm. quartz cell for spectrophotometer. The wavelength of theophylline solution was scanned from wavelength 200 nm. to 400 nm. at scan speed 50 nm./min. The optimum wavelength in which both theophylline and internal standard contained high absorptivity value would be selected and used in the analysis.

2. The Internal Standard Selection

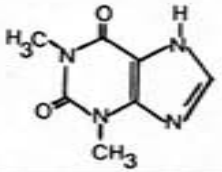
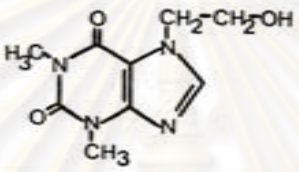
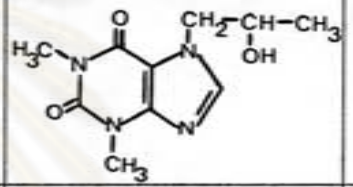
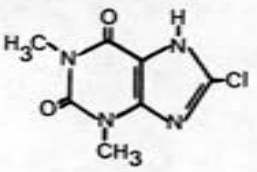
Internal standard is essential in HPLC analytical technique, because internal standard is the controller of many affected variations of the determination. Theoretically,the internal standard which is appropriate for one analysis should have physicochemical properties similar to that of the drug, would never be found in the sample and must have retention characteristic similar to the drug analyzed, but must be well resolved from all other peaks in chromatogram. Moreover,it should be highly pure and preferably readily available. (Smith and Stewart,1981).

In this study, three chemical compounds as homologue of theophylline [β-Hydroxyethyltheophylline (IS_1.), β-Hydroxypropyl theophylline (IS_2) and 8-Chlorotheophylline (IS_3)] (Table 1) were compared under the retention characteristics; the resolution of the compound from theophylline, the pattern of chromatogram obtained and the time of chromatographic analysis. Only the most appropriate compound according to the characteristics studied, would be selected as the internal standard for this study. Besides, internal standard consideration, caffeine which was frequently contained in many agents, dietaries and beverages was also included for interferent checking. Any compound interferring with caffeine peak would be excluded from the selection.

3. Validation of the Analytical Method

In general, method validation is a kind of method evaluation in which the precision and accuracy of any particular analytical method was determined for any specific applications. With this purpose, various topics on method validation were experimented in this study, according to the analytical methods validation under the agreement for bioavailability, bioequivalence and pharmacokinetics studies. (Shah et al., 1992) They were included the linearity, the lower limit of detection, the lower limit of quantitation, the selectivity, the accuracy and the precision of the method.

Table 1 Physicochemical properties of theophylline and theophylline homologues used in internal standard selection

Property	Theophylline	Etophylline	Proxyphylline	8-Cl-theophylline
structural formular				
chemical name	1,3-Dimethyl-xanthine	β-Hydroxyethyl theophylline	β-Hydroxyethyl theophylline	-
molecular formular&wt.	$C_7H_8N_4O_3$ 180.17	$C_9H_{12}N_4O_3$ 224.22	$C_{10}H_{14}N_4O_3$ 238.24	$C_7H_7ClN_4O_2$ 214.63
solubility	1 g dissolves in 120 ml H_2O, 80 ml EtOH, about 110 ml $CHCl_3$, sol.in hot H_2O, sparingly sol. in ether	freely sol.in H_2O,moderately sol. in alcohol	1 g dissolves in about 1 ml H_2O, in 14 ml abs. EtOH more sol. in boiling alcohol	-
pKa	8.77	-	-	-
m.p.(range)	270-274^{0}C	158^{0}C	135-136^{0}C	290^{0}C(dec.)
λ max	270 nm	270 nm	273 nm	-

(Windholz et. al., 1983)

3.1 Linearity

The linear range in which peak area ratio (PAR) is linearly related to concentration according to Beer's Law was determined.

A. Preparation of Calibration Solutions

The solutions of theophylline used for constructing the calibration curves were prepared by serial dilution of a stock solution. A series of calibration solutions containing theophylline in concentration of 0.02, 0.05, 0.1, 0.25, 0.5 and 1.0 mg./ml. for plasma calibration solutions and 0.02, 0.05, 0.1, 0.25, 0.4 and 0.5 mg./ml. for saliva calibration solutions were prepared from the stock solutions every two weeks.

A working solutions of the internal standard (β-hydroxyethyltheophylline) were also prepared every two weeks. One millilitre of internal standard stock solution was transferred into 250.0 ml. volumetric flask, adjusted to volume with methanol. The final concentration of internal standard for analysis in plasma was 4 mcg./ml.

For analyzing theophylline in saliva the concentration of internal standard used was diluted to be 2.0 mcg./ml. Calibration and internal standard solution were kept in refrigerator during the experimentation and used within two weeks.

Analytical method procedure

Either plasma or saliva theophylline was analyzed according to the following scheme.

Spike a 10.0 mcl. of theophylline calibration solution*

into a 500 mcl. of blank plasma or saliva in screwed-cap glass tube

⇓

vortexed- mixed

⇓

add 100 mcl. of 10% w/v zinc sulfate aqueous solution

⇓

vortexed- mixed

⇓

add 750 mcl. of the internal standard working solution

(4.0 mcg./ml. for plasma, 2.0 mcg./ml. for saliva)

⇓

vortex mixed for 20 seconds

⇓

Then, centrifuged at 1400 x g for 15 minutes

Transfer approximately 200 mcl. of supernate into clean sample vial in which 20 mcl. were autoinjected into HPLC

(*in case of samples, 10.0 mcl. of distilled water was used instead)

Chromatographic condition

Column : Bondclone 10 C_{18}, 300 x 3.9 mm.I.D.

Mobile phase : Acetonitrile in 0.01 M. Sodium acetate buffer pH 3.8 in the ratio of 1:9.

Flow rate : 1.5 ml. per minute

Pressure : 1500-2000 psi.

Temperature : ambient

Detector : UV detector at 272 nm.

Integrator : attenuation 4
chart speed 2.5 minute per cm.

B. Plasma and Saliva Curve and Calibration

Linearity of the assay were established in the range of 0-20 mcg./ml. for plasma and 0-10 mcg./ml. for saliva by analyzing spiked blank plasma and saliva samples covering these ranges according to aforementioned procedure.

The calibration curve was constructed by plotting the ratio of peak area of plasma or saliva standard theophylline to that of internal standard against the added standard theophylline concentrations. Theophylline in plasma and saliva sample were determined by extrapolating via the linear regression equations.

3.2 Lower Limit of Detection (LLD)

The limit of detection defined as the lowest concentration of analyte in a sample which can be responsed by the detector. It is expressed in terms of concentration which gives a response of the analyte more than 2 times of a response of noise at baseline (S/N $\geq$ 2) and has the percentage coefficient of variation of S/N ratio less than 10%. (Smith and Stewart, 1981; Silva, 1985)

procedure

The lowest concentration of theophylline solution which gives the S/N ratio of more than 2:1 was spiked into blank plasma and saliva and then, were analyzed according to the aforementioned method, at the lowest attenuation of integrator.

At this concentration, the sample was repeatly analyzed in ten replicates and the coefficient of variations of the ratio were determined.

3.3 Specificity

The specificity of the method was determined by comparing chromatograms obtained from analysis of blank plasma and saliva, blank plasma and saliva spiked with theophylline and IS, plasma and saliva from volunteer and patient administered the drug and standard solution of drug and IS in term of retention time and the occurance of interferences.

3.4 Accuracy

The exactness of the analytical method was determined in terms of physical recovery and analytical recovery. (Szepesi, 1990) The value from physical recovery study was used to evaluate the efficiency of the analytical seperation. The whole analytical process was evaluated in term of analytical recovery. (Silva, 1985)

3.4.1 Physical recovery

Peak areas of each theophylline and IS concentration in spiked plasma and saliva within the range of calibration curve (n=5) were compared to the peak areas of each couple in the calibration solution in which the percentage recovery was calculated according to the following equation.

$$\% \text{ physical recovery} = \frac{\text{peak area of theophylline or IS in plasma or saliva} \times 100}{\text{peak area of theophylline or IS in standard solution}}$$

3.4.2 Analytical Recovery

Nine plasma and saliva samples were received and analyzed concurrently with the series of calibration solutions. The concentrations of theophylline in samples were interpolated from linear regression equation of calibration curves. The percentage analytical recovery of drug at each concentration was calculated as:

$$\% \text{ analytical recovery} = \frac{\text{concentration of theophylline detected} \times 100}{\text{concentration of theophylline added}}$$

3.5 Precision

Precision is a measure of the degree of reproducibility of the analytical method under normal operating circumstances. (Szepesi, 1990) The precision of the sample preparation procedure and chromatography was evaluated by analyzing three replicate calibration curves of plasma and saliva on the same day to determine the within-run precision and six replicate calibration curves on different days to determine the between-run precision within four months.

The peak-area ratio of theophylline to the internal standard for each concentration was compared over the calibration curves and the percentage coefficient of variation (%C.V.) for each concentration was determined.

Part 2 Experiment in Volunteers

Thirty-six healthy Thai volunteers were used in this study, 18 males and 18 females with the ages ranged from 20 to 40 years. Every subject must not have the history or evidence of asthma, upper respiratory diseases, renal and hepatic diseases. Also they should not be on therapy with any particular drug especially one week before the experiment. To ensure that the subjects were normal, physical examination and standard laboratory screen were performed prior to the study.

Coffee, tea, chocolate, cola, cocoa, other foods or beverages containing xanthines and alcohol were withheld for at least twenty-four hours before and during the study. Food was abstained at least eight to ten hours before starting the experiment and two hours after drug administration. The method and conditions of the study were explained to all subjects. Informed consent was signed and obtained from each subject prior to entering the experiment.

On the day of experiment, blank blood and saliva were collected from each volunteer subject about one hour prior to drug ingestion. Every subject was suggested to throughly clean his or her mouth with water before starting each sample collection schedule.

Immediately after the administration of conventional theophylline tablet as a single oral dose of 5.0 mg./kg. body weight with 200 ml. of drinking water, exactly the same time schedule, blood and saliva

samples were collected concomitantly at 0.0, 0.5, 1.0, 1.5, 2.0, 4.0, 8.0 and 12.0 hours post dose. The crystals of citric acid were used for stimulating saliva, which was expectorated into a clean test tube in the volume of 5.0 ml. At the same moment, 5.0 ml of blood sample was withdrawn from a forearm vein through a heparin lock.

Both blood and saliva sample were centrifuged at 1400 x g for 10 minutes. Plasma and clear supernated saliva were seperated into the other tubes and kept frozen for subsequent analysis by ultilizing the aforementioned modified analytical method within one week.

Part 3 Data Analysis

Every plasma and saliva concentrations obtained from sample analysis was included in the determination of saliva-plasma concentration ratio. The correlation coefficient of theophylline saliva concentration and theophylline plasma concentration were determined. Two-way analysis of variance was the statistical method used in detecting the interaction between sex and sampling time to the calculated saliva-plasma ratio at significant level=0.05. Any statistically significance observed would be followed-up by one-way analysis of variance and Scheffe procedure, respectively.

Since some of the subjects in this study have the smoking habit, the effect of smoking on the saliva-plasma ratio was also determined.

Part 4 Application to Patients.

The saliva-plasma ratio establishing in this study was ultilized in predicting theophylline concentration in patient's plasma by analyzing saliva sample received.

The twelve Thai-outpatients both male and female from Pediatrics and Medicinal Departments, Chulalongkorn Hospital were involved in this study. They were ages ranged from 5 to 60 years. Any concurrent illness was not concerned in selecting patient. However, every patient must be on-therapy with theophylline for at least two weeks before entering this study. Co-administration of other bronchodilator or antihistamine drugs were also accepted.

Every patient was cleary explained about the objectives of study as well as the process performed on the day of experiment. Xanthine containing foods or beverages were also withheld for all patients.

Blood and saliva collection procedure the same as use in volunteers were performed on the day of experiment. Only one pair of blood and saliva sample from each patient were collected. The period of time between the last dose administration and sample collection for each patient was not fixed for the convenience of each patient.

The concentration of theophylline in patient's saliva was substitued into the saliva-plasma concentration ratio from volunteers to calculate for the plasma theophylline concentration. The calculated

plasma theophylline concentration from each patient was compared to the concentration of theophylline in analyzed plasma sample.