CHAPTER II

MATERIALS AND METHODS

Materials

1. Chemical reagents

1.1 Reference standards

1.1.1 Tumeric extracted powder, Lot no. 420724, TCF, Thailand

1.1.2 Mefenamic acid, purity 99.58%, Ningbo Smart Chemical & Pharmaceutical

1.1.3 Curcuminoids tablets; Faculty of Pharmaceutical Science, Mahidol University

1.2 The other chemical reagents

1.2.1 Methanol, HPLC grade; Burdick & Jackson, USA

1.2.2 Acetonitrile, HPLC grade; Burdick & Jackson, USA

1.2.3 Ethylacetate, AR grade; Fisher Scientific, UK

1.2.4 Acetic acid glacial, AR grade; Lab Scan Analytica Science; Thailand

2. Human plasma

Human plasma for method validation and analytical method modification was generously supplied from the Plasma Division. Thai Red Cross Society, Thailand.

3. Apparatus

3.1 High Performance Liquid Chromatography, HPLC, Waters Associates Pty, Ltd., Massachusetts. USA. Waters 2695 separations module composes of gradient pump, degasser, autosampler, Waters 2487 dual absorbance detector, Empower software.

3.2 UV-visible recording spectrophotometer; Shimadzu corporation, Japan

3.3 Microcentrifuge, Mikro 22R; Hettich , Germany

3.4 Vortex mixer, Vortex – Genie; Scientific, Germany

3.5 Vacuum concentrator; Heto model CT110, Denmark

3.6 Freezer -48°C, HLLF-240, Heto-Halten A/S, Denmark

3.7 Freezer -20°C, Sanyo SF-C95, Sanyo Universal Electric Co. Ltd., Thailand

3.8 pH-meter, 744 pH meter, Methrom ion analysis, Switzerland

3.9 Sonicator bath, RK 103H, Bandelin sonorex, Germany

3.10 HPLC column

3.10.1 SymmetryShield®, RP18, (150 x 3.0 mm, i.d.) 5 μm; Waters Associates Pty, Ltd., Massachusetts. USA

3.10.2 Guard column, (20 x 2.0 mm, i.d.) packed with Corosil® C18, 37-50 μm; Waters Associates Pty, Ltd., Massachusetts. USA

3.11 Micropipette; Socorex®, USA

4. Preparation of standard solutions

4.1 Stock solution of curcuminoids (0.15 mg/ml)

A 1.5 mg of curcuminoids was accurately weighed, dissolved and made up to 10 ml volume with methanol. The solution was light protected and used within a week.

4.2 Stock solution of 2-[(2,3-Dimethylphenyl)-amino]benzoic acid (0.012 mg/ml)

A 0.30 mg of IS was accurately weighed, dissolved and made up to 25 ml volume with methanol. The solution was light protected and used within a week.

Methods

To prevent the degradation of curcuminoids, the whole experiment in this study was performed in the room that illustrated with yellow light.

Four distinguished processes were performed in this study. They included

1. To set up the analytical method for determining curcumin in human plasma by HPLC
2. To perform the bioanalytical method validation
3. To determine the content of curcumin in curcuminoids tablet
4. Pharmacokinetic study of curcumin in Thai healthy volunteer

1. To set up the analytical method for determining curcumin in human plasma by HPLC

The analytical method reported by Heath et al., 2003, was used with modifications.

HPLC condition

Isocratic reversed-phase technique with following HPLC condition was performed.

Analytical column	: Water SymmetryShield®, C_{18}, (150 x 3.0 mm, i.d.) 5 μm
Guard column	: Corosil® C18 (20 x 2.0 mm, i.d.) 37-50 μm
Mobile phase	: ACN : MeOH : H_2O : Acetic acid (41 : 23 : 36 : 1)
Flow rate	: 0.5 ml/min
Detector	: according to the scanning spectra wavelength

1.1 To determine the detection wavelength for HPLC

Procedure

Standard methanolic curcumin solution (100 μg/ml) was spectrophotometrically scanned between the wavelength of 200 to 800 nm. The maximum absorption wavelength of curcuminoids would be used as the detection wavelength for curcumin in HPLC analysis.

1.2 Sample preparation

Procedure

The standard solution of curcumin (0.80 μg/ml) (n=3) was spiked into blank plasma. Curcumin in plasma sample was extracted into ethyl acetate (Heath et al., 2003). In this study the appropriate interval of extraction time was evaluated at 10, 20, 30 and 40 minutes for determining the highest efficiency of extraction in term of %recovery.

1.3 Internal standard (IS) selection

Theoretically, internal standard for bioanalytical method should not be the compound that usually found in human body, dietaries or beverages. Neither pharmaceuticals nor its metabolites can be used. Generally, the compound being selected as internal standard should contain similar physicochemical properties to the sample. If possible, the internal standard used, should be eluted later than the sample (Smith, 1981).

Many compounds were screened. They were

D(-)-threo-2-dichloroacetamido-1-p-nitrophenyl-1,3-propanediol (Chloramphenicol);

2-[(2,6-Dichlorophenyl)- amino]benzene acetic acid monosodium salt (Diclofenac);

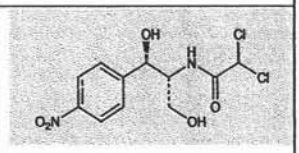
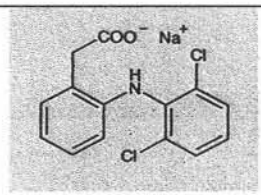
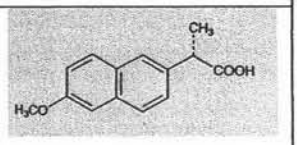
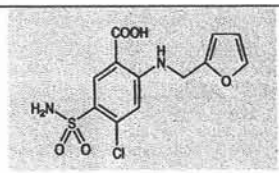
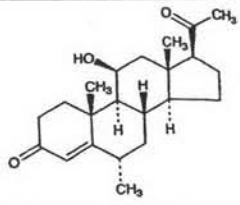
(S)-6-Methoxy-α-methyl-2-naphthalene acetic acid (Naproxen); 5-(Aminosulfonyl)-4-chloro-2-[(2-furanyl-methyl)amino] benzoic acid (Furosemide) and 2-[(2,3-Dimethylphenyl)-amino]benzoic acid (Mefenamic acid). These compounds exhibit the absorption wavelength in the UV absorption region of curcumin (282 nm). Besides, their physicochemical properties are not quite different.

Procedure

The retention time of each compound was compared to curcumin. The compound being selected as internal standard should have the similar maximum absorption wavelength to curcumin. Meanwhile its retention time under HPLC condition of curcumin, should be longer than curcumin.

Each individual methanolic solution of 1.0 μg/ml was injected into HPLC system under the condition of curcumin at the detector wavelength of 282 nm.

Table 1. Physicochemical properties of screening compounds as internal standard

Generic name	Chemical name	M.W.	Solubility	Wavelength	Structural
Chloramphenicol	D(-)-threo-2-dichloroacetamido-1-p-nitrophenyl-1,3-propanediol	323.1	Soluble in water, very soluble in methanol, ethanol, insoluble in benzene	274 nm	
Diclofenac	2-[(2,6-Dichlorophenyl)-amino]benzene-acetic acid monosodium salt	318.1	Solubility in water, very soluble in methanol	283 nm	
Naproxen	(S)-6-Methoxy-α-methyl-2-naphthalene acetic acid	230.3	Practically insoluble in water, Very soluble in ethanol (95%), methanol	271 nm	
Furosemide	5-(Aminosulfonyl)-4-chloro-2-[(2-furanyl-methyl)amino] benzoic acid	330.8	Slightly soluble in water and chloroform and ether, Soluble in acetone, methanol	288 nm	
Mefenamic acid	2-[(2,3-Dimethylphenyl)-amino]benzoic acid	241.3	Slightly soluble in methanol	279 nm	

2. To perform the bioanalytical method validation

The modified analytical method has to be validated to ensure the linearity, accuracy, precision, sensitivity, specificity and stability according to the criteria of bioanalytical method validation guidance. (Guidance for industry: Bioanalytical method validation, May 2001)

2.1 Linearity

Procedure

A series of standard plasma curcumin concentrations were analyzed according to the modified method. The pattern of linearity of the response to curcumin concentration was determined. The reproducibility of linearity pattern was confirmed by analizing the other three replications of curcumin standard plasma in the concentrations of 0, 0.01, 0.025, 0.050, 0.10, 0.25, 0.50 and 1.00 μg/ml. The slope and the intercept of the calibration curve were then calculated according to regression analysis.

2.2 Accuracy and precision

2.2.1 The intra-day accuracy and precision

Procedure

Six replications of spiked curcumin standard plasma in the concentrations of 0.04, 0.40 and 0.80 μg/ml were analyzed according to the modified method along with the series of standard plasma curcumin for constructing the calibration curve.

The accuracy of analytical method in term of %bias was determined according to equation (1).

$$\%bias = \frac{(analyzed\ conc. - added\ conc.)}{added\ conc.} \times 100 \quad .....(1)$$

The method was proven for its accuracy if the values of %bias were within ± 15%.

The precision of analytical method were determined in term of the percentage of relative standard deviation (%RSD) of curcumin concentration. The method is considered to be precise if the values of %RSD were within 15% for all curcumin concentrations studied.

2.2.2 The inter-day accuracy and precision

Procedure

The aforementioned procedure for intra-day analysis was followed but only one replication of standard plasma spiked curcumin was analyzed on six separately different days. Also, the accuracy and the precision were determined with the same criteria as the intra-day procedure.

2.3 Sensitivity

The sensitivity of an analytical method can be described in the term of the limit of quantitation (LOQ).

Procedure

Six replications of spiked curcumin standard plasma in the concentration of 0.01 μg/ml were analyzed according to the developed method along with the series of calibration curve. The variation of analysis in the term of %bias and %RSD would be within ± 20%.

2.4 Specificity

To determined the specificity of the analytical method, the retention time of curcumin and IS obtained from the analysis of standard solution and spiked plasma sample should be identical. In addition, endogenous peak should not be interfered with curcumin and IS

2.5 Stability study

The stability of plasma sample, in-processed analyte and stock solution were studied. The stability of plasma sample were determined at room temperature (25°C), storage temperature (-48°C) and freeze-thaw cycle. For in-processed analyte, the stability in autosampler was studied. In addition, the stability of curcumin stock solution as well as IS stock solution was also determined.

All stability programs were performed in three replications. Curcumin was considered stable if the analyzed curcumin concentration from the stability test were within ±10% of curcumin at zero time (the beginning)

2.5.1 Stability of curcumin plasma sample

Three different conditions, at room temperature, storage temperature and freeze-thaw cycle, were used for plasma curcumin stability studies as depicted in Table 1.

Procedure

On the day of analysis, standard plasma spiked curcumin at the concentrations of 0.04 and 0.80 μg/ml, were analyzed along with the calibration standard solution. The concentration of curcumin in plasma was determined from the calibration curve. The percentage of curcumin remained was calculated from equation (2).

$$\% \text{ curcumin remained} = \frac{Ct}{Co} \times 100 \qquad \text{.....(2)}$$

Ct was curcumin concentration at any time point or cycle

Co was curcumin concentration at zero time.

2.5.2 Stability of in-processed analyte in the auto sampler

Procedure

The procedure was similar with the stability of plasma sample but the condition and time schedule were difference as shown in Table 2.

2.5.3 Stability of stock solution of curcumin and IS

Procedure

Stability of stock solution

The methanolic solution of curcumin (0.15 mg/ml) and IS (0.012 mg/ml) were kept at -18°C and analyzed at 0, 2, 4, 6, 11 and 15 days. The peak area (PA) was used in determining the percentage of compound remaining comparing to the zero time as shown in equation (3).

$$\% \text{ curcumin remained} = \frac{PAt}{PAo} \times 100 \qquad \text{.....(3)}$$

PAt was PA of curcumin or IS at any time point and

PAo was PA of curcumin or IS at zero time.

Tabel 2. The stability study for curcumin in plasma sample

Stability program / Parameters	Room temperature	Storage temperature	Freeze – thaw cycle
Concentration of curcumin (μg/ml)	0.04 , 0.80	0.04 , 0.80	0.04 , 0.80
Temperature (°C)	25±2	-48±1	-48 and 25
Stability condition	Laboratory room	Freezer	Freezer and room temperature
Stability evaluation time	0, 3, 6 and 8 hrs.	0, 4, 6, 11 and 15 days	3 cycles with the time range of not less than 12 hrs.

Tabel 3. The stability study for in-processed analyte in the auto sampler

Stability program / Parameters	In-processed analyte in the auto sampler
Concentration of curcumin (μg/ml)	0.04, 0.80
Temperature(°C)	4±1
Stability condition	Auto sampler
Stability evaluation time	0, 5, 9 and 16 hrs.

3. To determine the content of curcumin in curcuminoids tablets

To confirm a specification of curcumin in curcuminoids tablets. The percentage of curcumin in curcuminoids tablets was determined by the same HPLC condition as plasma curcumin analysis.

3.1 To confirm the proportional ratio of curcuminoids tablet and extracted turmeric powder

Procedure

The slicing film coated tablets were powdered and accurately weighed equivalent to 100 mg of curcuminoids, dissolved and made up to 100 ml with methanol. The aliquot curcuminoids solution of 2.0 µg/ml was used for injecting into HPLC, comparing to tumuric powder standard solution (2.0 µg/ml).

The proportional ratio of curcuminoids was calculated according equation (4).

$$\text{Proportional ratio} = \frac{\text{PA of curcumin in curcuminoids tablets or extracted tumeric powder}}{\text{Total PA of curcuminoids in tablets or extracted tumeric powder}}$$

Or

$$= \frac{\text{PA of demethoxycurcumin in curcuminoids tablets}}{\text{Total PA of curcuminoids in tablets}}$$

Or

$$= \frac{\text{PA of bisdemethoxycurcumin in curcuminoids tablets}}{\text{Total PA of curcuminoids in tablets}} \quad \text{.....(4)}$$

3.2 Weight variation

Procedure

Weigh individually 20 whole tablets, and calculate the average weight of one tablet. The requirements are met if the weights of not more than 2 of the tablets differ from the average weight by more than the percentage listed in the accompanying table and no tablet differs in weight by more than double that percentage (USP 24, 2000). In this study, only 10 whole tablets was.

3.3 The content of curcumin in curcuminoids tablets

Procedure

Ten curcuminoids tablets were individually weighed pre- and post-slicing film coat. The slicing film coated tablets were powdered and accurately weighed equivalent to 100 mg of curcuminoids, dissolved and made up to 100 ml with methanol (n = 3). The aliquot curcuminoids solution of 2.0 μg/ml was used for injecting into HPLC, comparing to tumuric powder standard solution (2.0 μg/ml).

The content of curcumin in curcuminoids tablet was calculated according equation (5).

$$\% \text{ Content} = \frac{\text{PA of curcumin in curcuminoids tablets} \times 100}{\text{PA of curcumin in standard solution}} \quad \text{.....(5)}$$

4. Pharmacokinetic study of curcumin in Thai healthy volunteers

This part of the study was approved by the Ethic Committees for animal and human study, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

Protocol of study

All volunteers have to administer curcuminoids tablets in the dosage of 6 gram once a day in the morning before breakfast for 8 days. Blood sample was collected from the forearm vein before drug administration and after drug administration up to 12 hours on the first, seventh and eighth day of administration. On the second to the sixth day of experiment, drug compliance of each subject was followed and any adverse side effect was recorded.

4.1 Criteria for volunteers

Inclusion criteria

Thai volunteers in the age range of 20-50 years, body mass index (BMI) less than 30 kg/m^2, and passed the physical examination were included in the study. They have to sign the informed consent and follow the following recommendations.

- No taking of particular drug or curcumin supplement a week before the experiment.
- No alcohol or caffeine uptake on the day before experiment.
- Food has to obtained at least 8-10 hours before drug administration. Water was allowed.

Exclusion criteria

- Volunteers would be dropped out if fail to pass the physical examination.
- Not willing to continue the experiment

4.2 Plasma curcumin concentration determination

The validated method was used for analyzing curcumin in plasma samples. The quality control samples and calibration concentrations were analyzed along with the samples. The concentration of curcumin was calculated through the regression equation of the calibration curve.

4.3 Pharmacokinetic parameters determination

The plasma curcumin concentration was plotted against sampling time for determination C_{max} and T_{max} values. Other pharmacokinetics parameters AUC, $t_{1/2}$, V_{ss}, Cl, $C_{ss,av}$, R, were calculated via WinNonlin program version 1.0 in Noncormpartment: plasma extravascular input model. For statistical significant test, the SPSS program version 10.0 was used.

The equations for calculating these pharmacokinetic parameters were;

The area under the concentration – time curve (AUC) was determined from the linear trapezoidal method

$$AUC = \sum \frac{(C_2 + C_1) \times (t_2 - t_1)}{2} \quad \text{.....(6)}$$

$$AUC_{0->\alpha} = \sum \frac{(C_2 + C_1) \times (t_2 - t_1)}{2} + \frac{C_n}{\lambda_z} \quad \text{.....(7)}$$

$$AUMC_{0->\alpha} = \sum \frac{(t_2 - t_1) \times (C_2 t_2 + C_1 t_1)}{2} + \frac{t_{last} \times C_n}{\lambda_z} + \frac{C_n}{\lambda_z^2} \quad \text{.....(8)}$$

$$t_{1/2} = \frac{-\ln(2)}{\lambda_z} \quad \text{..... (9)}$$

$$MRT_{oral} = \frac{AUMC_{0->\alpha}}{AUC^2_{0->\alpha}} \quad \text{.....(10)}$$

$$V_{ss} = \frac{Dose \cdot AUMC_{0->\alpha}}{AUC_{0->\alpha}} \quad \text{.....(11)}$$

$$Cl = \frac{F \cdot Dose}{AUC_{0->\alpha}} \quad \text{.....(12)}$$

$$C_{ss,av} = \frac{AUC\ (single\ dose)}{\tau} \quad \text{.....(13)}$$

where λ_z: Lambda$_z$. First order rate constant associated with the terminal (log-linear) portion of the curve. It was estimated via linear regression of log concentration vs. time.

C_n denotes either the observed or predicted concentration at the last sampling time.