CHAPTER 2

MATERIALS AND METHODS

2.1 Study design

The study was randomized, open-labeled, crossover-three phase study, with washout period of at least 2 weeks. The study was approved by the Human Research Ethic Committee of the Faculty of Medicine, Chiang Mai University and was complied with the Helsinki Declaration.

2.2 Subjects

2.2.1 Inclusion criteria

A total of 12 postmenopausal women, age≥ 45 y and serum follicle -stimulating hormone concentration > 20 IU/L (270), were enrolled in this study. All had to be in good general health on the basis of medical history and physical examination. Routine blood examine including complete blood count (CBC), blood urea nitrogen (BUN), and serum creatinine (Cr) and liver function test (LFT) were screened to exclude subject with abnormal liver or kidney function and hematological diseases. Blood concentrations of calcium and phosphate had to be within normal ranges. The weight of subject had to be within ± 10% of the ideal body weight for age and height or body mass index was within 18-25 kg/m^2. The subjects were advised to maintain their usual diet and to avoid consuming high vitamin D, high calcium diet as well as soy food throughout the study. Supplement of vitamin D, calcium and isoflavones were not allowed throughout the study.

2.2.2 Exclusion criteria

Subjects with known premenopausal status (< 12 months since the last spontaneous menstrual bleeding and a serum follicle-stimulating hormone (FSH) concentration of≤ 20 IU/L) were excluded as well as those with known history of chronic renal, liver, pulmonary, or cardiovascular diseases, recent cigarette smoking, a history of substance abuse or addiction, use of antibiotic within the previous 6 wk, consumption of > 2 alcoholic drinks/d, regular use (> 1 dose/wk) of over-the-counter or prescription medications, as well as a history of malignancy, hyperparathyroidism or breast disease.

2.3 Isoflavones and vitamin D_3 plus calcium (D_3-calcium) supplement

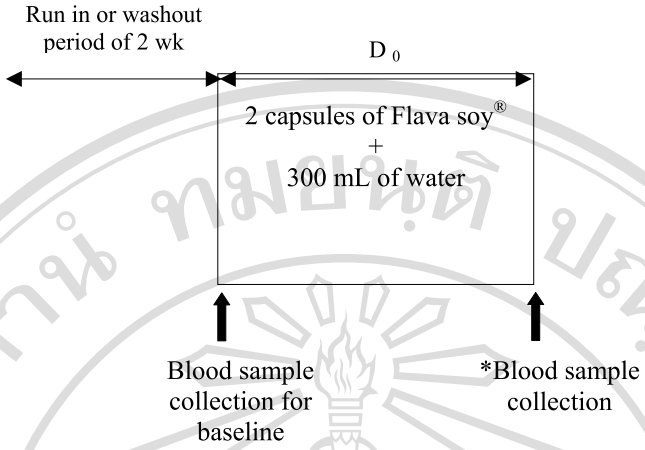
The isoflavone preparation used in this study was the commercial soy isoflavone capsule, Flava soy® (1 capsule contains not less than 25 mg isoflavones, manufactured by Thai Herbal Products Co., Ltd, Thailand). D_3 and calcium supplement was the commercial Caltrate 600 + D® (1 tablet contains vitamin D_3 200 IU plus calcium 600 mg, manufactured by Wyeth-Ayerst Co., Ltd, Thailand).

2.4 Dosage and drug administration

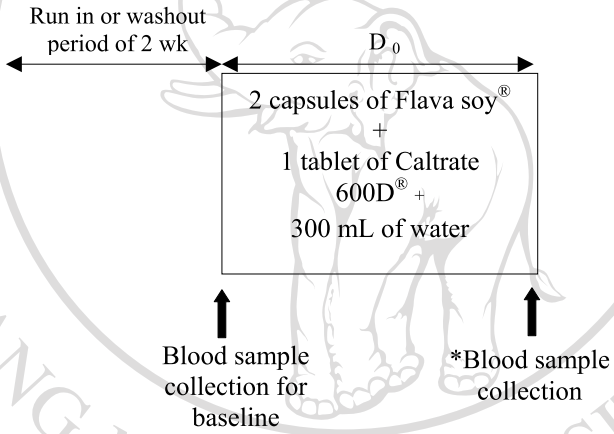
Subjects were admitted to the Clinical Pharmacology Unit, Faculty of Medicine, Chiang Mai University at 6:30 AM after an overnight fast of at least 8 h. Subjects were randomized to receive either: (A) a single dose of 2 capsules of Flava soy® ("single ISO"), (B) a single dose of 2 capsules of Flava soy® and 1 tablet of Caltrate 600 + D® ("single ISO + D_3-calcium"), or (C) 1 tablet of Caltrate 600 + D® twice a day after breakfast and dinner for 7 days, followed by 2 capsules of Flava soy® on 8th day

("continuous D_3-calcium/single ISO") (Figure 4 and Table 1). Subjects were asked to remain upright and were fast for 2 h after isoflavone administration. Water and lunch were served at 2 h and 6 h after dosing, respectively. Blood samples were collected at different time points (see below). After blood sample collection at 12 h postdose, subjects were discharged from the Clinical Pharmacology Unit and were asked to come back again on the next day to for blood sample collections at 24 h and 32 h postdose. While waiting for blood sample collections, subjects were allowed to perform any daily activities, except moderate-high degrees of exercises. After a washout period of at least 2 wk, subjects received the 2 remaining treatments according their randomized sequences. Subjects were required to refrain from drinking caffeine and alcohol containing beverages in order to standardize experimental conditions.

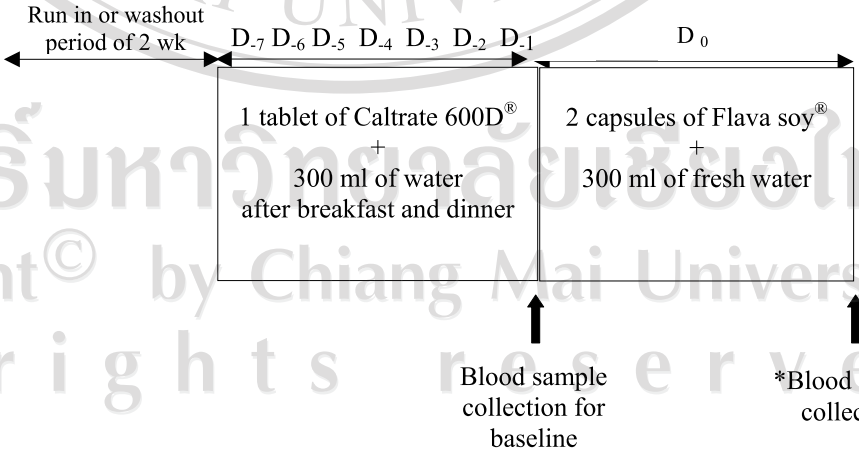
Phase A "single ISO ' (Reference treatment)



Phase B "single ISO + D_3-calcium"



Phase C "continouse D_3-calcium/single ISO"



* Blood sample collection: after administration at 1, 2, 4, 6, 8, 10, 12, 24 and 32 h

Figture 4 Schematic of single ISO and/or D_3-calcium treatments in phase A, B and C

Table 1 Randomized sequence of each subject

Subject No.	Randomized sequence of each subject		
1	Phase B	Phase C	Phase A
2	Phase C	Phase A	Phase B
3	Phase B	Phase C	Phase A
4	Phase A	Phase B	Phase C
5	Phase A	Phase B	Phase C
6	Phase C	Phase A	Phase B
7	Phase A	Phase B	Phase C
8	Phase B	Phase C	Phase A
9	Phase A	Phase B	Phase C
10	Phase C	Phase A	Phase B
11	Phase B	Phase C	Phase A
12	Phase C	Phase A	Phase B

2.5 Blood samples collection

Venous blood samples (10 mL/each) for determination of soy isoflavone were collected predose and then at 1, 2, 4, 6, 8, 10, 12, 24, 32 h after isoflavone administration. Samples were obtained from forearm by venipuncture through an indwelling intravenous catheter (BD Insyte®) and collected in heparinized Vacutainer (BD Insyte®). The blood collecting tubes were centrifuged at 2,500 rpm for 10 min and the plasma was separated and frozen at -80 ºC until analysis.

2.6 Determination of isoflavone concentrations

2.6.1 Chromatography condition

The assay of isoflavone content was modified from the HPLC method and conditions previously described by Thomas et al (271). Chromatographic separation was performed on 5 µm C18, 100x4.6 i.d. analytical and guard columns. The chromatography condition consisted of 2 mobile phases. Mobile phase A: 40 mM ammonium acetate/acetronitrile/methanol (250:105:105, v/v/v), added 20 µL perchloric acid and 250 µL of 1.44 mM sodium dodecyl. sulfate. Mobile phase B: 40 mM ammonium acetate/acetronitrile/methanol (250:200:250, v/v/v), added 20 µL perchloric acid and 250 µL of 1.44 mM sodium dodecyl sulfate. The HPLC system was run by 100% mobile phase A for 2.50 min and followed by 100% mobile phase B for 11.50 min. The flow rate was maintained at 1 mL/min, the column was maintained at 25ºC and the analytes were detected by UV absorption at 259 nm.

2.6.2 Sample preparation

Aliquot of 125 µL of plasma was transferred to a 1.5 mL plastic vial and treated with 0.25 mL of a mixture of β-glucuronidase/sulfatase from *Helix pomatia* (Sigma

G-0876) to hydrolyze glucuronide and sulfate conjugates of genistein and daidzein. The enzyme mixture was made up freshly and contained 0.1 g ascorbic acid in 10 mL of 0.1 M sodium acetate buffer, 0.01 g ethylenediaminetetraacetic acid (EDTA), pH 4.0, and 500 µL of *Helix pomatia* and the tubes were capped and heated overnight in water bath (15-18 h, 37 ºC). The tubes were removed from the water bath and allowed to cool to room temperature.

2.6.3 Determination of isoflavone concentrations in plasma

The assay was modified from protein precipitation procedure. Briefly, after enzymatic hydrolysis, plasma samples were spiked with 10 µL of internal standard (IS, 50,000 ng/mL fluorescein in 80 % methanol) and then deproteinated by mixing plasma sample with 500 µL acetonitrile, vortex mixing for 30 sec and centrifuged at 14,000 rpm for 10 min, respectively. An aliquot of the supernatant was removed and evaporated to vacuum dried for 2 h at 60ºC. The residue was dissolved in 50 µL of the mobile phase and 5 µL of sample was injected onto the HPLC system. The isoflavone contents of samples were determined by using a calibration curve of peak height ratios of isoflavones and IS versus respective isoflavone concentrations (37.5, 75, 150, 300, 600, 1200 and 2400 ng/mL) with the use of linear regression.

2.6.4 Specificity

The chromatograms of isoflavones and IS were compared to the chromatograms of 6 drug free plasma of different subjects. There must be no endogenous component peaks at the retention times of isoflavones and IS.

2.6.5 Recovery

Recovery was determined by comparing the peak height of isoflavones standard sample in mobile phase, with the peak height of isoflavones in plasma extracted from

5 sets of 3 different concentrations of QC samples (112.5, 1100, 2200 ng/mL).

2.6.6 Stability

Freeze and thaw stability were obtained by 3 repeating analysis of plasma concentrations of isoflavones in each 2 levels QC samples (112.5 and 2200 ng/mL) after 3 freeze-thaw cycle compared to the concentrations of isoflavones in QC samples without freeze-thaw processing.

2.6.7 Intra-day assay (within day) and inter-day assay (between days) precision

For intra-day validation, 5 samples from each of 3 quality control (QC) samples (112.5, 1100, 2200 ng/mL) were evaluated with a single calibration curve. For inter-day validation, 5 sets of the 3 different concentrations of QC samples (112.5, 1100, 2200 ng/mL) were studied on 5 independent days with concurrent 5 standard calibration curves.

The precision was reported as the percentage of coefficient of variation (% CV) which was calculated as follow:

$$\% \text{ CV} = \frac{SD}{\overline{X}} \times 100$$

Where SD = standard deviation

$\overline{X}$ = mean value of isoflavones concentration in plasma

The deviation was expressed as the percentage of inaccuracy calculated by the following equation:

$$\% \text{ Deviation} = \frac{(\text{Measured concentration} - \text{Spiked concentration})}{\text{Spiked concentration}} \times 100$$

2.6.8 Short-term stability

Short-term stability was determined by comparing the isoflavone concentrations in 3 aliquots of each of the low and high levels QC samples (112.5 and 2200 ng/mL) that kept at room temperature for 8 h with freshly prepared QC samples.

2.6.9 Post-preparative stability

Post-preparative stability was used to determine the time effect on isoflavones at the concentrations of 112.5 and 2200 ng/mL and IS after plasma preparation and waiting in the auto-sampler for injection onto the analytical column. The post-preparative stability time was a total analysis time of all samples in a running batch.

2.7 Data analysis and statistical methods

2.7.1 Pharmacokinetic parameters

Maximal plasma concentration (C_{max}, ng/mL) and time to reach peak concentration (T_{max}, h) were obtained directly by visual inspection of each subject's plasma concentration-time profile. The area under the plasma concentration-time curve from time 0-32 h and 0-∞ h (AUC_{0-32} and $AUC_{0-\infty}$, ng.h/mL), as well as half-life ($t_{1/2}$, h) were determined by noncompartmental analysis. The slope of the terminal log-linear portion of the concentration-time profile was determined by least-squares regression analysis and was used as the elimination rate constant (*Ke*). The elimination half-life was calculated as 0.693/*Ke*. The AUC from time zero to the last quantifiable point (AUC_{0-32}) were calculated using the trapezoidal rule. Extrapolated AUC from the last quantefiable time to infinity ($AUC_{0-\infty}$) was determined as Ct/*Ke*. Total $AUC_{0-\infty}$ was the sum of $AUC_{0-32} + AUC_{32-\infty}$. The calculation was performed by using the TopFit software version 2.0 for PC.

2.7.2 Statistical analysis

The pharmacokinetic parameters were presented as mean ± SD. The mean values of pharmacokinetic parameters obtained from treatment in phase A were compared to those of the 2 remaining treatments using Wilcoxon signed-rank test.