CHAPTER 2

MATERIALS AND METHODS

2.1 Study design

The study was an open-label, randomized two-phase crossover study with at least 10 days washout period. 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 healthy men, age 18-40 yr were enrolled in this study. The body mass index had to be within 18-25 kg/m^2. All had to be in good health on the basis of medical history and physical examination. Routine blood test including complete blood count (CBC), blood urea nitrogen (BUN), creatinine (Cr) and liver function test (LFT) were screened to exclude subjects with abnormal hematological diseases, kidney or liver functions. All subjects had to have normal blood pressure and heart rate. During screening phase subjects had to be able to retain the water enema for at least 5 min. Subjects included in the study were given thorough verbal and written information regarding the nature of the study. Signed informed consent of every subject was obtained prior to the study.

2.2.2 Exclusion criteria

Subjects who could not avoid foods or drinks that contain caffeine within the previous 10 day and during study period were excluded as well as those with known history of gastrointestinal diseases such as peptic ulcer, hemorrhoids, gut obstruction, diverticulitis, ulcerative colitis, Crohn's disease, irritable bowel syndrome (IBS), colostomy, recent bowel surgery and colorectal cancer. Other exclusion criteria were chronic renal, liver, neurological, pulmonary or cardiovascular diseases, recent cigarette smoking within the previous 3 months, history of substance abuse or addiction, use of any medication within the previous 1 month, and hypersensitivity to medications in xanthine group such as theophylline or aminophylline.

2.2.3 Withdrawal criteria

The withdrawal criteria of this study were subject who experiences adverse drug reactions during the study, subject who could not comply with the study protocol or wished to voluntarily withdraw from the study, and subject who required other medication during the study period.

2.3 Coffee procedures

2.3.1 Coffee enema and coffee enema procedure

Coffee solution used in enema procedure was prepared by mixing 4 gm of finely grounded coffee beans (VS coffee®, manufactured by V.S. coffee, Thailand) with 100 ml of purified water. The solution was boiled at 100 °C for 3 min and then simmered at 60 °C for approximately 15 min. Afterwards the solution was filtered by using a very fine sieve, adjusted total volume to 500 ml and allowed to cool to 37 °C.

The coffee enema devices used in this study were disposable commercial set (Cleansing enema set®, made in Mexico, imported by Thanyaphu Co. Ltd., Thailand) consisted of a plastic nozzle connected by a tube to a plastic bag containing the coffee enema fluid. The nozzle was lubricated with 2 drops of organic olive oil and then inserted 2 inches into the anus while subject was lying down on his left side, with their legs curled into their abdomen. The bed height is 3 feet above the floor, whereas the enema bag was hung 5 feet above the floor. The coffee solution filled in enema bag was completely infused within 5-10 min. The subject was requested to retain the coffee enema fluid for 10 min. During this period, subject was instructed to change his lying position to right side for 3.5 min and then switched to left side for 3.5 min and supine position for 3 min before defecation. The enema procedure was performed at the Clinical Pharmacology Unit, Department of Pharmacology, Faculty of Medicine, Chiang Mai University, with the assistance of the researcher.

2.3.2 Coffee consumption

The coffee used for consumption in this study was commercial ready to drink coffee. The net volume of 1 serving was 180 ml. Subject was instructed to consume coffee within 1 min followed by 100 ml of water.

2.3.3 Dose of coffee enema and coffee consumption

Subjects were requested to visit the Clinical Pharmacology Unit, Department of Pharmacology, Faculty of Medicine, Chiang Mai University on the days specified according to protocol schedule. In the first phase of the study, subjects were randomly assigned to receive either coffee enema (500 ml each time, every other days, in the morning for 6 visits) or coffee consumption (180 ml each time, 2 times/day, 30 min before breakfast and dinner, everyday for 11 days). After a washout period of at least

10 days, subjects were switched to the second phase receiving the alternative coffee preparation. All subjects were instructed to restrict consumption of fruit juice not more than 2 glass/day (total 300 mL) and not to change their dietary habits throughout of the study.

2.4 Blood sample collection

2.4.1 Blood sample collection for determination of caffeine pharmacokinetic parameters

At the first visit of both single coffee enema phase and coffee consumption phase, subjects were fasted overnight for at least 8 h and were admitted to the Clinical Pharmacology Unit, Department of Pharmacology, Faculty of Medicine, Chiang Mai University at 5.30 AM. Venous blood sample was taken via heparinzed IV catheter inserted into a forearm vein. Fifteen ml of blood sample each was drawn prior to either coffee enema or coffee consumption (6.00 AM.) and again at 10, 20, 30, 40, 60 min and 1.5, 2, 4, 8, 12 h after each procedure. After initiation of each coffee procedure, subjects were continued to be fast, then water and lunch were served 2 h (08.00 AM.) and 6 h (12.00 AM) afterwards, respectively. Identical meal and fluid were served during the 2 study phases. After the last blood sample collection at 12 h, only subjects in the coffee consumption phase received the evening dose of coffee. Then, all subjects were discharged from the Clinical Pharmacology Unit.

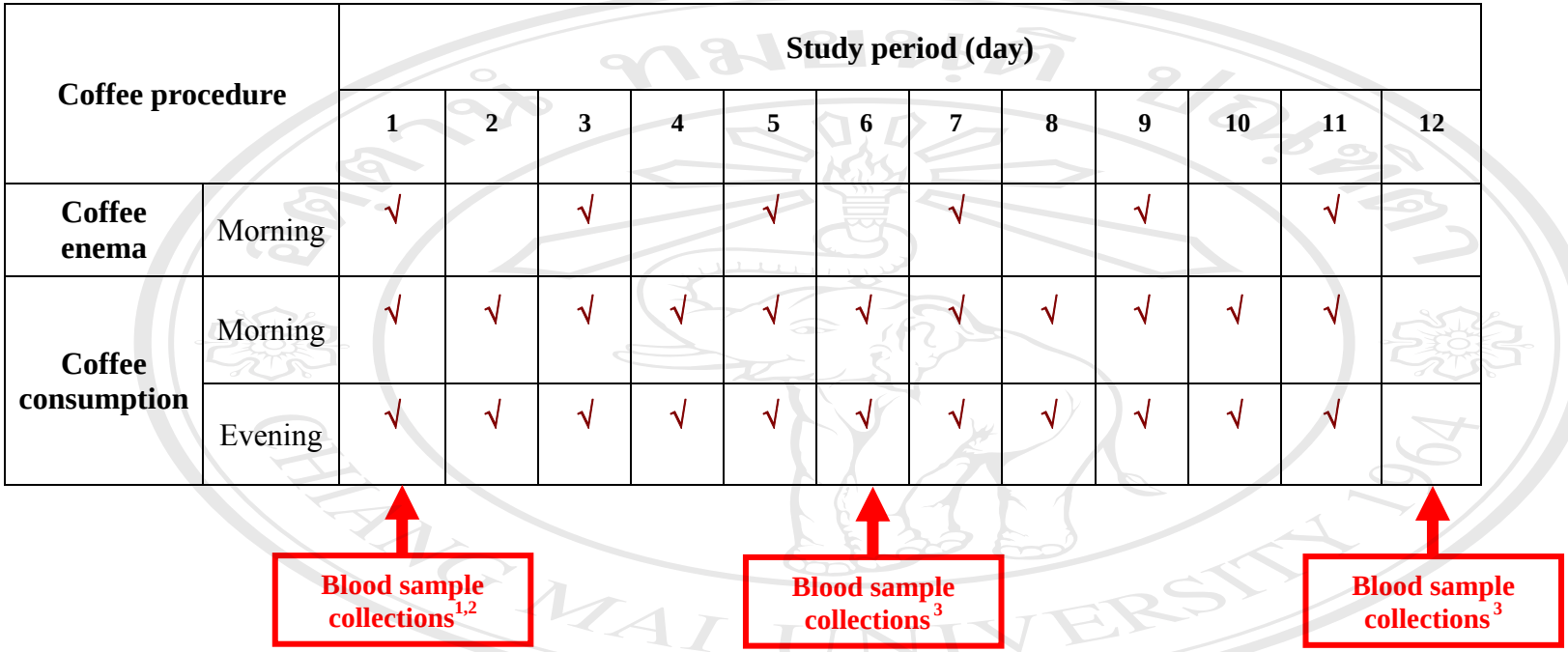
2.4.2 Blood sample collection for determination of antioxidant parameters

In this study, an acute antioxidant effects from single administration of either coffee enema or coffee consumption were determined by using serum levels of GSH, MDA and TEAC as antioxidant parameters. The blood samples used for determination of these parameters were the same as those used for pharmacokinetic study mentioned above.

The blood samples taken following multiple doses of either coffee procedure treatment were collected during the study period (day 6) and again after completion of either treatment phase (day 12). The antioxidant parameters were included serum levels of GSH and MDA and TEAC

Fifteen ml of blood were obtained by venipuncture at each time point mentioned above. Eight ml of blood samples were collected into test tubes containing heparin sodium for determination of plasma caffeine concentrations. Seven ml of these blood samples were collected into clot activator test tube for determination serum levels of GSH, MDA and TEAC.

Plasma and serum samples were separated within 1 h by centrifugation at 2,250 rpm for 30 min and were stored at -20 °C until analysis.



[1] Blood sample collection before the first coffee enema or coffee drink, for determination of basal caffeine level and basal antioxidant parameters
[2] Blood sample collections at 10, 20, 30, 40, 60 min and 1.5, 2, 4, 8, 12 hour after the first coffee enema or coffee consumption, for determination of caffeine pharmacokinetics and antioxidant parameters
[3] Blood sample collections in the morning for determination of antioxidant parameters following multiple doses of coffee enema or coffee consumption

Figure 5. Schedule of administration of coffee procedures and blood sample collections during both phases of the study

Table 1. The randomized sequence of coffee procedures in each subject

Subject		Study period 1		Study period 2
1	Run-in period	Coffee consumption	Wash-out period	Coffee enema
2		Coffee enema		Coffee consumption
3		Coffee enema		Coffee consumption
4		Coffee enema		Coffee consumption
5		Coffee consumption		Coffee enema
6		Coffee consumption		Coffee enema
7		Coffee enema		Coffee consumption
8		Coffee consumption		Coffee enema
9		Coffee consumption		Coffee enema
10		Coffee enema		Coffee consumption
11		Coffee consumption		Coffee enema
12		Coffee enema		Coffee consumption

2.5 Determination of caffeine concentrations

2.5.1 Determination of caffeine concentrations in coffee solutions

The assay of caffeine content was modified from the HPLC method and conditions previously described by Tanaka E (63). Six servings of each coffee solution were mixed and diluted with 10% methanol to approximate 10 fold of caffeine concentration and spiked with 10 µL of internal standard (IS, 100,000 ng/mL acetaminophen). Five microliter of standard or sample solution was separately injected into HPLC system. Chromatographic separation was performed on 5 µm C18, 100x4.6 i.d. analytical and guard columns. The mobile phase A used was 1

mmol/L perchloric acid/ isopropanol (1000/56, v/v)/ 2.2 mmol/L sodium dodecyl sulfate which was pumped through the column at a flow rate 1 ml/ min for 7 min. Then the mobile phase B used was 1 mmol/L perchloric acid/ isopropanol (1000/88, v/v)/ 3 mM sodium dodecyl sulfate which was pumped through the column at a flow rate 1 ml/ min for 8 min and the analytes were detected by UV absorption at 274 nm, and the column was maintained at 40 ºC. The caffeine contents of unknown samples were determine by using a calibration curve of peak height ratios of caffeine and IS versus respective caffeine concentrations (2500, 6250, 12500, 25000, 50000 and 100000 ng/mL) with the use of linear regression.

2.5.2 Determination of caffeine concentrations in plasma

The assay was modified from protein precipitation procedure previously described by Schreiber-Deturmeny E *et al* (64). Briefly, sample plasma was spiked with 10 µL of internal standard (IS, 100,000 ng/mL acetaminophen). Then, deproteinated by mixing plasma sample with 380 µL of acetonitrile and stand at room temperature 20 min. After vortex mixing, the proteins were removed by centrifugation at 14000 g (room temperature) for 5 min. An aliquot of the supernatant (600 µl) was removed and evaporated to vacuum dried for 2 hr at 60 ºC. The residues were reconstituted with 50 µL of mobile phase B and span with vortex for 20 sec, and then injected 5 µL of the standard sample into the HPLC system described above. Plasma concentrations of caffeine were determined by interpolating the peak height ratios of the caffeine and IS versus respective caffeine concentrations (100, 250, 500, 1000, 2000 and 4000 ng/mL). The details of validation of HPLC method were discussed in "Chapter 3".

2.6 Determination of blood pressure and heart rate

Blood pressure and heart rate were measured by using Omron digital blood pressure monitor (Intelli Sense^{TM}, Model HEM-711, Omron Healthcare, Inc.) prior to coffee enema or coffee consumption and again at 10, 20, 30, 40, 60 min, 1.5, 2, 4, 8, 12 h following coffee enema or coffee consumption. Subjects were maintained a relaxed, semi-recumbent position for 5-min stabilization period before each measurement.

2.7 Determination of serum glutathione (GSH) concentrations

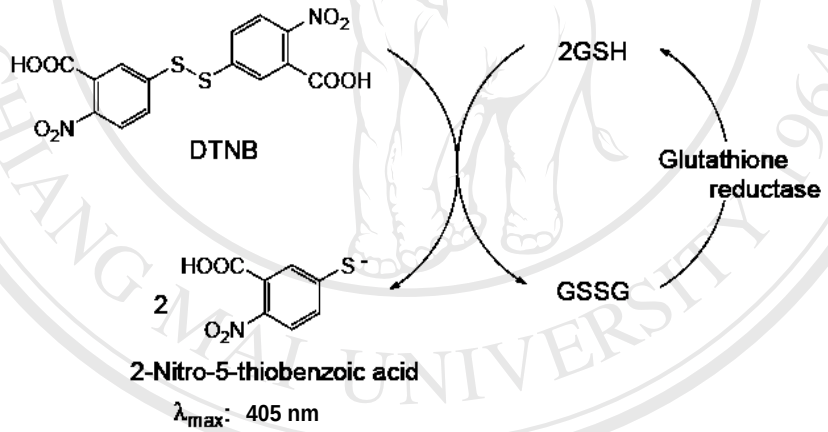


Figure 6. Enzymatic recycling method for the quantification of GSH concentration

GSH level was measured by the method described by Ellman (65) and deproteinization was achieved by a modification of the method of Fariss and Reed by using metaphosphoric acid (MPA). Ten percent of MPA was added into serum to precipitate protein followed by centrifugation at 10,000 rpm at 4 ºC for 20 min. The deproteinized supernatant was taken and stabilized by adding 4 mol/L triethanoamine (TEAM) reagent. The deproteinized supernatants can be stored under -20 ºC up to six

months for assay. Enzymatic recycling method was used glutathione reductase, for the quantification of serum GSH concentrations (Figure 6). Duplicates of 25 µL of deproteinized supernatant or standard or blank were assayed at 405 nm in a 150 µL of reaction mixture consisting of 4 mmol/L â–Nicotinamide adenine dinucleotide phosphate (NADPH), 10 mmol/L sodium phosphate buffer containing EDTA (PBE, pH 7.5) and 6 U/mL glutathione reductase (GR), and were incubated at 37 °C for 5 min. Then, 6 mmol/L of 5-5-Dithio-bis (2-Nitrobenzoic acid) (DTNB; Ellman's reagent) solution was added and incubated at room temperature for 30 min. The absorbance of mixture was measured at 405 nm. The GSH level was obtained from a standard curve prepared by using 4, 8, 16, and 32 µmol/L of standard reduced GSH in deionized water.

2.8 Determination of serum malondialdehyde (MDA) concentrations

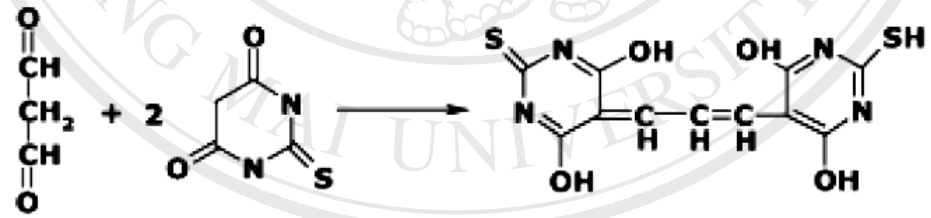


Figure 7. Reaction of MDA with thiobarbituric acidb (TBA) forming thiobarbituric acid reactive substances (TBARs)

MDA assay was based on the reaction of MDA with thiobarbituric acid (TBA) forming thiobarbituric acid reactive substances (TBARs) (Figure 7). MDA level in serum was measured by modified procedure according to Smith *et al* (66). Briefly, sample serum was prepared by using 4 mL of 3N sulfuric acid (H_2SO_4) was added to

200 µL of serum and the mixture was gentle shaken. Then 500 µL of 10% phosphotungstic acid was added and mixed. After standing at room temperature for 5 min, the mixture was centrifuged at 3000 rpm for 15 min. The pellet was mixed with 2.0 mL of 3 N H_2SO_4 and 300 µL of 10% phosphotungstic acid. The mixture was centrifuge at 3000 rpm for 15 min. After removing supernatant, the sediment was suspended in 4.0 mL of deionized water. Two milliliters of 0.33% of TBA reagent was added in standard MDA and serum. After agitation in a vortex mixer, the reaction mixture was heated for 1 hr in a boiling water bath. Five milliliter of *n*-butanol was added and the mixture was shaken vigorously after cooling to room temperature and centrifuge at 3000 rpm for 15 min. The pink chromogen of *n*-butanol layer was taken for read at 530 nm in a double-beam spectrophotometer. The MDA level was obtained from a standard curve prepared by using 0.01, 0.02, 0.04, and 0.08 mmol/L.

2.9 Determination of serum trolox equivalent antioxidant capacity (TEAC)

Serum TEAC concentrations were determined according to ABTS radical cation ($ABTS^{\bullet+}$) decolorization assay (67-68). Stock ABTS solution (7 mmol/L) was prepared by dissolving 98 mg of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) diammonium salt (ABTS) in 5 mmol/L PBS (a final volume of 25 ml) and stored at 4 $^{\circ}$C. Because ABTS and potassium persulfate react stoichiometrically at a ratio of 1:0.5, this will result in incomplete oxidation of the ABTS. Oxidation of the ABTS commenced immediately, but the absorbance was not maximal and stable until more than 6 h had elapsed. ABTS radical cation ($ABTS^{\bullet+}$) generates a blue-green colored complex with a maximal absorption at 734 nm, and is stable for more than two days at room temperature when stored in the dark. The $ABTS^{\bullet+}$ solution was

produced by reacting the stock ABTS solution with 2.45 mmol/L potassium persulfate (a final concentration) (1:0.5, *v*/*v*) and allowing to stand in the dark at room temperature for 12-16 h before use. Then, $ABTS^{\bullet+}$ solution was diluted with PBS, pH 7.4 solution until optical density at 734 nm (A_{734nm}) reaches 0.700 (±0.020). Antioxidants reduce the color intensity in proportion to their anti-oxidative capacity. In the assay 1.0 mL of working $ABTS^{\bullet+}$ solution was mixed with 10 µl of serum or PBS-buffered trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) solution at 30 °C for exactly 6 minutes, the absorbance was immediately measured at 734 nm against reagent blank. %Inhibition values of samples and standards were calculated, and trolox equivalent antioxidant capacity (TEAC) was determined from the calibration curve. After 1.0 ml of diluted $ABTS^{\bullet+}$ solution was added to 10 ml of standard trolox solutions (final concentrations of 0-15 mmol/L), absorbance was measured between 1-6 min. Three independent triplicate determinations were carried out for both standard trolox and serum samples. Capacity of reducing the $ABTS^{\bullet+}$ (a decrease of A_{734nm}) by antioxidant was shown as percent inhibition (%Inhibition) value calculated from the following formula,

$$\% \text{ Inhibition} = \left(\frac{A_{734nm} \text{ by reagent blank} - A_{734nm} \text{ by standard trolox}}{A_{734nm} \text{ by reagent blank}} \right) \times 100$$

A calibration curve of TEAC assay was made by plotting %Inhibition (y-axis) against trolox concentrations (x-axis) of 0.05, 0.75, 1.00 and 1.00 mmol/L. Antioxidant activity of the serum samples was determined from the calibration curve.

2.10 Data analysis and statistical methods

All statistical analyses were performed by using the SPSS (version 16.0) package for Windows and Statdirect 2.5.6. All data were compared with two-side test. Differences was considered statistically significant at $p< 0.05$

2.10.1 Caffeine pharmacokinetic parameters

The maximal plasma concentration (C_{max}, μg/mL) and time to the maximal 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 0-12 h (AUC_{0-12}, μg.h/ml) and from time 0 to infinity ($AUC_{0-\infty}$, μg.h/ml) as well as the half life ($t_{1/2}$, h) were determined by non-compartmental analysis using the TopFit software version 2.0 for personal computer. The slope of the terminal log-linear portion of the concentration-time curve was determined by least-squares regression analysis and was used as the elimination rate constant (k_e). The elimination $t_{1/2}$ was calculated as $0.693/k_e$. The AUC from time zero to the last quantifiable point (AUC_{0-12}) was calculated using the trapezoidal rule. Extrapolated AUC from time t to infinity ($AUC_{0-\infty}$) was determined as Ct/k_e. Total AUC was sum of $AUC_{0-12} + AUC_{12-\infty}$. In this study, the sampling time was continued for more than 3 times the $t_{1/2}$ therefore, the AUC_{0-12} was sufficient to cover at least 80% of the total AUC.

The pharmacokinetic parameters and cardiovascular parameters were presented as mean ± SD. The differences of the mean values of C_{max}, T_{max}, $t_{1/2}$, AUC_{0-12}, $AUC_{0-\infty}$, systolic and diastolic blood pressure, and heart rate between baseline and at any time points were compared by using one-way ANOVA with

repeated measurement, whereas the differences in mean change from baseline of these parameters between single administration of both coffee procedures were statistically analyzed by using paired t-test.

2.10.2 Antioxidant parameters

The mean serum concentrations of GSH, MDA and TEAC between baseline and at any time points, including day 6 and day 12, were compared by using one-way ANOVA with repeated measurement. In addition, the mean changes from baseline of these parameters at day 6 and day 12 between both coffee procedures were compared by using paired t-test.