CHAPTER 2
MATERIALS AND METHODS

2.1 Quantitative analysis of ganoderic acids A and F in Ling Zhi preparations

2.1.1 Ling Zhi preparations

Seventeen commercial Ling Zhi preparations in various brands and dosage forms including capsules containing crushed Ling Zhi, Ling Zhi extract or spore (8 preparations), tea bag or instant tea (5 preparations), and sliced fruiting bodies (4 preparations) were randomly purchased from different stores in Chiang Mai and Bangkok, Thailand. Each preparation was given the sample code instead of its trade name. In addition, the sliced fruiting bodies (MG2FB) and MG2FB-WE kindly provided by Muang Ngai Special Agricultural Project under the patronage of Her Majesty Queen Sirikit, Chiang Dao, Chiang Mai, were also included in the determination of ganoderic acids A and F.

2.1.2 Chemicals and reagents

The reference substances of ganoderic acids A and F (Figure 2), including cortisone 21-acetate (Figure 4) used as internal standard (IS) were kindly provided by Assoc. Prof. Noppamas Soonthornchareonnon, Department of Pharmacognosy, Faculty of Pharmacy, Mahidol University. HPLC-grade methanol and acetonitrile were purchased from Burdick and Jackson, Honeywell International Inc. Ammonium acetate obtained from Fisher Scientific International Inc., and perchloric acid obtained from Panreac Química SA, were of analytical grade. Deionized water used for HPLC analyses was supplied by the Laboratory Unit of the Medical Science Research Equipment Center, Faculty of Medicine, CMU.

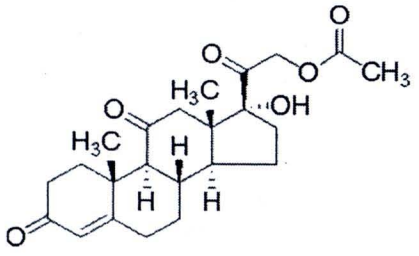

Figure 4 Structure of cortisone 21-acetate (IS).

2.1.3 HPLC system and conditions for analysis of ganoderic acids A and F in Ling Zhi preparations

A Shimadzu HPLC system (Shimadzu Ltd., Kyoto, Japan) equipped with two LC-10ADvp pumps, DGU-14A degasser, SIL-10ADvp auto injector, CTO-10ASvp column oven, SPD-M10Avp diode array detector, and SCL-10Avp system controller was used. Its stationary phase consisted of an Inertsil ODS-3-C_{18} analytical column (250 × 4.6 mm, 5 μm) connected to an Inertsil ODS-3 guard column (50 × 4 mm, 5 μm) and maintained at 50 °C. The detecting wavelength was set at 252 nm. Two mobile phases consisted of solvent A [20 mM ammonium acetate and 0.2 mM perchloric acid in deionized water/acetonitrile/methanol (250/60/1.5, v/v/v)] and solvent B [20 mM ammonium acetate and 0.2 mM perchloric acid in deionized water/acetonitrile/methanol (250/150/1.5, v/v/v)]. A gradient elution of solvent A : B was scheduled as 65 : 35 for the first 15 min, followed by 40 : 60 at 15-35 min, and 0 : 100 at 35-60 min. The flow rate was maintained at 1.0 mL/min. Chromatographic data were analyzed using the Shimadzu Class-VP software.

2.1.4 Preparation of standard solutions

The stock solutions of ganoderic acids A and F were prepared by dissolving each reference substance in 90% methanol to final concentration of 500,000 ng/mL, and then the stock solutions were subsequently diluted in the same diluent to give 7 respective concentrations (2.50, 5.00, 10.00, 25.00, 50.00, 100.00 and 200.00 μg/mL) for establishing calibration curve. The IS stock solution was prepared by dissolving cortisone 21-acetate in the same diluent at a concentration of 100,000 ng/mL. All the stock solutions were stored at -20 °C.

2.1.5 Sample preparation of Ling Zhi products

Sample extraction was modified from the method described by Wang *et al* [102]. Briefly, 50 mg of Ling Zhi from each preparation was extracted with 1 mL of 95% methanol in an ultrasonic water bath for 60 min. The extraction solution was then centrifuged at 14,000 rpm for 5 min at room temperature. Thereafter, 10 μL of clear supernatant was spiked with 10 μL of IS (20.00 μg/mL of cortisone 21-acetate) and diluted with 30 μL of mobile phase B. Aliquot of 10 μL of each sample solution was injected into the HPLC system. The contents of ganoderic acids A and F in each Ling Zhi preparation were determined from a calibration curve and linear regression of the seven known concentrations of ganoderic acids A and F, *versus* the peak area ratios of corresponding ganoderic acids and IS.

2.1.6 Assay validation of HPLC method

The assay validation such as specificity, linearity, limits of detection (LOD) and quantification (LOQ), including intra- and inter-day precision were performed. Intra-day precision was determined by analyzing 5 repetitions of the standard mixtures of ganoderic acids A and F at 3 different concentrations (7.50, 90.00, 180.00 μg/mL) on the same day. Inter-day precision was determined by analyzing 5 repetitions of these standards on the 3 independent days. The percentage of coefficient of variation (% CV), calculated from standard deviation/mean of the measurements x 100, was taken as a measure of precision.

2.1.7 Statistical analysis

The individual and total contents of ganoderic acids A and F in each Ling Zhi preparation was presented as mean ± standard deviation (SD) and descriptive analysis was used to summarize the data and consolidate a mass of numerical data into significant information. Correlation coefficient value (r^2) calculated by linear regression analysis was used to evaluate the correlation between total contents of ganoderic acids A and F in 17 commercially available Ling Zhi preparations and their prices.

2.2 Pharmacokinetic study of ganoderic acids A and F after an oral administration of Ling Zhi preparation

2.2.1 Study design

The study was a single-dose, open-label, randomized, two-phase crossover study with at least 2-wk washout period. This study was approved by the Human Research Ethic Committee of the Faculty of Medicine, CMU, and was complied with the Declaration of Helsinki.

2.2.2 Subjects

Inclusion criteria

Twelve healthy Thai male subjects aged between 18-40 y whose body mass index (BMI) were within the normal range (18-25 kg/m^2), were enrolled into the present study. They were considered healthy on the basis of their medical history and physical examination. The results of routine laboratory tests including complete blood count, liver function test, blood urea nitrogen and creatinine had to be within normal limits. Subjects included in the study were given both verbal and written information regarding the nature and purpose of the study. Informed consent was voluntarily obtained from each subject prior to study participation.

Exclusion criteria

Subjects with known hypersensitivity to Ling Zhi were excluded as well as those with known medical history of neurological, pulmonary, kidney, liver or cardio-vascular diseases including malignancy. Other exclusion criteria were recent cigarette smoking within the previous 3 months, use of alcohol, substance abuse, any Ling Zhi preparation or other medications (except acetaminophen) within the previous 1 month.

Withdrawal criteria

The withdrawal criteria of this study were subject who experienced severe adverse events during the study, subject who could not comply with the study protocol or wished to voluntarily withdraw from the study, including subject who required other medications during the study period.

2.2.3 Dosage and administration

Eligible subjects were admitted to the Clinical Pharmacology Unit, Faculty of Medicine, CMU, at 6.30 am after an overnight fast of at least 8 h. Each subject was randomized (Table 2) to receive a single oral dose of MG2FB-WE either under fasted condition, or immediately after a Melander type standard breakfast (fed condition). The standard breakfast consists of 150 mL semi-skimmed milk, 100 mL orange juice, 1 hard-boiled egg, 2 pieces of whole wheat bread, 5 g margarine, 20 g orange marmalade and 20 g hard cheese [103]. The 3,000 mg of MG2FB-WE in granular formulation containing 1,417.80 ± 40.74 μg/g of ganoderic acid A and 224.15 ± 8.02 μg/g of ganoderic acid F, was dissolved in 200 mL of warm water before oral administration.

Table 2 The randomized sequence of oral Ling Zhi administration in each subject

Subject No.	Study period 1		Study period 2
1	Fasted condition	Wash-out period of at least 2 wk	Fed condition
2	Fed condition		Fasted condition
3	Fed condition		Fasted condition
4	Fasted condition		Fed condition
5	Fasted condition		Fed condition
6	Fed condition		Fasted condition
7	Fed condition		Fasted condition
8	Fasted condition		Fed condition
9	Fed condition		Fasted condition
10	Fasted condition		Fed condition
11	Fasted condition		Fed condition
12	Fed condition		Fasted condition

All subjects were instructed to remain upright without intake of any food or beverage 2 h after Ling Zhi administration. Water and lunch were served at 2 and 4 h after dosing, respectively. Serial blood samples were collected at different time points as described in Section 2.2.4. While waiting for blood sample collections, subjects were allowed to perform any daily activities, except moderate to high degree of exercise. After blood sample collection at 8 h post-dose, all subjects were discharged from the Clinical Pharmacology Unit.

After a washout period of at least 2 wk, the subjects were crossed over to receive the same oral dose of Ling Zhi preparation after an alternative (fasted or fed) condition. The blood sample collection and other study conditions in the 2nd study period were as same as the previous study period. An identical meal and fluid were served on both study days. All subjects were instructed to avoid consumption of Ling Zhi or any Ling Zhi preparation throughout of the study.

2.2.4 Blood sample collection

Serial blood sample collections (10 mL each) were obtained before oral administration of the Ling Zhi preparation, and at 5, 10, 15, 30, 45 and 60 min, then at 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, and 8 h, respectively, after dosing for determination of the plasma concentrations of ganoderic acids A and F. The blood samples were obtained from forearm by venipuncture through an indwelling intravenous catheter (BD Insyte®) and collected into heparinized vacutainers (BD Insyte®). The blood collecting tubes were centrifuged at 2,500 rpm for 15 min at 4 °C and the plasma was then separated and frozen at -20 °C until analysis.

2.2.5 LC-MS system and conditions for analysis of ganoderic acids A and F in plasma samples

LC: Agilent technologies model 1100 Series, Germany
Zorbax SB-C_{18} analytical column (4.6 x 150 mm, 5 μm)
Phenomenex C_{18} guard column (4.0 x 3.0 mm)
Wavelength of 252 nm with diode array detector
Mobile phases;
A = 10 mM of amonium formate pH 4.00
B = Acetonitrile
Ratio constant A : B (40 : 60) flow rate 1.0 mL/min
Column oven 40 °C, volume injection 15 μL, runtime 20 min

Mass detector: Agilent technologies model LC/MSD G1956B, USA
Positive API-ES ionization mode
Gas temperature 350 °C
Drying gas (nitrogen) flow rate 13 L/min

Nebulizer pressure 50 psi

Quadrupole temperature 100 °C

Volt capillary positive 4000 V

Volt capillary negative 3500 V

Selected ion monitoring mode;

499.40, 555.30 m/z for ganoderic acid A

403.20, 441.20 m/z for IS

571.30, 572.30 m/z for ganoderic acid F

2.2.6 Preparation of plasma samples

The plasma sample extraction for quantitative determination of ganoderic acids A and F was performed by using protein precipitation method. Concisely, 250 µL of each plasma sample was spiked with 25 µL of IS (2.50 ng/mL of cortisone 21-acetate), and subsequently deproteinated by mixing with 500 µL of 1% acetic acid in 50% methanol/acetonitrile and then kept at room temperature for 20 min. The proteins in plasma sample were separated by centrifuge at 14,000 rpm for 10 min at room temperature. Thereafter, an aliquot of the supernatant was removed, and evaporated to dryness by the concentrator at 60 °C for 1.5 h. The residues were then dissolved in 50 µL of mobile phase and a 15 µL of the sample was injected into LC-MS system. Plasma concentrations of ganoderic acids A and F were determined by using a calibration curve of the peak area ratios of each ganoderic acid and IS, *versus* respective ganoderic acid concentrations with the use of linear regression analysis.

2.2.7 Assay validation of LC-MS method

The pre-study validation such as specificity, lower limit of quantification (LLOQ), linearity, precision and accuracy, recovery as well as stability was performed following the U.S. Food and Drug Administration (FDA) guidance for bioanalytical method validation [104].

Specificity

Specificity is the ability of an analytical method to differentiate and quantify the analyte of interest in the presence of interfering substances including impurities, degradants, or matrix components. In present study, the analyte was referred as

ganoderic acids A and F. A chromatogram of plasma sample spiked with both ganoderic acids and IS was compared with those of blank plasma sample to ensure that the signals measured from ganoderic acids A and F as well as IS were free from endogenous interference at the retention times of these substances.

LLOQ

LLOQ is the lowest concentration of the analyte in sample that can be quantitatively determined with acceptable precision and accuracy. In our study, this test was assessed by analyzing a series of 5 aliquots of 0.50 ng/mL of ganoderic acids A and F in plasma. Precision, reported as % CV, and accuracy had to be within the range of ± 20% and of 80-120%, respectively. The calculation of precision was performed according to equation in Section 2.1.6, whereas accuracy was determined as mentioned below.

Linearity of calibration curve

Linearity of an analytical method is its ability to obtain test results that are directly proportional to the concentration of analyte in sample within the range of standard curve. For the construction of a calibration curve in present study, 7 concentration levels (0.50, 1.00, 2.50, 5.00, 7.50, 10.00, and 20.00 ng/mL) from standard mixture of ganoderic acids A and F were analyzed, and peak area ratios of each ganoderic acid to IS were plotted against the concentrations of corresponding ganoderic acids in the plasma sample. The linearity of calibration curve was calculated by least squares linear regression analysis reported as r^2 value, and the high value ($r^2 \geq 0.99$) indicated that the method had a good linearity.

Precision and accuracy

Precision of an analytical method describes the closeness of repeated individual measures of the analyte, whereas accuracy describes the closeness of the determined value obtained by the method to the true value of the analyte. In present study, precision and accuracy were performed to find out intra-day and inter-day variations by using 3 different concentrations (1.50, 9.00 and 18.00 ng/mL) of quality control (QC) samples of ganoderic acids A and F in plasma. The intra-day precision and accuracy were assessed by analyzing 5 replicates of each concentration within one day, whereas the inter-day precision and accuracy were determined daily with one sample of each concentration for 3 independent days. Precision was reported as % CV,

whereas accuracy was presented as % accuracy, measured from calculated concentration/spiked concentration x 100. The % CV and % accuracy had to be within the range of ± 15% and 85-115%, respectively.

Recovery

Recovery pertains to the extraction efficiency of the developed method within the limits of variability. In our study, this test was determined by comparing the peak area of each ganoderic acid and IS that extracted from plasma, with those of unextracted standard sample in mobile phase from 5 sets of 3 different concentrations (1.50, 9.00 and 18.00 ng/mL) of QC samples. It was reported as % recovery which need not be 100%, but the extent of recovery of ganoderic acids A and F and of IS must be consistent, precise, and reproducible.

Stability

Analyte stability in a biological fluid is a function of the storage conditions, the chemical properties of analyte, the matrix, and the container system. Conditions used in stability experiments reflect situations likely to be encountered during actual sample handling and analysis.

Freeze/thaw stability

Stability of ganoderic acids A and F in plasma was determined after 3 freeze/thaw cycles. It was obtained by using 3 repeating analysis of plasma ganoderic acid concentrations in each 2 levels (1.50 and 18.00 ng/mL) of QC samples stored at -20 °C after 3 unassisted freeze/thaw cycles, comparison to the concentration of these ganoderic acids in freshly prepared QC samples without freeze/thaw processing. The freeze/thaw stability was reported as % remaining of each ganoderic acid.

Short-term stability

Short-term stability of ganoderic acids A and F was determined by comparing the concentration of each ganoderic acid in 3 aliquots of each of the low and high (1.50 and 18.00 ng/mL) QC samples that thawed and left to stand at room temperature for 8 h (which exceeded the expected duration that samples could be maintained at room temperature after thawing until they were analyzed) with those of freshly prepared QC samples.

Long-term stability

Long-term stability was determined by storing 3 aliquots of each of the low and high (1.50 and 18.00 ng/mL) QC samples under the same conditions as the study samples for 1.5 months (the time between the first sample collection and the last sample analysis). The ganoderic acids A and F concentrations of long-term stability samples were compared to those from the first day of this testing.

Post-preparative stability

Post-preparative stability is the stability of extracted plasma samples and the time in the autosampler. In present study, this test was evaluated by comparing the concentration of each ganoderic acid in 3 aliquots of each of the low and high (1.50 and 18.00 ng/mL) QC samples immediately after preparation and at 18 h after preparation (duration that cover the expected runtime for the analytical batch of plasma samples).

2.2.8 Data and statistical analysis

Pharmacokinetic parameters

The C_{max} (ng/mL) and T_{max} (h) of ganoderic acids A and F were evaluated directly by visual inspection of each subject's plasma concentration-time profile. The AUC_{0-8} and $AUC_{0-\infty}$ (ng.h/mL) as well as half-life ($t_{1/2}$, h), were determined by non-compartmental analysis. The slope of the terminal log-linear portion of the concentration-time profile was determined by least-squares regression analysis and used as the elimination rate constant (K_e). The elimination $t_{1/2}$ was calculated from the ratio of $0.693/K_e$. The AUC from time zero to the last quantifiable point (AUC_{0-8}) was calculated by using the trapezoidal rule and the extrapolated AUC from time t to infinity ($AUC_{t-\infty}$) was determined as C_t/K_e. Total AUC was the sum of AUC_{0-8} + $AUC_{8-\infty}$. The calculation was performed by using the Topfit software version 2.0 for personal computer.

Statistical analysis

The pharmacokinetic parameters were presented as mean ± SD. The differences in the mean values of C_{max}, T_{max}, $t_{1/2}$, AUC_{0-8} and $AUC_{0-\infty}$ between the fasted and fed conditions were analyzed by using paired Student's *t* test and were considered statistically significant if $p < 0.05$.