MATERIALS AND METHODS

Chemicals and Reagents

The standard mefloquine HCl (Ro 21-5998/001) and mefloquine metabolite (Ro 21-5104) were kindly donated from F-hoffmann-La Roche Ltd., Basel, Switzerland. Mefloquine (MEQUIN, 250 mg/tablet, Lot No.010192) was kindly given from the Insect Prevention Center, Songkhla, Thailand and ketoconazole (Ketazol, 200 mg/tablet, Lot No.1A918/31) was purchased from the Central Poly Trading Co.,Ltd.,Bangkok,Thailand. The HPLC grade of acetronitrite and methanol were purchased from J.T.Baker (Phillipsburg, NJ, USA). Anhydrous sodium sulfate (analytical grade), 85% phosphoric acid (analytical grade) and zinc sulfate (pro analytical grade) were bought from Merck Darmstadt, Germany and Carlo Erba, Italy, respectively. Water was purified for HPLC by the Milli Q Water Purification System (Millipore, Milford, MA, USA.)

Instrumentation

The HPLC system consists of Waters 2695 pump and autusampler (Water Associates, Milford, MA, USA) and a Water 2487 UV detector. Detection was done with the variable- wavelength UV detector set at 222 nm. The column was reverse-phase Novapak C_{18} (3.9 mm x 150 mm HPLC column, particle size 4 µm, Water Associates, Milford, MA, USA). A guard-pak precolumn module was used to obviate the effect of rapid column degeneration.

Methods

1. Subjects

The volunteers were given a detailed explanation of the purpose, protocol, and risk of the study, and each was given a written consent that was approved by the Ethics Committee, Faculty of Medicine, Prince of Songkla

University, Hat Yai, Thailand. Eight Thai male volunteers, age 16-39 years old (mean age 29.5 ± 8.4 yrs) and weighed 56-64 kilograms (mean weight 61.5 ± 2.6 kgs) participated in the study. Prior to the study, a medical history, physical examination, standard biochemical and hematological screening test (CBS, FBS, BUN, creatinine, SGOT, SGPT, direct bilirubin and albumin/globulin) were done in each volunteer. None of volunteers was a smoker or used continuous medications. Drinking of alcoholic beverages, coffee and tea were not allowed at least 1 month prior to and during the entire of study.

2. Protocol

The study was an open-label, two-phase cross over design with a 1-month washout period.

Phase 1

In the morning after an overnight fast, each subject received a single oral dose of 500 mg mefloquine (2 tablets of 250 mg mefloquine tablet). The drug was administered with a glass of water (200 ml) under supervision. No food was taken at least 2 hours after ingestion of the drug.

A catheter was inserted into a forearm vein for the collection of blood sample, and was maintained patent using 1 ml of a dilute heparin solution (100 unit/ml) after each sample. Venous blood sample (5 ml) were collected in heparinized tubes before drug administration and at 0,0.5,1,2,3,4,6,8,10,12 hours, and 2,3,4,7,14,21,35,49 and 56 days post drug administration. Samples were centrifuged not later than 30 minutes after collection, and the plasma was separated and stored at –60 ^{0}C until analysis.

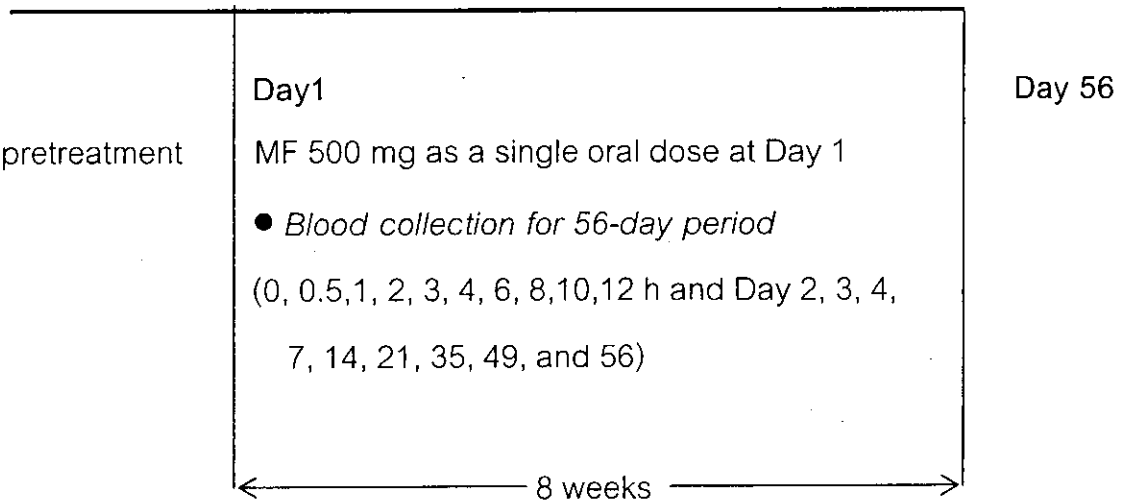
Phase 2

After 1 month of being free, all subjects received a 400 mg of orally ketoconazole (2 tablets of 200 mg ketoconazole tablet) once daily before breakfast for 5 days prior to mefloquine administration. In the morning of day 1

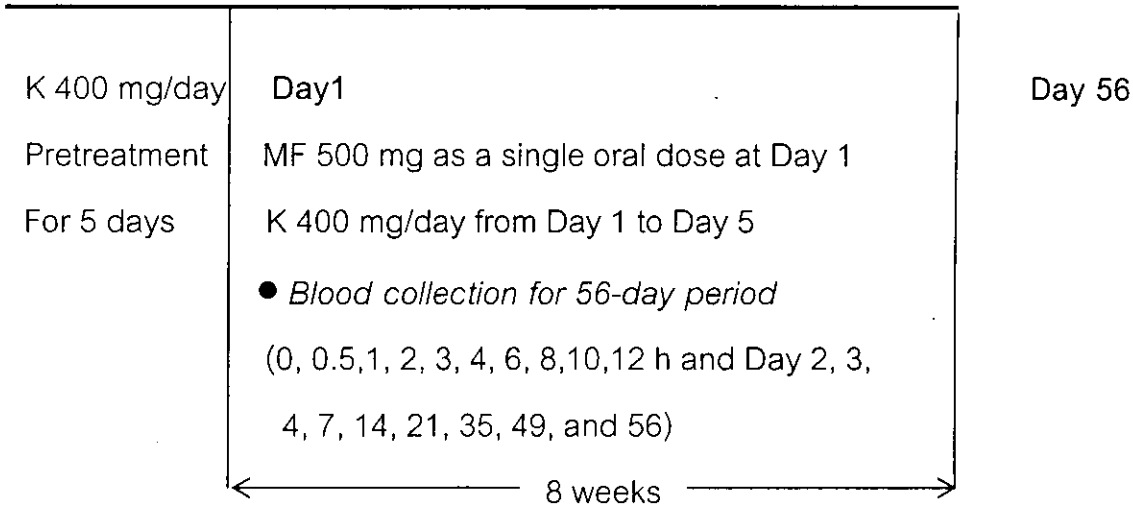
(after ketoconazole pretreatment for 5 days), after an overnight fasting, each volunteer took 500 mg mefloquine orally. Venous blood sample were collected at the time interval before and after mefloquine administration as previously done in phase 1. However, ketoconazole was continued to orally administered 400 mg daily before breakfast from day 1 to day 5 after mefloquine administration.

Schematic plan of the study: Effect of ketoconazole on pharmacokinetic profiles of a single oral dose of mefloquine (500 mg) in healthy subjects.

Phase 1: A single oral dose of mefloquine (MF) alone



Phase 2 : Ketoconazole (K) and a single oral dose of mefloquine (MF) after ketoconazole pretreatment



3. Sample Analysis

The plasma mefloquine and mefloquine carboxylic metabolite concentrations were measured by a high performance liquid chromatographic (HPLC) method (Crevoisier, et al, 1997; Ridtitid, et al, 2000).

3.1 Mobile phase

The mobile phase consisted of 50 mM/L sodium sulfate: methanol: acetonitrile (50:34:16 vol/vol/vol) and adjusted to pH 3.07 with 85% phosphoric acid. The mobile phase was freshly prepared daily and filtered through 0.45 micropore filter (Nylon 66), then degases by sonification for 10 minutes before using. The flow rate was 1.5 ml/min. All analyses were performed at room temperature (25 ± 1^{0}C).

3.2 Stock Standard Solution

A stock standard solution at a concentration of 400 µg/ml was prepared by dissolving 4 mg of standard mefloquine in 10 mM/L hydrochloric acid. The standard mefloquine carboxylic metabolite was dissolved in 10 mM/L sodium hydroxide solution. All solutions were adjusted to 10 ml in a 10 ml volumetric

flask. The stock solutions were stable for at least 6 months at 4^0C (Edstein, et al., 1991). Working standard solution used to prepare a calibration curve day by day were prepared by appropriate dilution of the stock standard solution with a blank plasma (Appendix-1).

3.3 Calibration Curves

Calibration curves were prepared by adding a standard mefloquime and mefloquine metabolite solution to blank human plasma so that the final concentrations in plasma were 62.5, 125, 250, 500 and 1000 ng/ml. The calibration curves for mefloquine and mefloquine metabolite were linear in the range of 62.5 - 1000 ng/ml. The lower of quantitation (LOQ) for mefloquine and mefloquine metabolite was 62.5 ng/ml.

3.3.1 Recovery

Potential loss of mefloquine and mefloquine metabolite during the precipitation by acetronitrile was determined by comparing the peak area of mefloquine and mefloquine metabolite precipitated from plasma samples in range of 62.5-1000 ng/ml and the equal concentration of standard mefloquine prepared in mobile phase. The percent of recovery was calculated as following.

$$\frac{\text{Peak area of standard mefloquine or mefloquine metabolite in plasma}}{\text{Peak area of standard mefloquine or mefloquine metabolite in mobile phase}} \times 100$$

3.3.2 Precision and variability

To determine intra-day precision and variability, the standard mefloquine and mefloquine metabolite were spiked in blank plasma at 62.5, 125, 500 and 1000 ng/ml concentrations and 5 replications of each were carried out on one day. Accuracy should be ± 10 % of spiked value and the percent of coefficient of variation (%CV) of each concentration should be less than 10 %.

To determine inter-day precision and variability, the standard mefloquine and mefloquine metabolite were spiked in blank plasma at 62.5,

125, 500 and 1000 ng/ml concentrations and 10 replications of each were carried out on different ten days. Accuracy should be ± 10 % of spiked value and the percent of coefficient of variation (%CV) of each concentration should be less than 10 %. The percent of coefficient of variation (%CV) was calculated as following formula.

$$\%CV = \frac{\text{standard deviation (SD) of peak area of mefloquine or metabolite}}{\text{Mean peak area of mefloquine or metabolite}} X100$$

3.4 Sample preparation

A 200 µl of plasma sample or spiked standard plasma was used and 50 µl of 0.2 M zinc sulfate solution was added drop wise to polypropylene tubes containing sample plasma or spiked plasma and vortex mixing for 30 seconds. A 500-µl volume of acetonitrile was then added drop wise during vortex mixing for 30 seconds. After 15 minutes the tubes were centrifuged at 10,000 g for 5 minutes. The supernatant (600 µl) was transferred into polypropylene tubes and evaporated to dryness at 55^0C for 3-4 hours under a stream of air. The residue was reconstituted in 200 µl of mobile phase via ultrasonication (3 minutes) and 100 µl of the solution was injected into the HPLC system (Appendix-2). The chromatographic conditions used in this study were good to separate mefloquine and mefloquine metabolite from other endogenous substances in plasma.

4. Data Analysis

4.1 Pharmacokinetic Calculations

The pharmacokinetic parameters were analyzed by one-compartment methods, with the use of WinNonlin version 4.1 (Pharsight, Mountain View, CA). The total area under the plasma concentration – time curve (AUC) was calculated by use of the linear trapezoidal rule. The elimination rate constant (Ke) was estimated from the least – squares regression slope of the terminal

plasma concentrations. The AUC from time 0 to infinity ($AUC_{0-\alpha}$) was calculated as follows:

$$AUC_{0-\alpha} = AUC + Ct/Ke$$

in which Ct is the last plasma concentrations measured. The half – life ($t_{1/2}$) of mefloquine and mefloquine metabolite were calculated with the following equation

$$t_{1/2} = \ln 2 / Ke$$

The maximum plasma concentration (C_{max}) and the time to reach C_{max} (t_{max}) were estimated from the plasma concentration – time data.

The pharmacokinetic parameters included the area under the concentration time curve from time zero to the end time of the collection interval (AUC_{0-last}), the area under the concentration-time curve extrapolated to infinity ($AUC_{0-\alpha}$), the absorption rate constants (Ka), the elimination rate constants (Ke), the maximum plasma concentration (C_{max}), the time to reach C_{max}(T_{max}), and the elimination half life ($t_{1/2}$). The apparent oral clearance (Cl/f) was calculated as dose/(AUC_{0-last}x body weights). The apparent volume of distribution (Vd/f) was calculated as Cl/f divided by Ke.

4.2 Statistical Analysis

All results were expressed as mean ± S.D. Differences in mefloquine and mefloquine metabolite pharmacokinetic parameter among control and treatment groups were tested by one – way ANOVA with $P< 0.05$ taken as the minimum level of significant. The effect of period, sequence and interaction were evaluated with the use of two- way ANOVA analysis.

Table 1. The intra-day variance of four different mefloquine concentrations in plasma[a]

Concentration (ng/ml)	Mean peak area (n=5)	Standard deviation (SD)	CV (%)[b]
62.5	24995.20	1300.61	5.20
125	58257.40	2684.25	4.60
500	223809.20	12012.50	5.36
1,000	453584.00	7294.70	1.60

[a]Various concentrations of standard mefloquine were added to drug-free human plasma samples prior to precipitation as described in the text.

[b]Standard deviation divided by mean, expressed in percent.

Table 2. The intra-day variance of four different mefloquine metabolite concentrations in plasma[a]

Concentration (ng/ml)	Mean peak area (n=5)	Standard deviation (SD)	CV (%)[b]
62.5	22081.20	840.83	3.80
125	41893.00	3802.06	9.07
500	185468.60	6387.38	3.44
1,000	347194.00	5731.98	1.65

[a]Various concentrations of standard mefloquine metabolite were added to drug-free human plasma samples prior to precipitation as described in the text.

[b]Standard deviation divided by mean, expressed in percent.

Table 3. The inter-day variance of four different mefloquine concentrations in plasma[a]

Concentration (ng/ml)	Mean peak area (n=10)	Standard deviation (SD)	CV (%)[b]
62.5	24817.60	1922.95	7.74
125	54270.10	4650.14	8.56
500	214831.00	19333.03	8.99
1,000	437406.50	21018.50	4.80

[a]Various concentrations of standard mefloquine were added to drug-free human plasma samples prior to precipitation as described in the text.

[b]Standard deviation divided by mean, expressed in percent.

Table 4. The inter-day variance of four different mefloquine metabolite concentrations in plasma[a]

Concentration (ng/ml)	Mean peak area (n=10)	Standard deviation (SD)	CV [b] (%)
62.5	21076.10	2152.61	10.21
125	46949.40	4317.80	9.19
500	164294.40	13196.11	8.03
1,000	348742.32	12246.10	3.51

[a]Various concentrations of standard mefloquine metabolite were added to drug-free human plasma samples prior to precipitation as described in the text.

[b]Standard deviation divided by mean, expressed in percent.

Table 5. Relative percent recovery of standard mefloquine in human plasma

Concentration (ng/ml)	Mean peak area in mobile phase[a] (n=5)	Standard deviation (SD)	Mean peak area in plasma[b] (n=5)	Standard deviation (SD)	% Recovery[c]
62.5	32123.30	1321.65	31253.45	1423.96	97.30
125	61673.20	2743.76	58257.40	2684.25	94.46
500	269032.03	969.84	223809.20	12012.50	83.19
1000	510235.10	4086.70	488923.40	22803.82	95.82

[a]Various concentrations of standard mefloquine in mobile phase were directly injected.

[b]Various concentrations of standard mefloquine were added to drug-freehuman plasma samples prior to precipitation.

[c]Mean peak area in plasma divided by mean peak area in mobile phase, expressed in percent.

Table 6. Relative percent recovery of standard mefloquine metabolite in human plasma

Concentration (ng/ml)	Mean peak area in mobile phase[a] (n=5)	Standard deviation (SD)	Mean peak area in plasma[b] (n=5)	Standard deviation (SD)	% Recovery[c]
62.5	24123.85	1296.46	23998.74	1523.79	99.48
125	47322.62	2584.46	46949.40	4317.80	99.21
500	206796.20	4835.15	185468.60	6387.38	89.68
1000	410884.20	9297.51	383987.0	10708.81	93.45

[a]Various concentrations of standard mefloquine metabolite in mobile phasewere directly injected.

[b]Various concentrations of standard mefloquine metabolite were added to drug-free human plasma samples prior to precipitation.

[c]Mean peak area in plasma divided by mean peak area in mobile phase, expressed in percent.