CHAPTER 3

MATERIALS AND METHODS

Chemicals and Reagents

Standard quinine and quinidine hydrochloride were purchased from SIGMA® Chemical Co. (St Louis, MO, U.S.A.). Quinine sulphate (300 mg tablet Lot No. 98098) was obtained from General drug House Co., Ltd., Bangkok Thailand. Ketoconazole (Nizoral®, Lot No. B181297) and itraconazole (Sporal®, Lot No. 384037) were obtained from OLIC (Thailand) Limited, under license of Janssen Pharmaceutica Ltd. Acetonitrile (HPLC-grade) and Triethylamine (analytical grade) were obtained from J.T. Baker Inc. Phillipsburg, U.S.A. and Fluka, Messerchmittstr, Switzerland, respectively. Water was purified for HPLC by the Milli Q Water Purification System (Millipore, Milford, MA, U.S.A).

Equipments

The HPLC system composed of Waters 510 pump and the automated injection system, Waters 717 plus Autosampler (Waters Associates, Milford, MA, U.S.A.). The detector was Jasco 821-FP intelligent Spectrofluorometer (Japan Spectroscopic Co., Ltd., Tokyo, Japan). The integrator was the Jasco model 807-IT (Japan Spectroscopic Co., Ltd., Tokyo, Japan). A μ-Bondapak C_{18} (30 cm x 3.9 mm I.D., particle size 10 μm, Waters Associates, Milford,

MA, U.S.A) was used as the column. A Guard-pak precolumn module was used to obviate the effect of rapid column degeneration.

Methods

1. Subjects

The Human Ethics Committee Faculty of Science, Prince of Songkla University, Hat-Yai, Thailand, approved the study. All subjects gave written informed consent before study. Nine Thai male healthy volunteers, aged 16-37 years old, weighing 47-70 kilograms (mean weight 61.3 ± 7.52 kgs) participated in the study. Prior to the study, a medical history, physical examination, standard biochemical and hematological screening were performed in each subject. Neither any drugs were taken in the month preceding nor during the study. All of them are non-smokers. Drinking of alcoholic beverages, coffee and tea are not allowed at least 2 weeks prior to and during the entire period of study.

2. Protocol

Three phases of the studies were randomized crossover study designed with two weeks wash out period. In each phase, three subjects received a single 300 mg dose of quinine sulphate orally with 150 ml of water. The rest of two groups (3 subjects of each group) received 400 mg ketoconazole (Nizoral®) or 200 mg itraconazole (Sporal®) orally with breakfast and 150 ml of water for 4 days and followed by a single 300 mg dose of quinine

sulphate orally in the study day 4. A serial blood draw was done in the study day 4 before and after a single oral dose of 300 mg quinine sulphate ingestion. All volunteers were overnight fast before the study day 4.

2.1. Blood Sample Collection

The heparin-lock catheter was placed in a forearm vein of each subject. In the study day 4 after administration of 300 mg quinine sulphate, serial blood samples (5 ml) were drawn immediately before and at 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 2.0, 2.5, 3, 4, 6, 8, 24 and 48 hours after quinine sulphate administration. Plasma was separated from blood sample by centrifugation at 1,000 g for 15 minutes and aliquots of the plasma were stored at –70 °C until analysis.

2.2 EKG and Blood Pressure Monitoring

The EKG, heart rate and blood pressure were monitored immediately before and at 0.5, 1.0, 1.5, 2.5, 4, 8 and 24 hours after 300 mg quinine sulphate administration.

3. Analytical Methods

Plasma samples were analyzed by high performance liquid chromatography for quinine and quinidine (internal standard) by methods previously described by Lehmann *et al.* (1986); Edsteine *et al.* (1990); and Supanaranond *et al.* (1991) with a slight modification using reverse-phase column (μ-Bondapak C_{18}) and a fluorescence detector (an excitation and emission wavelength were set at 340 and 425 nm, respectively).

3.1 Mobile Phase

A 10 ml of triethylamine was added to 900 ml deionized water and the pH was adjusted to 2.5 with 85% phosphoric acid. The mixture was added with deionized water to a final volume of 930 ml, and 70 ml of acetonitrile (CH_3CN) was added in the last step. The final component of mobile phase consisted of 91.4% of deionized water : 1% of triethylamine : 0.6% of 85% phosphoric acid : 7% of acetonitrile (vol/vol/vol/vol). The mobile phase was filtered through 0.45 micropore filtered paper (Nyron 66), then degassed by ultrasonicator for 9 minutes before using. The mobile phase was freshy prepared daily. The flow rate was 1.5 ml/min. All analysis were performed at room temperature (about 24 $\pm$ 2 °C).

3.2 Stock Standard Solution

Stock standard solution of quinine and quinidine hydrochloride at a concentration of 100 μg/ml were prepared by dissolving 10 mg of standard quinine in 1 ml methanol and added with the mobile phase to a final volume of 10 ml in volumetric flask and covered it with foil to protect from light. Then, they were diluted to 100 μg/ml with the mobile phase for stock standard solutions and store at –20 °C.

Working standard solutions used to prepare a calibration curve were freshly prepared by appropriate dilution of the stock standard solution with blank plasma.

3.3 Calibration Curve

Calibration curve was prepared by adding a standard quinine and internal standard quinidine solution to blank human plasma so that final

concentrations in plasma was 0.5, 1, 2.5, 5 and 10 μg/ml. The calibration curve plotted using of quinine peak height ratio of quinine to quinidine and concentration was linear in the concentration range of 0.5 to 10 μg/ml (Figure 9). The lower detection limit for quinine was 0.2 μg/ml.

3.3.1 Precision and Variability

To determine intra-day precision and variability, the standard quinine was spiked in blank plasma at the concentration of 1, 2.5, 5 and 10 μg/ml, and internal standard quinidine at concentration of 25 μg/ml was spiked in each concentration of quinine in plasma and eight replicated of each were carried out on one day.

Inter-day precision and variability was done as intra-day but carried out on different ten days. Accuracy should be of ± 10% of spiked value and the CV% of each concentration should be less than 10%.

3.3.2 Recovery

Potential loss of quinine during the precipitation by acetonitrile was determined by comparing the peak height of quinine from plasma sample in the concentration range of 1 to 10 μg/ml and the equal concentration of standard quinine prepared in the mobile phase. The potential loss of internal standard was also determined by the same method. The percent recovery was calculated as the following formulae.

$$\% \text{ Recovery} = \frac{\text{peak height of quinine in plasma} \times 1.5}{\text{peak height of quinine in mobile phase}} \times 100$$

3.4 Sample preparation

Adding a 400 μl of quinine standard and 100 μl internal standard (25 μg/ml quinidine hydrochloride in mobile phase) into a propylene tube, then vortex mixing for 30 seconds. Add acetonitrile 250 μl to the mixture and vortex mixing for 30 seconds. After 10 minutes the tube was centrifuged at 14,000 rpm for 15 minutes. The supernatant (40 μl) were injected into the HPLC system by an automated injection.

4. Data Analysis

4.1 Pharmacokinetic Calculations

The following parameters were calculated by using Winnonlin® solfware program, 1995; the maximum plasma quinine concentration (C_{max}), the time to reach C_{max} (t_{max}), the absorption rate constant (Ka), the elimination rate constant (Ke), the elimination half-life ($t_{1/2}$), the area under the concentration time curve (AUC) and lag time.

The apparent oral clearance (CL/f) was calculated as dose/(AUC×body weight). The apparent volume of distribution (Vd/f) was calculated as CL/f divided by Ke.

4.2 Pharmacodynamic study

Automatic Digital Blood Pressure Monitor, Model HEM-703C (Omron Corporation, Tokyo, Japan), measured the systolic and diastolic blood pressure and pulse rate. The EKG was measured by polygraph and QT_c was calculated by measurement QT interval divided by square root of R-R interval.

4.3 Statistical Analysis

All results were expressed as means ± S.D. Differences in quinine pharmacokinetic and phamacodynamic parameters among control and treatment groups were tested by analysis of variance (ANOVA) *P* value less than 0.05 taken as the minimum levels of significant. Student's pair *t*-test was used to test for significant differences between means.

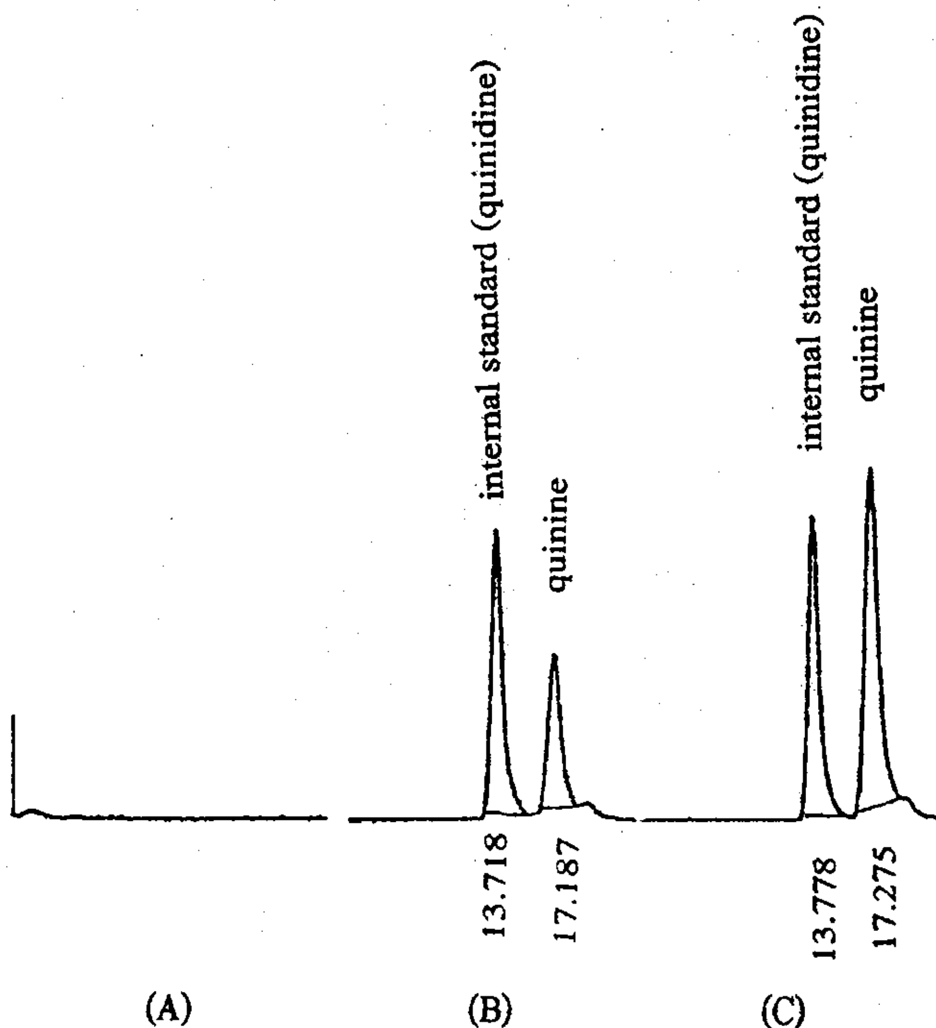

Figure 5 Representative chromatograms of 40 µl human plasma samples. Key: (A) blank human plasma; (B) and (C) spiked with quinine 5 and 10 µg/ml, respectively. The mobile phase consisted of deionized water-triethylamine-85%phosphoric acid-acetonitrile (91.4: 1: 0.6: 7 vol/vol/vol/vol) pH 2.5 at a flow rate of 1.5 ml/min. Chart speed and attenuation were 1 mm/min and 128 mVF.S, respectively.

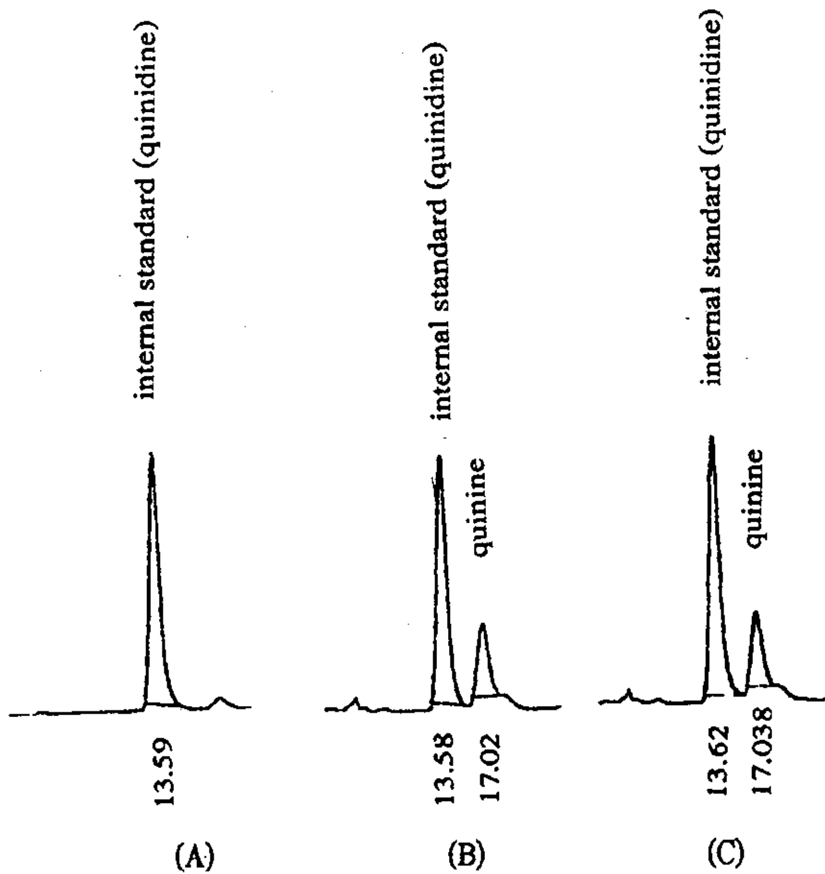

Figure 6 Representative chromatograms of 40 µl human plasma samples. Key: (A) spiked with internal standard 25 µg/ml quinidine hydrochloride; (B) and (C) plasma obtained from a subject receiving 300 mg quinine alone at 0.75 and 2.5 hours, respectively. The mobile phase consisted of deionized water - triethylamine - 85%phosphoric acid - acetonitrile (91.4: 1: 0.6: 7 vol/vol/vol/vol) pH 2.5 at a flow rate of 1.5 ml/min. Chart speed and attenuation were 1 mm/min and 128 mV F.S., respectively.

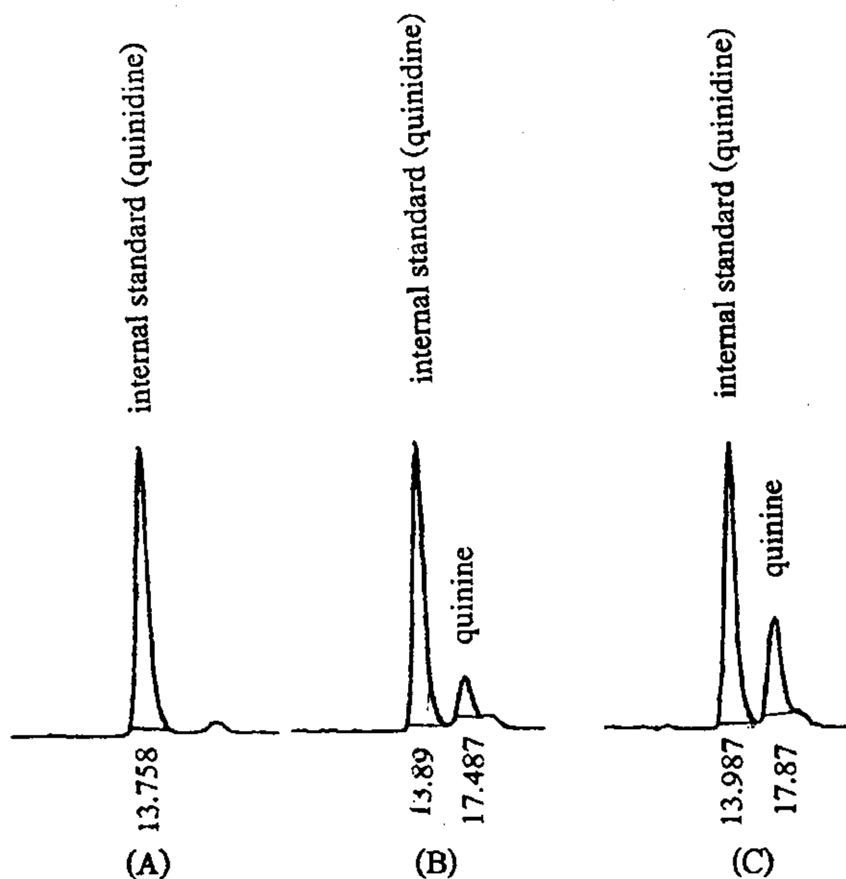

Figure 7 Representative chromatograms of 40 µl human plasma samples. Key: (A) spiked with internal standard 25 µg/ml quinidine hydrochloride; (B) and (C) plasma obtained from subjects after pretreatment with 400 mg ketoconazole for 4 days at 0.75 and 2.5 hours, respectively after an oral administration of 300 mg quinine sulphate. The mobile phase consisted of deionized water-triethylamine-85% phosphoric acid-acetonitrile (91.4: 1: 0.6: 7 vol/vol/vol/vol) pH 2.5 at a flow rate of 1.5 ml/min. Chart speed and attenuation was 1 mm/min and 128 mVF.S., respectively.

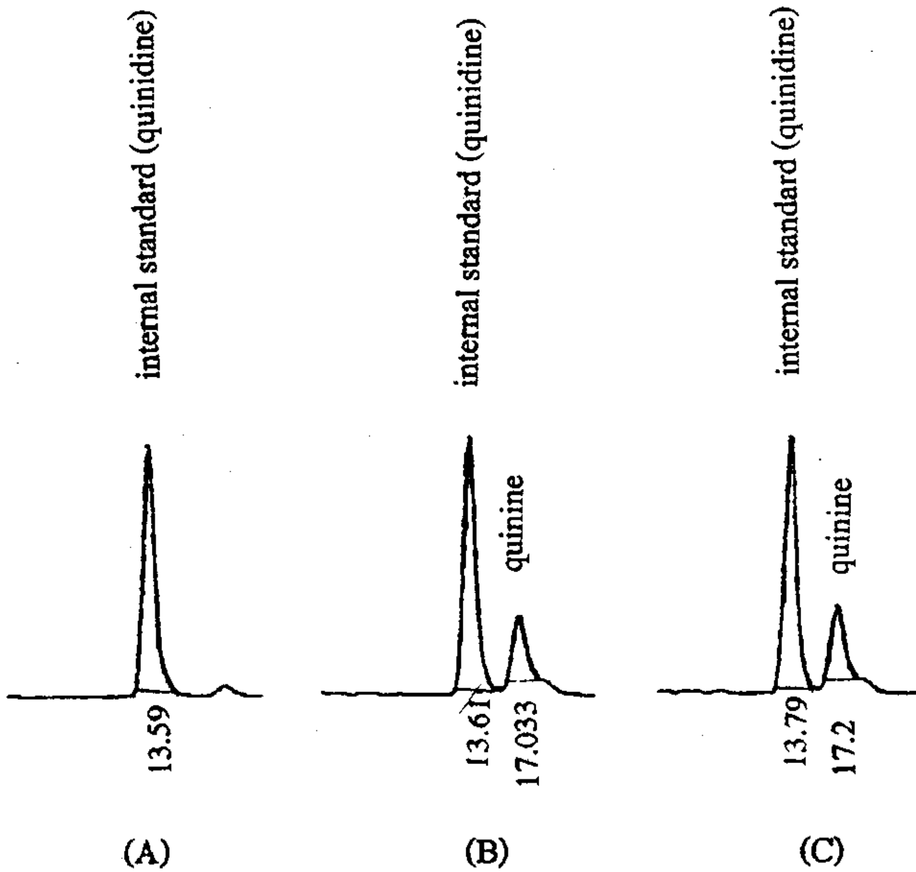

Figure 8 Representative chromatograms of 40 µl human plasma samples. Key: (A) spiked with internal standard 25 µg/ml quinidine hydrochloride; (B) and (C) plasma obtained from a subject after pretreatment with 200 mg itraconazole for 4 days at 0.75 and 2.5 hours, respectively after an oral administration of 300 mg quinine sulphate. The mobile phase consisted of deionized water-triethylamine-85% phosphoric acid-acetonitrile (91.4: 1: 0.6: 7 vol/vol/vol/vol) pH 2.5 at a flow rate of 1.5 ml/min. Chart speed and attenuation was 1 mm/min and 128 mVF.S, respectively.

Table 3 The intra-assay variance of four different quinine concentrations in human plasma

Concentration[a] (µg/ml)	Mean peak height ratio of quinine to quinidine ± S.D. (n = 8)	CV(%)[b]
1	0.13 ± 0.002	1.59
2.5	0.32 ± 0.011	3.48
5	0.66 ± 0.020	3.03
10	1.34 ± 0.014	1.05

[a]Various concentrations of standard quinine and internal standard quinidine 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 Inter-assay variance of four different quinine concentrations in human plasma

Concentration[a] (μg/ml)	Mean peak height ratio of quinine to quinidine ± S.D. (n = 10)	CV(%)[b]
1	0.15 ± 0.098	6.67
2.5	0.34 ± 0.018	5.31
5	0.66 ± 0.031	4.69
10	1.30 ± 0.051	3.92

[a]Various concentrations of standard quinine and internal standard quinidine 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 quinine in human plasma

Concentration (µg/ml)	Mean peak height in mobile phase[a] ± S.D. (n = 5)	Mean peak height in human plasma[b] ± S.D. (n = 5)	% Recovery[c]
1	5.8 ± 0.11	3.48 ± 0.109	89.38
2.5	13.4 ± 0.42	8.40 ± 0.96	94.03
5	26.1 ± 1.08	16.00 ± 0.35	91.95
10	51.8 ± 2.25	31.64 ± 1.01	91.62

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

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

[c]Mean peak height of quinine in plasma multiplied by 1.5 and divided by mean peak height of quinine in mobile phase, expressed in percent.