CHAPTER 3

MATERIALS AND METHODS

Chemicals and Reagents

The standard praziquantel (Lot No. 28H5003) and diazepam were purchased from Sigma Chemical Co (St. Louis, MO, USA). Praziquantel (Praquantel, 600 mg/tablet, Lot No. 000194) and rifampicin (Rifagen, 300 mg/capsule, Lot No. 99649) were purchased from Atlantic Laboratories Co. Ltd., Bangkok, Thailand, and General Drug House Co. Ltd., Bangkok, Thailand, respectively. An HPLC grade of acetonitrile and methanol were bought from J.T. Baker (Phillipsburg, NJ, USA). Zinc sulphate (pro analytical grade) was purchased from Carlo Erba, Italy. Water was purified for HPLC by the Milli Q Water purification system (Millipore, Milford, MA, USA).

Instrumentation

The HPLC system composed of Waters 515 pump and a Waters 717 plus autosampler (Waters Associates, Milford, MA, USA). The column was reverse-phase Spherisorb ODS 2 (5 μm, 250 x 4.6 mm i.d., Waters Associates, Milford, MA, USA). A Guard-pak precolumn module was used to obviate the effect of rapid column degeneration. The praziquantel was detected by the UV detector (SpectroMonitor Model 3100, Milton Roy, Riviera Beach, FL, USA) at 217 nm.

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 participated in this study. Ten healthy male volunteers, aged 20-37 years old (mean 28.9 ± 6.38 yr.), weighing 54-68 kilograms (mean 59.3 ± 4.8 kg.) participated in the study. Prior to the study, a medical history, physical examination, standard biochemical and hematological screening tests were performed in each subject. Neither 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

The protocol was divided into 2 studies. Study 1, effect of rifampicin on pharmacokinetic profiles of single oral doses of praziquantel (40 mg/kg) in healthy subjects, was composed of 2 phases; and study 2, effect of rifampicin on pharmacokinetic profiles of multiple oral doses of praziquantel (25 mg/kg × 3) in healthy subjects, was composed of 2 phases. Two phases of each study were randomized crossover study designed with two weeks washout period.

Study 1 Effect of rifampicin on pharmacokinetic profiles of a single oral dose of praziquantel (40 mg/kg) in healthy subjects.

Phase 1 : A single oral dose of praziquantel (40 mg/kg) alone

In the morning after an overnight fast, each subject received a single oral dose of 40 mg/kg praziquantel. The drug was administered with a glass of water (250 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 samples, and was maintained patent using 1 ml of dilute heparin solution (100 units/ml) after each sample collecting. Venous blood samples. (5 ml) were collected in heparinized tubes before drug administration and at 0.25, 0.5, 0.75, 1, 2, 3, 4, 6, 8, 10, 12 and 24 hours post drug administration. Samples were centrifuged not later than 30 minutes after collection, and the plasma was separated and stored at -70°C until analysis.

Phase 2 : Rifampicin and a single oral dose of praziquantel (40 mg/kg)

The subjects received rifampicin capsules at an oral dose of 600 mg (2 capsules of 300 mg rifampicin capsule) once daily after breakfast for 5 days prior to praziquantel administration. In day 6 (after rifampicin pretreatment for 5 days), after an overnight fast, all subjects ingested a 40 mg/kg praziquantel. Venous blood samples were collected at the time interval before and after praziquantel administration as previously done in phase 1.

Study 1 Effect of rifampicin on pharmacokinetic profiles of a single oral dose of praziquantel (40 mg/kg) in healthy subjects.

Phase 1 : A single oral dose of praziquantel (40 mg/kg) alone

Day

1 2 3 4 5 6

● P40

Blood collection for 24 hr

(at 0, 0.25, 0.5, 0.75, 1, 2, 3,

4, 6, 8, 10, 12 and 24 hr)

Phase 2 : Rifampicin and a single oral dose of praziquantel (40 mg/kg)

Day

1 2 3 4 5 6

▲ ▲ ▲ ▲ ▲ ● P40

R R R R R Blood collection for 24 hr

(at 0, 0.25, 0.5, 0.75, 1, 2, 3,

4, 6, 8, 10, 12 and 24 hr)

Remark : P40 = Praziquantel (40 mg/kg, orally)

R = Rifampicin (600 mg, orally once daily for 5 days)

Study 2 Effect of rifampicin on pharmacokinetic profiles of a multiple oral dose of praziquantel (25 mg/kg × 3) in healthy subjects.

Phase 1 : A multiple oral dose of praziquantel (25 mg/kg) alone

Each subjects received 2 doses of 25 mg/kg praziquantel in the same day at 12.00 AM before lunch and 6.00 PM before dinner. On the next day, after an overnight fast the last dose was administered at 7.00 AM. The drug was administered with a glass of water (250 ml) under supervision. No food was taken at least 2 hours after ingestion of the drug. Venous blood samples were collected at the time interval before and after praziquantel administration as previously done in study 1.

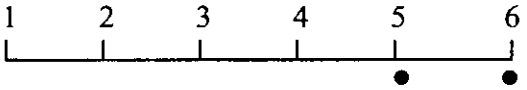
Phase 2 : Rifampicin and a multiple oral dose of praziquantel (25 mg/kg × 3)

The subjects received rifampicin at an oral dose of 600 mg (2 capsules of 300 mg rifampicin) once daily after breakfast for 5 days prior to praziquantel administration. On day 5, (after rifampicin pretreatment) the subjects received 2 doses of 25 mg/kg praziquantel at 12.00 AM before lunch and 6.00 PM before dinner. On day 6, after an overnight fast the last dose was administered at 7.00 AM. Venous blood samples were collected at the time interval before and after praziquantel administration as previously done in study 1.

Study 2 Effect of rifampicin on pharmacokinetic profiles of a multiple oral dose of praziquantel (25 mg/kg × 3) in healthy subjects.

Phase 1 : A multiple oral dose of praziquantel (25 mg/kg) alone

Day



P25 × 2 P25 × 1 (at 7:00 AM)

(at 12:00 AM

and 6:00 PM

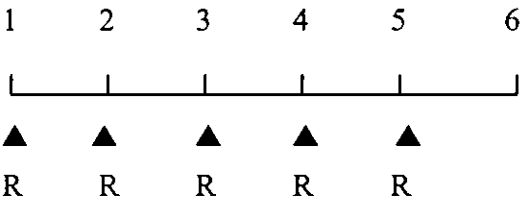
◆ Blood collection for 24 hr

(at 0, 0.25, 0.5, 0.75, 1, 2, 3,

4, 6, 8, 10, 12 and 24 hr)

Phase 2 : Rifampicin and a multiple oral dose of praziquantel (25 mg/kg × 3)

Day



P25 × 2 P25 × 1 (at 7:00 AM)

(at 12:00 AM

and 6:00 PM)

◆ Blood collection for 24 hr

(at 0, 0.25, 0.5, 0.75, 1, 2, 3,

4, 6, 8, 10, 12 and 24 hr)

Remark : P25 = Praziquantel (25 mg/kg, orally)

R = Rifampicin (600 mg, orally once daily for 5 days)

3. Analytical Methods

The plasma praziquantel concentrations were measured by a high performance liquid chromatography (HPLC) method (Ridtitid *et al.*, 2002).

3.1 Mobile Phase

The mobile phase consisted of a mixture of deionized water : acetonitrile : methanol (54 : 36 : 10 vol/vol/vol). The mobile phase was freshly prepared daily and was filtered through a 0.45 μm membrane filter (Nyron 66, Millipore, Milford, MA, USA) and degassed by ultrasonification (Tru-Sweep, ETL Testing Laboratories, Cortland, NY, USA) for 15 minutes before use. The flow rate was 1.5 ml/min. All analyses were performed at room temperature.

3.2 Stock Standard Solution

The stock standard solution at a concentration of 40 μg/ml was prepared by dissolving 2 mg of standard praziquantel in deionized water and adjusted to 50 ml in a 50 ml volumetric flask and covered it with foil to protect from light. Then they were diluted to 4 μg/ml with the deionized water for stock standard solutions and stored at 4 °C.

Working standard solutions used to prepare a calibration curve were freshly prepared by appropriate dilution of the stock standard solution with blank plasma (Appendix-1).

3.3 Stock Internal Standard Solution

A stock diazepam solution at a concentration of 250 μg/ml was prepared by dissolving 2.5 mg of standard diazepam in mobile phase and adjusted to 10 ml in a 10 ml volumetric flask. Then they were diluted to 25 μg/ml with the deionized water for stock standard solutions and stored at

4 °C. The stock diazepam solution was diluted to a concentration of 840 ng/ml for working solutions. (Appendix-1).

3.4 Calibration Curve

Calibration curve was prepared by adding a standard praziquantel to blank human plasma so that the final concentrations in plasma were 100, 200, 400, 800, 1600 and 2000 ng/ml. The calibration curve for praziquantel was linear in the range of 100 to 2000 ng/ml. The average coefficient of variations (CV) should be less than 10%. The lower detection limit for praziquantel was 12.25 ng/ml.

3.4.1 Recovery

Potential loss of praziquantel during the precipitation by zinc sulphate and acetonitrile was determined by comparing the peak height ratio of praziquantel to diazepam from plasma samples in the concentration range of 100-2000 ng/ml and the equal concentration of standard praziquantel prepared in mobile phase. The percent recovery was calculated as follow:

$$\% \text{ recovery} = \frac{\text{peak height ratio of praziquantel to diazepam in plasma}}{\text{peak height ratio of praziquantel to diazepam in mobile phase}} \times 100$$

3.4.2 Precision and variability

To determine intra-day precision and variability, the standard praziquantel was spiked in blank plasma at 100, 200, 400, 800, 1600 and 2000 ng/ml, and internal standard diazepam at concentration of 60 ng/ml was spike in each concentration of praziquantel in plasma and 5 replicates of each were carried out on one day. All should be of ±10% of spiked value and the

coefficient of variation (CV) of each concentration should be less than 10%.

To determine inter-day precision and variability, the standard praziquantel was spiked in blank plasma at 100, 200, 400, 800, 1600 and 2000 ng/ml, and internal standard diazepam at concentration of 60 ng/ml was spiked in each concentration of praziquantel in plasma and each concentration was carried out on 10 different day. Accuracy should be of ±10% spiked value and the coefficient of variation (CV) of each concentration should be less than 10%. The validity of the bioanalytical results was monitored by analysing quality control (QC) samples at 3 concentration levels (low, medium, high) together with the known study samples.

3.5 Sample preparation

A 200 µl of plasma sample or spiked standard plasma in a 2-ml stoppered microcentrifuge tube was added with a 50 µl of diazepam internal standard solution (840 ng/ml) and 50 µl of .02 M zinc sulphate solution drop-wise, and mixed for 30 seconds on a vortex mixer (Vortex Genie-2, Scientific Industries, Bohemia, NY, USA). A 400 µl of acetonitrile was then added drop-wise and shaken thoroughly on a vortex mixer for 30 seconds. The final concentration of diazepam internal standard in the mixer used in this study was 60 ng/ml. After 15 minutes the mixture was centrifuged at 14,000 rpm for 10 minutes, A 100 µl of supernatant was injected into HPLC system. (Appendix-2).

4. Data Analysis

4.1 Pharmacokineic Calculations

The pharmacokinetic parameters were analyzed by noncompartment

methods (Gibaldi, 1991), using WinNonlin software (Scientific Consulting, Inc.). The area under the concentration-time curve from time zero to the end time of the collection interval (AUC_{0-12}), the area under the concentration-time curve was extrapolated to infinity (AUC_{∞}), the elimination rate constants (λ_z), the terminal disposition half-life ($t_{1/2,z}$) and the mean residence time (MRT).

Maximum plasma concentration (C_{max}) and time to reach C_{max} (T_{max}) were read directly from the plasma concentration-time profile. The apparent oral clearance (Cl/f) was calculated as Dose/AUC_{∞}×body weight. The apparent volume of distribution (V_z/f) was calculated as Cl/f divided by λ_z. In subjects whose praziquantel plasma concentrations could not be detected, the mean C_{max} of praziquantel was estimated under the condition that the individual C_{max} value was assumed to be equal to the lower detection limit of praziquantel (12.25 ng/ml), whereas the individual AUC_{0-12} of praziquantel was determined by the trapezoidal rule using the lower detection limit of praziquantel (12.25 ng/ml) for calculation.

4.2 Statistical Analysis

All results were expressed as mean ± SD. Difference in pharmacokinetic parameters of praziquantel among control and treatment groups were tested for statistical significance by Student's paired *t*-test with *P* value less than 0.05 taken as the minimum levels of significance.

Table 2 The intra-day assay coefficient variation (CV) of six different praziquantel concentrations in mobile phase.

Concentration[a] (ng/ml)	Peak height ratio of praziquantel to diazepam (Mean ± SD, n = 5)	CV (%)[b]
100	0.068 ± 0.001	1.91
200	0.139 ± 0.006	4.18
400	0.264 ± 0.003	1.02
800	0.488 ± 0.030	6.15
1,600	1.102 ± 0.014	1.29
2,000	1.375 ± 0.026	1.92

[a]Various concentrations of standard praziquantel in mobile phase were directly injected into HPLC system

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

Table 3 The inter-day assay coefficient variation (CV) of six different praziquantel concentrations in mobile phase.

Concentration[a] (ng/ml)	Peak height ratio of praziquantel to diazepam (Mean ± SD, n = 10)	CV (%)[b]
100	0.071 ± 0.005	7.05
200	0.145 ± 0.011	7.61
400	0.285 ± 0.023	8.08
800	0.598 ± 0.033	5.52
1,600	1.281 ± 0.088	6.87
2,000	1.622 ± 0.103	6.35

[a]Various concentrations of standard praziquantel in mobile phase were directly injected into HPLC system

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

Table 4 The intra-day assay coefficient variation (CV) of six different praziquantel concentrations in human plasma.

Concentration[a] (ng/ml)	Peak height ratio of praziquantel to diazepam (Mean ± SD, n = 5)	CV (%)[b]
100	0.087 ± 0.003	3.81
200	0.134 ± 0.008	6.12
400	0.288 ± 0.006	2.18
800	0.587 ± 0.012	2.04
1,600	1.141 ± 0.017	1.51
2,000	1.465 ± 0.038	2.59

[a]Various concentrations of standard praziquantel and internal standard diazepam 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 The inter-day assay coefficient variation (CV) of six different praziquantel concentrations in human plasma.

Concentration[a] (ng/ml)	Peak height ratio of praziquantel to diazepam (Mean ± SD, n = 10)	CV (%)[b]
100	0.078 ± 0.004	4.87
200	0.149 ± 0.012	8.07
400	0.298 ± 0.016	5.38
800	0.618 ± 0.025	4.04
1,600	1.235 ± 0.109	8.82
2,000	1.540 ± 0.105	6.82

[a]Various concentrations of standard praziquantel and internal standard diazepam 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 6 Relative recovery of standard praziquantel in plasma.

Concentration (ng/ml)	Peak height in mobile phase[a] (Mean ± SD, n=10)	Peak height in Plasma[b] (Mean ± SD, n=10)	% Recovery[c]
100	0.071 ± 0.005	0.078 ± 0.004	110.0
200	0.145 ± 0.011	0.149 ± 0.012	102.9
400	0.285 ± 0.023	0.298 ± 0.016	104.6
800	0.598 ± 0.033	0.618 ± 0.025	103.4
1,600	1.281 ± 0.088	1.235 ± 0.109	96.4
2,000	1.622 ± 0.103	1.540 ± 0.105	95.0

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

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

[c]Mean peak height of praziquantel to diazepam in plasma divided by mean peak height of praziquantel to diazepam in mobile phase, expressed in percent.