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), Ketoconazole (Ketazol, 200 mg/tablet, Lot No. 1A918/31) and Itraconazole (Sporal, 100 mg/capsule, Lot No. 1A546/33) were purchased from Atlantic Laboratories Co. Ltd., Shiwa Chemical Co. Ltd. and Janssen-Cilag Ltd. Bangkok, Thailand, respectively. The HPLC grade of acetonitrile and methanol were purchased 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 consist 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 Waters 2487 Dual λ absorbance detector (Waters Associates, Milford, MA, USA) at 217 nm.

Methods

1. Subjects

The volunteers signed informed consents after detailed explanation of the purpose, protocol, and risk of the study. Ten healthy male volunteers results were expressed as mean ± SD., aged 22-39 years old (mean aged 30.9 ± 1.8 yrs.), weighing 55-70 kilograms (mean weight 61.4 ± 1.6 kgs.) participated in the study. Prior to the study, a medical history, physical examination, standard biochemical and hematological screening tests (CBC, FBS, BUN, creatinine, SGOT, SGPT, direct bilirubin and albumin/globulin) were done in each volunteer. None of the volunteers was a smoker or used continuous medications. Drinking of alcoholic beverages, coffee and tea were not allowed at least 2 weeks prior to and during the entire period of study.

2. Protocol

The protocol has been approved by the Human Ethics Committee, Faculty of Science, Prince of Songkla University, Hat Yai, Thailand

The protocol was an open and conventional randomized crossover study design with 3 phases. The volunteers were random sampling without replacement in each phase. The interval between each phase was 2 weeks.

Phase 1 : A single oral dose of praziquantel (20 mg/kg) alone

In the morning after an overnight fasting, each volunteer received a single oral dose of 20 mg/kg praziquantel. The medication was with a glass of water (200 ml). A standard meal and water were served 2 hours after praziquantel administration and a standard lunch was served 4 hours after drug ingestion.

A catheter was inserted into a forearm vein for the collection of blood samples. Venous blood samples (5 ml) were collected in heparinized tubes before drug administration and at 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 10, 12 and 24 hours post drug administration. Samples were centrifuged at 2,500 rpm for 15 min not later than 30

minutes after collection, and the plasma was separated and stored at –70 °C until analysis.

Phase 2 : Ketoconazole and a single oral dose of praziquantel (20 mg/kg)

After 2 weeks of being drug free, each volunteer ingested ketoconazole at the dose of 400 mg once daily after breakfast (at 7.00 AM.) for 5 days prior to praziquantel administration. On day 6 (after pretreatment with ketoconazole for 5 days), after an overnight fasting, each volunteer took 20 mg/kg praziquantel. The medication was with a glass of water (200 ml). A standard meal and water were served 2 hours after praziquantel administration, and a standard lunch was served 4 hours after drug administration. Venous blood samples were collected at the time interval before and after praziquantel administration as previously done in phase 1.

Phase 3 : Itraconazole and a single oral dose of praziquantel (20 mg/kg)

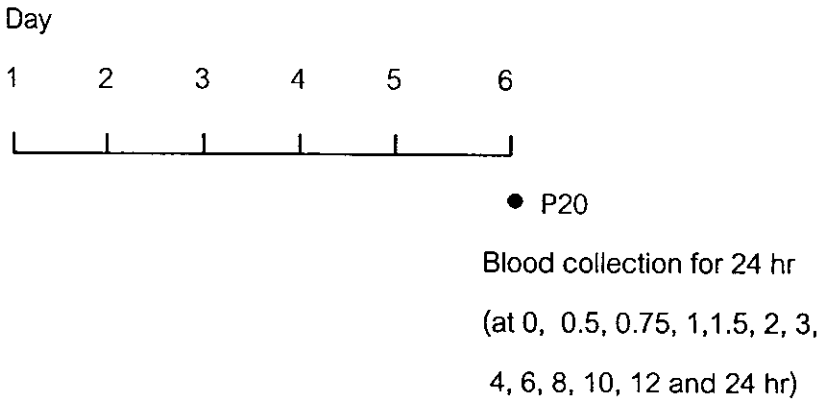
This phase was done in the same protocol as in phase 2 except that each volunteer received an oral dose of 200 mg itraconazole once daily instead of ketoconazole after breakfast (at 7.00 AM.) for 5 days prior to praziquantel administration.

Table 1 Illustrates randomized crossover study design with 3 phases

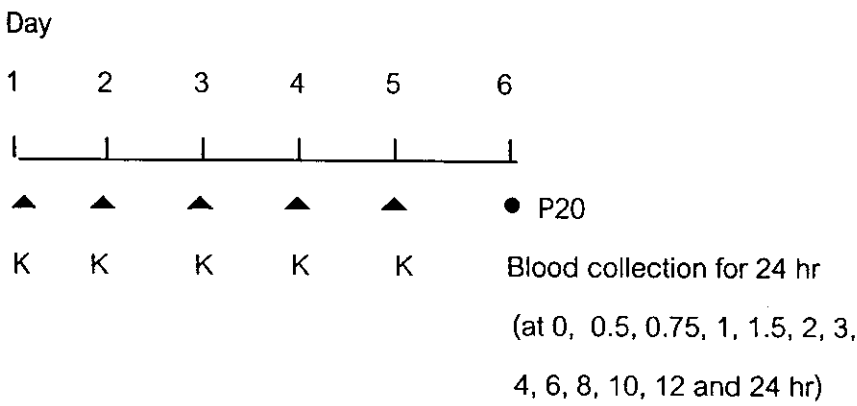
Sequence	Phase		
	1	2	3
1	S1	S7	S9
2	S6	S5	S4
3	S10	S8	S2
4	S3	S4	S3
5	S8	S2	S5
6	S9	S10	S7
7	S7	S6	S1
8	S5	S9	S10
9	S2	S3	S8
10	S4	S1	S6

Schematic plan : Effects of ketoconazole and itraconazole on pharmacokinetic profiles of a single oral dose of praziquantel (20 mg/kg) in healthy subjects.

Phase 1 : A single oral dose of praziquantel (20 mg/kg) alone.



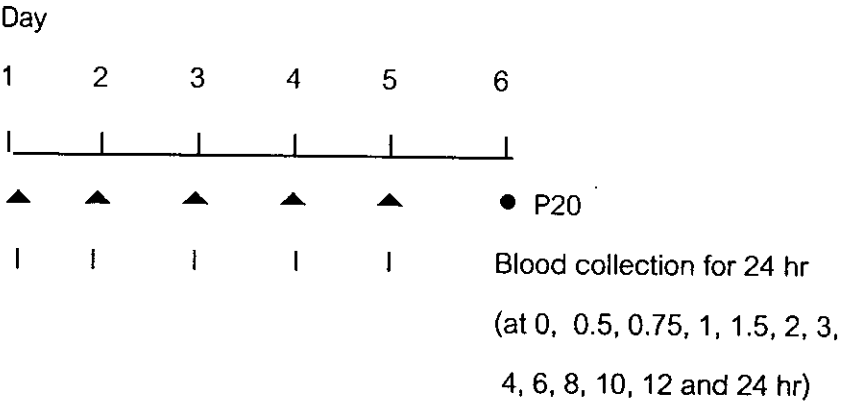
Phase 2 : Ketoconazole and a single oral dose of praziquantel (20 mg/kg).



Remark : P20 = Praziquantel (20 mg/kg, orally)

K = Ketoconazole (400 mg, orally once daily for 5 days)

Phase 3 : Itraconazole and a single oral dose of praziquantel (20 mg/kg).



Remark : P20 = Praziquantel (20 mg/kg, orally)

I = Itraconazole (200 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 (56 : 34 : 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 using. The flow rate was 1.5 ml/min. All analyses were performed at room temperature (about $24 \pm 2^{\circ}$C).

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 was covered with foil to protect from light. Then it was diluted to 4 μg/ml with 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 25 ml in a 25 ml volumetric flask. Then it was 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 2700 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 12.25, 100, 200, 400 and 800 ng/ml. The calibration curve for praziquantel was linear in the range of

12.25 to 800 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 of praziquantel precipitated from plasma samples in the range of 12.25-800 ng/ml and the equal concentration of standard praziquantel prepared in mobile phase. The percent recovery was calculated as following formula.

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

3.4.2 Precision and variability

To determine intra-day precision and variability, the standard praziquantel was spiked in blank plasma at 12.25, 100, 200, 400 and 800 ng/ml, and internal standard diazepam at the final concentration of 150 ng/ml was spike in each concentration of praziquantel in plasma and 5 replicates of each were carried out in one day. Accuracy should be $\pm$ 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 praziquantel was spiked in blank plasma at 12.25, 100, 200, 400 and 800 ng/ml, and internal standard diazepam at the final concentration of 150 ng/ml was spiked in each concentration of praziquantel in plasma and each concentration was carried out on different ten days. Accuracy should be $\pm$ 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.

$$\%CV = \frac{\text{standard deviation (SD) of peak height ratio of praziquantel to diazepam} \times 100}{\text{Mean of peak height ratio of praziquantel to diazepam}}$$

3.5 Sample preparation

A 400 μl of plasma sample or spiked standard plasma in a 1.5 ml stoppered microcentrifuge tube was added with a 50 μl of diazepam internal standard solution (2700 ng/ml) and 50 μl of 0.4 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 150 ng/ml. After 15 minutes the mixture was centrifuged at 14,000 rpm for 10 minutes, A 200 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 and log-linear trapezoidal method (Gibaldi, 1991), using WinNonlin software version 1.1 (Scientific Consulting, Inc.). The pharmacokinetic parameters included the area under the concentration-time curve from time zero to the end time of the collection interval (AUC_{0-24}), the area under the concentration-time curve extrapolated to infinity ($AUC_{0-\infty}$), the elimination rate constants (λ_z), the terminal disposition half-life ($t_{1/2,z}$) and the mean residence time (MRT). The MRT is an estimate of the average time a drug molecule resides in the body and encompasses absorption, distribution and elimination processes.

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/($AUC_{0-\infty} \times$ body weight). The apparent volume of distribution ($V_{z/f}$) was calculated as Cl/f divided by λ_z.

4.2 Statistical Analysis

All results were expressed as mean $\pm$ SD. Differences 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 five different praziquantel concentrations in mobile phase.

Concentration[a] (ng/ml)	Peak height ratio of praziquantel to diazepam (Mean ± SD, n = 5)	CV (%)[b]
12.25	0.033 ± 0.002	6.061
100	0.266 ± 0.003	1.128
200	0.534 ± 0.008	1.498
400	1.155 ± 0.024	2.078
800	2.333 ± 0.010	0.429

[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 five different praziquantel concentrations in mobile phase.

Concentration[a] (ng/ml)	Peak height ratio of praziquantel to diazepam (Mean ± SD, n = 10)	CV (%)[b]
12.25	0.041 ± 0.004	9.756
100	0.258 ± 0.009	3.488
200	0.557 ± 0.030	5.386
400	1.116 ± 0.045	4.032
800	2.191 ± 0.067	3.058

[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 five different praziquantel concentrations in human plasma.

Concentration[a] (ng/ml)	Peak height ratio of praziquantel to diazepam (Mean ± SD, n = 5)	CV (%)[b]
12.25	0.040 ± 0.003	7.500
100	0.282 ± 0.004	1.418
200	0.539 ± 0.005	0.928
400	1.166 ± 0.028	2.401
800	2.257 ± 0.038	1.684

[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 five different praziquantel concentrations in human plasma.

Concentration[a] (ng/ml)	Peak height ratio of praziquantel to diazepam (Mean ± SD, n = 10)	CV (%)[b]
12.25	0.039 ± 0.004	10.256
100	0.267 ± 0.016	5.992
200	0.542 ± 0.019	3.506
400	1.075 ± 0.036	3.349
800	2.137 ± 0.084	3.931

[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 ratio in mobile phase[a] (Mean ± SD, n=10)	Peak height ratio in Plasma[b] (Mean ± SD, n=10)	% Recovery[c]
12.25	0.041 ± 0.004	0.039 ± 0.004	95.122
100	0.258 ± 0.009	0.267 ± 0.016	103.488
200	0.557 ± 0.030	0.542 ± 0.019	97.307
400	1.116 ± 0.045	1.075 ± 0.036	96.326
800	2.191 ± 0.067	2.137 ± 0.084	97.535

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