CHAPTER 3

MATERIALS AND METHODS

Chemicals and Reagents

The standard DEC (Lot No. 35F-0262) was purchased from the Sigma® chemical company, USA. The quinidine sulfate was purchased from the Nutritional Biochemicals Corp. DEC tablets (Lot No. 10303123) were kindly given from the Insect Prevention Center, Songkhla, Thailand. Rifampicin capsule (Lot No. K470138) and ketoconazole tablets (Lot No. A 18288.58.) were purchased from Songklanakarind Hospital. The HPLC grade of methanol and dichloromethane were purchased from J.T. Baker (Phillipsburg, NJ, USA.). Potassium dihydrogen phosphate (KH_2PO_4) and disodium hydrogen phosphate (Na_2HPO_4) were purchased from Merck Darmstadt, Germany. Water was purified for HPLC by the Milli Q Water Purification System (Milipore, Milford, MA, USA.).

Methods

1. Subjects

All volunteers were given the detailed explanation of the purpose, protocol, and risk of the study, and each was given a written consent which was approved by the Ethics Committee of the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai, Thailand (Appendix E). Twelve Thai male healthy volunteers, age 22-40 years old (mean age $\pm$ SD. 28.5 $\pm$ 5.78 years) and weighed 51-70 kilograms (mean weight $\pm$ SD. 60.46 $\pm$ 5.37 kilograms) participated in the study. Prior to the study, the medical history, physical examination, standard biochemical and hematological screening tests (CBC, FBS, BUN, serum creatinine, SGOT, SGPT, direct bilirubin, total protein and albumin) were done in each volunteer. The volunteers were not allowed to take any medications, to smoke cigarettes, or to ingest alcoholic, caffeine-containing beverages for one month before starting the study and during the study period.

2. Study protocols

The study was an open-labeled, randomized, three-phase crossover design with a one month separated period.

In this study was separated into 3 periods. The twelve healthy subjects were divided into 3 groups (4 subjects of each group). The first period, all subjects in 3 groups were experimented as in phase 1, phase 2, and phase 3 at the same time. The second and third period, all subjects in 3 groups were crossed until complete.

2.1 Phase 1

In the morning after an overnight fasting, each subject received a single oral dose of 6 mg/kg DEC (50 mg/tablet). The drug was administered with a glass of water (200 ml) under supervision. No food was taken at least 2 h after ingestion of the drug.

A catheter was inserted into a forearm vein for the collection of blood sample, and was maintained patent using 50 μl of a heparin solution (5,000 unit/ml) after each blood sample. Venous blood samples (5 ml) were collected in heparinized tubes before drug administration and at 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 24, 36 and 48 h post drug administration. Samples were centrifuged not later than 30 minutes after collection, and the plasma was separated and stored at -60 $^{\circ}$C until analysis. Urine samples were collected in container before drug administration and at 1, 4, 12, 24, 36 and 48 h post drug administration for measuring urine pH.

2.2 Phase 2

After 1 month of being free from any medication, all subjects received a 600 mg rifampicin orally (2 capsules of 300 mg rifampicin capsule) once daily before breakfast for 5 days prior to DEC administration. In the morning of day 6 (after rifampicin pretreatment for 5 days), after an overnight fasting, each volunteer took 6 mg/kg DEC orally. Venous blood samples and urine samples were collected at the time interval before and after DEC administration as previously done in phase 1.

2.3 Phase 3

After 1 month of being free from any medication, all subjects received a 400 mg ketoconazole orally once daily before breakfast for 5 days prior to DEC administration. In the morning of day 6 (after ketoconazole pretreatment for 5 days), after an overnight fasting, each volunteer took 6 mg/kg DEC orally. Venous blood samples and urine samples were collected at the time interval before and after DEC administration as previously done in phase 2.

Schematic plan of the study: Effects of rifampicin and ketoconazole on pharmacokinetic profiles of a single oral dose of DEC (6 mg/kg) in healthy subjects.

Phase 1 : A single oral dose of DEC alone

Day
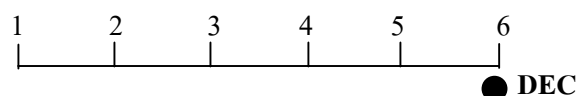
```
1       2       3       4       5       6
|_____|_____|_____|_____|_____|
                                        ● DEC
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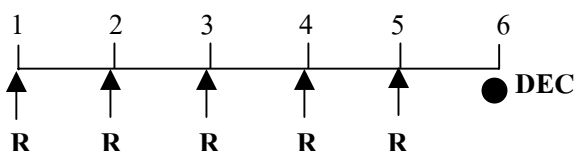
Blood collection for 48 h

(0, 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 24, 36 and 48 h)

Urine collection for 48 h (0, 1, 4, 12, 24, 36 and 48 h)

Phase 2 : Rifampicin (R) and a single oral dose of DEC

Day

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1       2       3       4       5       6
|_____|_____|_____|_____|_____|
↑       ↑       ↑       ↑       ↑        ● DEC
R       R       R       R       R
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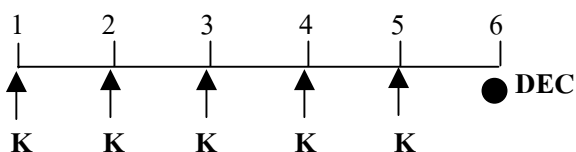
Blood collection for 48 h

(0, 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 24, 36 and 48 h)

Urine collection for 48 h (0, 1, 4, 12, 24, 36 and 48 h)

Phase 3 : Ketoconazole (K) and a single oral dose of DEC

Day

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1       2       3       4       5       6
|_____|_____|_____|_____|_____|
↑       ↑       ↑       ↑       ↑        ● DEC
K       K       K       K       K
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Blood collection for 48 h

(0, 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 24, 36 and 48 h)

Urine collection for 48 h (0, 1, 4, 12, 24, 36 and 48 h)

Remarks: **DEC = Diethylcarbamazine (6 mg/kg, orally)**

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

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

3. Analytical procedures

The plasma DEC was measured by the HPLC method (Bolla *et al*., 2002).

3.1 Instrumentation

The HPLC system consisted of Waters 2695 pump and autosampler (Waters Associates, Milford, MA, USA) and a Water 474 electrochemical detector. Detection was done with the potential stat +1150 mV and I-range set at 100 nA. The column was reverse-phase Nova-Pak 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.

3.2 Mobile phase

The mobile phase consisted of methanol: 0.2 M phosphate buffer pH 6.5 (55:45 vol/vol). The mobile phase was freshly prepared daily and filtered through 0.45 micrometer filtered paper (Nylon 66), then degassed in the ultrasonic bath for 10 minutes before using. The flow rate was 0.5 ml/min. All analyses were performed at room temperature (25 ± 1 ºC).

3.3 Stock Standard and Internal Standard Solution

The stock standard solution and internal standard solution at a concentration of 1 mg/ml was prepared by dissolving 10 mg of standard DEC or quinidine sulfate in methanol. The solutions were adjusted to 10 ml in 10 ml volumetric flasks. The stock solutions were stable for at least 3 months at -20 ºC (Miller *et al*., 2001). Standard and internal standard solutions used to prepare a standard curve, day by day, were prepared by appropriate dilution of the stock standard solution with blank plasma.

3.4 Calibration Curves

Calibration curves were prepared by adding standard DEC solutions to blank human plasma so that the final concentrations in plasma were 25, 50, 100, 250, 500, 1000 and 2000 ng/ml. The calibration curves for DEC was linear in the range of 25-2000 ng/ml. The lower limit of detection (LOD) and lower limit of quantification (LOQ) for DEC were approximately 10 and 25 ng/ml, respectively. LOD was considered by a signal-to-noise ratio of 3:1 and LOQ was considered by a signal-to-noise ratio of 10:1 (Swartz and Krull, 1997).

3.5 Sample preparation

A 500 µl of plasma in screw-capped test tube, were added with 100 µl of 10 µg/ml quinidine sulfate dissolved in methanol, 200 µl of 2 M NaOH, and 5 ml dichloromethane. The mixture was placed on a mechanical shaker for 15 minutes and then centrifuged for 20 minutes. The lower organic layer was transferred to another screw-capped test tube and was evaporated to dryness. The residue was reconstituted in 100 µl of methanol by vortex mixing for 15 seconds. 20 µl was injected into the HPLC system for analysis.

4. Method validation

4.1 Recovery

To determine the recovery of DEC at three different concentrations (100, 500 and 2000 ng/ml), the samples (five replicates each) were extracted and analyzed. Recovery was calculated by comparing analyte concentrations observed in spiked plasma samples with those obtained from the analysis of unextracted methanol standards.

$$\frac{\text{Peak area ratio of standard DEC extraction}}{\text{Peak area ratio of standard DEC unextraction}} \text{ X } 100$$

4.2 Precision and Accuracy

To determine intra-day precision and accuracy, the standard DEC was spiked in blank plasma at 100, 500 and 2000 ng/ml concentrations and five replications of each were carried out in one day.

To determine inter-day precision, the standard DEC was spiked in blank plasma at 100, 500 and 2000 ng/ml concentrations and five replications of each were carried out in ten days.

The percent of coefficient of variation (%CV) of each concentration should be less than 15% (FDA Thailand, 2004).

$$\text{Coefficient variation (\%CV)} = \frac{\text{Standard deviation (SD)}}{\text{Mean value}} \text{ X } 100$$

$$\text{Accuracy (\%)} = \frac{\text{Concentration of DEC found in sample}}{\text{Nominal concentration of DEC spiked samples}} \text{ X } 100$$

5. Data Analysis

5.1 Pharmacokinetic Calculations

The pharmacokinetic parameters were analyzed by one-compartment model, with the use of WinNonlin version 3.1 (Pharsight, Mountain View, CA). The plasma DEC concentration-time curve (semilogarithmic curve) fitted with one-compartment model. The area under the concentration-time curve from time zero to the end time of the collection interval (AUC_{0-48}), the area under the concentration-time curve extrapolated to infinity ($AUC_{0-\infty}$), the absorption rate constants (k_a), the elimination rate constants (k_e), the terminal disposition half-life ($t_{1/2}$), maximum plasma concentration (C_{max}), time to reach C_{max} (t_{max}), the apparent oral clearance (Cl/F), the apparent volume of distribution (V_d/F) were obtained.

5.2 Statistical Analysis

All pharmacokinetic parameters were expressed as mean ± SD. The differences in pharmacokinetic parameters among these groups were tested by using two-way ANOVA, with significance level of 0.05. The effects of sequence, period, and subjects were determined. The (treatment*sequence) interaction was not affect the treatment.