CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals and reagents

The standard Risperidone : 3-{2-[4-(6-fluoro-1,2-benzisoxazol-3-yl)-1-piperidinyl]ethyl}-6, 7, 8, 9-tetrahydro-2-methyl-4H-pyrido[1,2-a] pyrimidin-4-one was purchased from the Janssen Research Foundation (Beerse, Belgium). The internal standard, Clozapine (Clopaze® 100 mg/tablet, Lot No.1A25/42) was purchased from the Pharminar Co., Ltd.,Bangkok,Thailand. The HPLC grade of acetronitrile and methanol were purchased from J.T. Baker (Phillipsburg, NJ, USA). Potassium phosphate monobasic was obtained from Carlo Erba (Milan, Italy), triethylamine and sodium hydroxide from Sigma–Aldrich (Milan, Italy), diisopropyl ether, isoamylalcohol, from J.T. Baker (Deventer, The Netherlands). Water was deionized and purified by using a Milli-Q system (Millipore, Milford, MA, USA).

3.2 Instrumentation

The HPLC system consists of Waters 2695 pump, autosampler (Water Associates, Milford, MA, USA) and a Waters 2487 UV detector. Detection was done with the variable-wavelength UV detector set at 278 nm. The column was reverse-phase Symmetry C_{18} (4.6 mm x 250 mm HPLC column, particle size 5 μm, Waters Associates, Milford, MA, USA). A guard-pak precolumn module was used to obviate the effect of rapid column degeneration.

3.3 Methods

3.3.1 Subjects

The volunteers were given a detailed explanation of the purpose, protocol, and risk of the study, and each volunteer was given a written inform consent that was approved by the Ethics Committee, Faculty of Medicine, Prince of Songkla University, Hat Yai, Thailand. Ten healthy Thai male volunteers, age 20-45 years old and body mass index 18-24 kg/m^2 were

enrolled in this study. All were in good health on the basis of medical history and physical examination. Routine blood test including CBC with differential, white blood cell count, BUN, creatinine, SGOT, SGPT, direct bilirubin and albumin/globulin were screened to exclude subject with abnormal hematological, liver, or kidney functions. None of the volunteers was a smoker or used any medications continuously. Subject with known contraindication or hypersensitivity to antipsychotic agent was excluded as well as those with known history of alcoholism or drug abuse. Drinking of alcoholic beverages, coffee and tea were not allowed at least 1 month prior to and during the entire period of study.

3.3.2 Protocol

The study was an open-labeled, randomized two-phases cross over designed with a 2 weeks washout period.

Phase 1: A single oral dose of risperidone (4 mg) alone.

In the morning after an overnight fasting, each subject received a single oral dose of 4 mg risperidone (2 tablets of 2 mg Risperdal®). 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 samples (5 ml) were collected in heparinized tubes before drug administration and at 10, 20, 30, 45 min, 1, 1.5, 2, 4, 6, 8, 12, 24 and 48 hours post drug administration. Samples were centrifuged not later than 30 minutes after collection, and the plasma was separated and stored at $-70\ ^{0}C$ until analysis.

Phase 2: Rifampin and a single oral dose of risperidone (4 mg) .

After 2 weeks of being free from the drug, the subjects recived rifampin capsules at an oral dose of 600 mg (2 capsules of 300 mg rifampin capsules) once daily after breakfast for 5 days prior to single oral dose of risperidone administration. In day 6 (after rifampin pretreatment for 5 days), after an overnight fasting, all subjects took 4 mg risperidone orally. Venous blood samples were collected at the time interval before and after risperidone administration as previously done in phase 1.

3.3.3 Sample analysis

The plasma risperidone concentrations were measured by a high performance liquid chromatographic (HPLC) method (applied form Avenoso *et al*., 2000).

Mobile phase

The mobile phase consisted of 0.05 M potassium dihydrogenphosphate : acetonitrile (68:32 vol/vol) and adjusted to pH 3.80 with 25% phosphoric acid. The mobile phase was freshly prepared daily and filtered through 0.45 micropore filter (Nylon 66), then degased by sonification for 10 minutes before using. The flow rate was 1 ml/min. All analyses were performed at room temperature (25 ± 1^0C).

Stock standard solution

A stock standard solution at a concentration of 1 μg/ml was prepared by dissolving 1 mg of standard risperidone in 0.1 N HCl. The solution was adjusted to 10 ml in a 10 ml volumetric flask. The stock solution was stable for at least 4 months at -20^0C (Avenoso *et al*., 2000). Working standard solution used to prepare a calibration curve day by day were prepared by appropriate dilution of the stock standard solution with blank plasma.

Calibration curves

Calibration curves were prepared by adding a standard risperidone solution to blank human plasma so that the final concentrations in plasma were 2, 5, 10, 20, 50 and 100 ng/ml. The calibration curves for risperidone were linear in the range of 2 - 100 ng/ml. The lower limit of quantitation (LOQ), expressed as the lowest concentrations at which percent deviation from the accuracy and precision are less than 15% for risperidone was 2 ng/ml.

Recovery

Potential loss of risperidone during the extraction by diisopropyl ether–isoamyl alcohol was determined by comparing the peak area of risperidone from plasma samples in the range of 2-100 ng/ml and the equal concentration of standard risperidone prepared in 0.1 M potassium dihydrogenphosphate pH 2.2 mobile phase. The percent of recovery was calculated as follows.

$$\text{Recovery (\%)} = \frac{\text{Peak area of standard risperidone in plasma}}{\text{Peak area of standard risperidone in mobile phase}} \times 100$$

Precision and variability

To determine intra-day precision and variability, the standard risperidone were spiked in blank plasma at 5, 20 and 100 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 15 % .

To determine inter-day precision and variability, the standard risperidone were spiked in blank plasma at 5, 20 and 100 ng/ml concentrations and 5 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 15 %. The percent of coefficient of variation (%CV) was calculated as following formula.

$$\%CV = \frac{\text{standard deviation (SD) of peak area of risperidone}}{\text{Mean peak area of risperidone}} \times 100$$

3.3.4 Sample preparation

20 µl of clozapine (1µg/ml) as internal standard (I.S.) and 1 ml NaOH (2 M) were added to 1 ml of plasma. The tubes were vortex-mixed for 10 seconds and 4 ml of diisopropyl ether–isoamylalcohol (99 : 1, vol/vol) was added as extraction solvent. After 10 min shaking, the mixture was centrifuged at 3000 *g* for another 10 min at 4° C and the organic phase (upper phase) was transferred to tubes containing 150 µl of KH_2PO_4 (0.1 M, pH 2.2 with 25% H_3PO_4), mixed for 1 min, and centrifuged at 3000 *g* for 5 min. The upper organic layer was carefully aspirated and 1 ml of diethyl ether was added. After gentle mixing, the ether phase was eliminated and a 60 µl aliquot of the remaining acid solution was injected into the HPLC system. The chromatographic conditions used in this study were good to separate risperidone from other endogenous substances in plasma.

3.4 Data Analysis

3.4.1 Pharmacokinetic calculations

The pharmacokinetic parameters(AUC_{0-48}, $AUC_{0-\infty}$, K_e, $t_{½}$, C_{max}, $t_{max,}$, Cl/f, Vd/f, MRT) were analyzed by non-compartment method, with the use of WinNonlin version 4.1 (Pharsight, Mountain View, CA).

3.4.2 Statistical analysis

All results were expressed as mean ± S.D. or S.E. Differences in risperidone pharmacokinetic parameter among control and treatment phases were tested by Wilcoxon signed rank test with *P* value less than 0.05 taken as minimum levels of significance.