

## CHAPTER 3

### MATERIALS AND METHODS

#### 3.1 Chemicals and reagents

##### Drug formulations

The innovator product: Seroquel<sup>®</sup> 200 mg lot no. CT523 was purchased from the AstraZeneca UK, Manufactured date: 06/2005, Expire date: 06/2008.

The generic product: Quantia 200<sup>®</sup> 200 mg lot no. T02/6-066 was obtained from Unison Laboratories Co., Ltd. (Thailand), Manufactured date: 9-2-06,

The standard quetiapine (Lot No. AH 5-16564) was obtained from Medline Co., Ltd. (Thailand), risperidone (Batch No. RISP 005/0403) was used as the internal standard (I.S).

The HPLC grade of acetonitrile 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 were purchased from Sigma-Aldrich (Milan, Italy), diisopropyl ether and isoamyl alcohol were purchased 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 consisted of a Waters 2695 pump, an autosampler (Waters Associates, Milford, MA, USA) and a Waters 2487 variable wavelength UV detector set at 225 nm. The column was reverse-phase Symmetry C8 (particle size 5  $\mu\text{m}$ ; column size 250 mm  $\times$  4.6 mm i.d.) A guard-pak precolumn module was used to obviate rapid column degeneration.

### 3.3 Methods

#### 3.3.1 Subjects

The subjects of this study were physically and mentally normal male volunteers, aged 18-45 years old with BMI 18-25 kg/m<sup>2</sup>. All were in good health on the basis of medical history and physical examination, routine blood test including CBC, white blood cell count, BUN, creatinine, SGOT, SGPT, ALP, bilirubin, total protein and albumin. The subjects who have abnormal hematological, liver or kidney functions were excluded from this study. Subjects with known contraindication or hypersensitivity to antipsychotic drug were 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. After complete explanation of the study, written informed consent was obtained from all subjects. The study protocol was approved by the Ethics Committee, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla, Thailand.

#### 3.3.2 Sample size

The following example illustrate the calculation of sample size to achieve an 80% power at the 5% nominal level when  $\theta = 5\%$ . According to the current FDA guidelines,  $\nabla$  is usually set to be  $\pm 20\%$  of the average reference bioavailability in most BE studies. CV is the coefficient of variation, which is the intrasubject variability expressed in percentage of the average reference bioavailability, CV=20%. The sample size for each sequence group is approximately:

$$n \geq [t_{\alpha, 2n-2} + t_{\beta, 2n-2}]^2 [CV/(\nabla - \theta)]^2$$

$$n \geq [1.69 + 0.85]^2 [0.2/(0.223 - 0.0513)]^2$$

$$n \geq [6.45][1.35] = 8.7 \approx 9$$

Thus  $n = 9$  per sequence or 18 subjects in total, in this study used for 24 subjects.

### 3.3.3 Protocol

The study was an open-label, randomized, two-phase crossover design with a 2 weeks washout period.

#### Study design

The BE of two formulations of quetiapine fumarate 200 mg was conducted using an experimental design of two-way crossover, single blind, two-periods, two-sequences, and randomized study with a 2-week washout period. During the first period, volunteers from group I received a single 200-mg dose of Seroquel<sup>®</sup> (reference product) whereas volunteers from group II received a single 200-mg dose of Quantia 200<sup>®</sup> (test product). During the second period, the procedure was repeated on the groups in reverse.

The drugs were administered to the volunteers in the morning, after an overnight fasting, with 200 ml of water. No food was taken at least 4 h after ingestion of the drug. Volunteers were not allowed to ingest any alcoholic drink or coffee during the trial.

Blood samples were collected at 0 (pre dose) and at 0.33, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 12, 24 and 48 h post dose. The samples were centrifuged and the plasma was collected and stored at -70°C until analysis.

#### Sample analysis

The plasma quetiapine concentrations were measured by an HPLC method which was modified from the method of Mandrioli *et al.* (2002).

#### Mobile phase

The mobile phase composed of 0.05 M potassium dihydrogen phosphate (containing 11.5 mM triethylamine), acetonitrile and methanol in a 55:18:22 (v/v/v) ratio and adjusted to pH 2.5 with 85% phosphoric acid. The mobile phase was freshly prepared daily and filtered through 0.2 µm filter (Nylon 66, Whatman International Ltd. Maidstone, England), then degassed in the ultrasonic bath

for 15 min before using. The flow rate was 0.7 ml/min. all analyses were performed at room temperature ( $25^{\circ}\pm 1^{\circ}\text{C}$ ).

### **Stock standard and internal standard solution**

The stock standard solution (quetiapine) and internal standard (IS) solution (risperidone) at a concentration of 1 mg/ml was prepared by dissolving 10 mg in 10 ml methanol. Stock solutions were stable for at least 2 months when stored at  $-20^{\circ}\text{C}$  (Saracino *et al.*, 2006). Working standard solution, which was used for preparation of a calibration curve, was freshly daily prepared by diluting the stock solution with blank plasma.

### **Calibration curves**

Calibration curves was prepared by adding various concentrations of standard quetiapine fumarate solution to blank plasma so that the final concentration in plasma were 9, 35, 109, 217, 434, 869 and 1,737 ng/ml.

### **Sample preparation**

Quetiapine and the added IS risperidone were extracted from 1 ml of human plasma, alkalinized with 1 ml of NaOH (2 M) and vortex-mixed for 20 sec. The mixture was added 4 ml of di-isopropylether:isoamylalcohol (99: 1, v/v) shaken for 5 min, and was centrifuged at 2,500 g for 10 min. The organic phase (upper phase) was transferred to tubes which containing 150  $\mu\text{l}$  of  $\text{KH}_2\text{PO}_4$  (0.1M, pH 2.2), mixed for 20 sec and centrifuged at 2,500 g for 5 min. The upper organic layer was carefully aspirated and 1 ml of diethyl ether was added, vortex-mixed for 1 min, then shaking for 5 min and centrifuged at 2,500 g for 10 min. The upper ether phase was eliminated and a 70- $\mu\text{l}$  aliquot of the remaining acid solution was injected into the HPLC system for analysis.

### **3.4 Method validation (Thai FDA, 2006)**

#### **3.4.1 Specificity**

Specificity was evaluated by analyzing six blank plasma samples and looking for interfering peaks at the retention times of quetiapine and IS

#### **3.4.2 Lower limit of quantification (LLOQ)**

The LLOQ of the assay is the smallest analytical concentration defined by the concentrations which give rise to peaks height with a signal to noise ratio of 5 times of the blank plasma. In addition, the analyte peak in LLOQ sample should be identifiable, discrete and reproducible with a precision of 20% and accuracy within 80-120%.

#### **3.4.3 Linearity/standard calibration curve**

Linearity was evaluated using freshly prepared dilution of plasma samples to the concentrations of 9, 35, 109, 217, 434, 869 and 1,737 ng/ml. Samples were quantified using the ratio of peak area of quetiapine to that of IS as the assay parameter. Standard curves were derived from the equation  $y = mx + c$  using weighted least square regression. A correlation of more than 0.99 was desirable for all the calibration curves.

#### **3.4.4 Accuracy and precision**

Intra-day accuracy and precision were evaluated by replicated analysis of quetiapine at different concentrations in human plasma. The run consisted of a calibration curve plus five replicates each of low (13 ng/ml), medium (347 ng/ml) and high (1,042 ng/ml) quality control (QC) samples. The inter-day accuracy and precision were assessed in a similar manner by analysis of low, medium and high QC samples for quetiapine on five separate occasions. A comparison was made between the experimental values obtained and actual values. The evaluation of precision was based on the criteria that, the CV for each concentration level should not be more than 15%. Similarly, for accuracy, the mean value should not deviate by  $\pm 15\%$  of the actual concentration.

$$\text{Accuracy (\%)} = (\text{calculated concentration} / \text{actual concentration}) \times 100$$

### 3.4.5 Recovery

The extraction efficiency of quetiapine from human plasma was evaluated by comparing the mean detector responses of five processed QC samples of low (13 ng/ml), medium (347 ng/ml) and high (1,042 ng/ml) concentrations to mean detector responses for five standard solutions of equivalent concentration. As per the acceptance criteria the recovery of the analyte need not be 100%, but the extent of recovery of an analyte and of IS should be consistent, precise and reproducible.

### 3.4.6 Stability

**3.4.6.1 Freeze-thaw stability:** Effect of three freeze and thaw cycles on the stability of frozen plasma sample containing quetiapine was determined to establish the ruggedness of the method. Three aliquots each of low (13 ng/ml), medium (347 ng/ml), and high (1,042 ng/ml) extracted QC samples were stored at  $-70^{\circ}\text{C}$  and subjected to three freeze-thaw cycles. After the completion of third cycle the samples were processed, analyzed and results were compared with nominal values. The values must not deviate were than  $\pm 15\%$  of the actual concentration.

**3.4.6.2 Long-term stability:** To determine the long-term stability of quetiapine in human plasma three aliquots of each low (13 ng/ml), medium (347 ng/ml), and high (1,042 ng/ml) QC samples were kept in deep freezer at  $-70^{\circ}\text{C}$  for 1 month. The samples were analyzed and concentrations obtained were compared with the nominal values of QC set and all values must not deviate more than  $\pm 15\%$  of the actual value is qualified the test.

**3.4.6.3 Short-term stability:** Six aliquots each of the low (13 ng/ml), medium (347 ng/ml), and high(1,042 ng/ml) unprocessed QC samples were kept at ambient temperature ( $20\text{--}30^{\circ}\text{C}$ ) for 2 and 6 h in order to establish the short-term stability of quetiapine in human plasma. Thereafter, the samples were analyzed and the concentrations obtained were compared with the actual values of QC samples. The values must not deviate were than  $\pm 15\%$  of the actual concentration.

**3.4.6.4 Post-preparative stability (Auto-sampler stability):** In order to establish the auto-sampler stability of quetiapine in human plasma matrix, three aliquots of low (13 ng/ml), medium (347 ng/ml), and high (1,042 ng/ml) QC samples were stored in auto-sampler for 6 h. Thereafter, samples were analyzed and concentrations were compared with the actual values. The samples met the criteria of stability if the deviation was within  $\pm 15\%$ .

**3.4.6.5 Stock solution stability:** Quetiapine and IS were prepared by dissolving suitable amount of each pure substance in methanol and kept at  $-70^{\circ}\text{C}$  for 14 days. Stock solutions were diluted with the mobile phase. Thereafter, the mean detector response of quetiapine and IS from three replicate chromatographic runs were compared to that of freshly prepared solutions of the same concentration. The samples met the criteria of stability if the deviation was within  $\pm 2\%$ .

### 3.5 Data Analysis

#### 3.5.1 Pharmacokinetic analysis

The pharmacokinetic parameters, namely;  $C_{\max}$ ,  $T_{\max}$ ,  $AUC_{0-t}$ ,  $AUC_{0-\infty}$ , and  $T_{1/2}$  were computed for the test and reference drugs using WinNonlin<sup>®</sup> Professional Software Version 1.1 (Pharsight, Mountain View, CA) by non-compartment model.

#### 3.5.2 Statistical analysis

The comparison of the pharmacokinetic parameters and ANOVA for ln-transformed pharmacokinetic parameters:  $C_{\max}$ ,  $AUC_{0-t}$ , and  $AUC_{0-\infty}$ . The evaluation criteria were based on the statistical results of 90% CI for the difference in the means of the ln-transformed data using the following equation:

$$90\% \text{ CI} = \Delta \pm t_{0.10, v} \sqrt{EMS(2/n)}$$

Where  $\Delta$  was a difference in means of ln-transformed pharmacokinetic parameters ( $C_{\max}$  or  $AUC_{0-48}$  or  $AUC_{0-\infty}$ ) between the test product and the reference,

$t_{0.10, v}$  is the tabulated two-tail t values for a 90% CI,  $v$  is a degree of freedom of the error mean square obtained from the ANOVA table, EMS is the error mean square from the ANOVA table and  $n$  is the number of subjects. Antilogarithm of the calculated CI will yield an exact CI for the ratio.

BE would be concluded if the 90% CI fell within the BE range of 80.0-125.0% for  $C_{\max}$ ,  $AUC_{0-t}$  and  $AUC_{0-\infty}$ .