CHAPTER III

RESEARCH METHODOLOGY

MATERIALS AND METHODS

Chemicals and materials

Amlodipine besylate was obtained from Unison Laboratories Co. Ltd. (Thailand). Sertraline HCl was obtained from IRCS Co. Ltd. Nortriptyline HCl, 7-chloro-4-nitrobenz-2-oxa-1, 3-diazole (NBD-Cl) were purchased from Sigma (USA). Sodium acetate anhydrous was from Sigma-Aldrich (Germany). Potassium dihydrogen phosphate, Sodium hydroxide and 25% ammonia solution were purchased from Merck (Germany). HPLC grade acetonitrile, methanol, diethyl ether were purchased from Lab-Scan (Lab-Scan Asia, Bangkok, Thailand). Formic acid was obtained from Asia Pacific Specialty Chemicals Ltd. (Australia). n-Heptane was purchased from Fisher Chemicals (UK).

C2 SPE filled with 500 mg sorbent mass in 3 ml cartridge was purchased from Alltech (USA).

Apparatus

The HPLC-UV system consisted of LC-10 AT VP pump, SPD-10 A VP UV detector and SCL-10 A VP controller (Shimadzu, Japan). The rheodyne injection port with 100 µl loop (USA) was used. The analyses were carried out on Hyperclone C18 BDS, 5 µm, 150 X 4.6 mm I.D. column (Phenomenex, USA). Class-VP software from Shimadzu (Japan) was used to control the system and integrate the data.

The HPLC-fluorescence system consisted of LC-10 AT VP pump, RF-10 A XL fluorescence detector and SCL-10 A VP controller (Shimadzu, Japan). The injection port with 100 µl loop was purchased from Rheodyne (USA). The Hypersil 5 phenyl, 5 µm, 250 X 4.6 mm I.D. (Phenomenex, USA) column was used. Class-VP software from Shimadzu (Japan) was used to control the system and integrate the data.

The LC-MS/MS system consisted of a model 1100 Series liquid chromatography equipped with a binary pump, a degasser, a built in oven-column compartment and an autosampler (Agilent Technologies, USA). The Clipeus C18, 5 μm, 150 X 3.0 mm I.D. column was used (Higgins Analytical, USA). Mass spectrometric detection was carried out on API4000 triple quadrupole instrument from Applied Biosystems/MDS Sciex (Canada) equipped with an ESI interface. Analyst version 1.1 software from Applied Biosystems/MDS Sciex was used to control the LC-MS/MS system and to integrate the data.

Standard solutions

1. Standard stock solutions

Amlodipine besylate was dissolved in 50% acetonitrile in water to give a final concentration of 50 μg/ml of amlodipine base. The stock solution of an internal standard (IS) was prepared by dissolving nortriptyline HCl or sertraline HCl in 50% acetonitrile in water to give a final concentration of 50 μg/ml of base.

2. Solutions used for method validation

Standard stock solution of amlodipine was diluted to achieve concentrations of 1, 10 and 100 ng/ml. These solutions were spiked into plasma to give final concentrations of 0.05, 0.1, 0.5, 1, 5, 8 and 15 ng/ml, These amlodipine solutions were used for plotting calibration curve. For method validation, amlodipine in plasma at concentrations of 0.3, 3 and 10 ng/ml were prepared.

Sample preparation for HPLC with UV detector analysis

A sample extraction method was used for cleaning a sample and reducing the interference in the sample before HPLC analysis. Various extraction procedures using both solid-phase extraction (SPE) and liquid-liquid partition (liq-liq) methods were tested. The procedures of sample extraction methods were as follows.

1. Solid phase extraction method

The C2 sorbent was pretreated with 2 ml of acetonitrile, 1 ml of water and 1ml of 0.025M pH 7.0 potassium dihydrogen phosphate. A 1.0 ml of 0.025M pH 7.0

potassium dihydrogen phosphate, nortriptyline solution (equivalent to nortriptyline 200 ng) and 1.0 ml of plasma were consecutively loaded into the cartridge. Then, the cartridge was washed with 2.0 ml of 10% acetonitrile in water and dried the under reduce pressure. Finally, the cartridge was washed with 1.5 ml of acetonitrile and eluted with 2.0 ml of 4% ammonia solution in acetonitrile. The eluent was evaporated under nitrogen gas and reconstituted with 200 µl of mobile phase before HPLC analysis.

2. Liquid-liquid partition method

The amount of 1.0 ml of plasma was transfered to a test tube. The internal standard solution (equivalent to 50 ng) was added following by 0.5 ml of 0.5 M sodium hydroxide solution. Then, the plasma was extracted with 5.0 ml of various organic solvents as follows: chloroform, dichloromethane, diethylether, n-heptane:isopropanol at the ratios of 99:1, 95:5, 90:10, 85:15 and 80:20 (v/v), and n-heptane:diethylether at the ratios of 95:5, 90:10, 80:20, 75:25, 70:30, 60:40 and 50:50 (v/v). The organic part was decanted and dried under nitrogen gas. The dried extracted sample was reconstituted with 200 µl of mobile phase.

Sample preparation for HPLC with fluorescence detector analysis

The internal standard solution equivalent to 200 ng of sertraline was added into 1.0 ml of plasma. After mixing, it was basified with 0.5 ml of 0.5 M of sodium hydroxide solution. Then the sample was extracted with 5.0 ml of n-heptane:diethylether 70:30 (v/v). The organic part was dried under nitrogen gas and reconstituted with 100 µl of 0.025 M pH 7.0 sodium dihydrogen phosphate solution. Then, 100 µl of NBD-Cl in methanol (6mg/ml) was added and the mixture was kept at 80^{o}C for 25 min. After the mixture was cool, 100 µl of 0.1 N hydrochloric acid was added and the sample was extracted again with 3.0 ml of ethyl acetate. The ethyl acetate part was dried under nitrogen gas and reconstituted with 200 µl of the mobile phase.

Sample preparation for HPLC with MS/MS detector analysis

The sample was prepared by transferring 0.2 ml of plasma to a test tube. The amount of 0.2 ml nortriptyline solution (1 ng/ml) was added followed by 0.2 ml of 0.01 M

sodium hydroxide solution. Then the sample was extracted with 4.0 ml of n-heptane:diethylether 80:20. The organic part was dried under nitrogen gas and reconstituted with 200 µl of 50% 0.1 M formic acid in acetonitrile.

Analytical methods

1. HPLC with UV detector analysis

The experiment was done in the isocratic mode. The mobile phase was a mixture of 0.1M potassium dihydrogen phosphate and acetonitrile in the ratio of 2.15:1 v/v. The pH was adjusted to 2.9 with phosphoric acid. The flow rate was set at 1 ml/min and the UV detector was used at 240 nm.

2. HPLC with fluorescence detector analysis

The experiment was done in gradient mode. The mobile phase was programmed as shown in Table 3. Solvent A was acetonitrile:0.1 M sodium acetate 1:1 pH 7.5. Solvent B was acetonitrile.

Table 3 Gradient elution program of the HPLC with fluorescence detector analysis.

Time (min)	A (%)	B (%)
0.01	90	10
5.00	90	10
5.05	100	0
15.95	100	0
16.00	50	50
20.00	50	50
20.05	100	0
35.00	100	0
35.05	0	100
45.00	0	100
45.05	100	0
50.00	100	0

The flow rate was set at 1 ml/min and the fluorescence detector was set at the excitation wavelength of 459 nm and the emission wavelength of 528 nm.

3. HPLC with MS/MS detector analysis

The experiment was done in the isocratic mode. The mobile phase was a mixture of 0.01M formic acid and acetonitrile in the ratio of 55:45 v/v. The flow rate was set at 0.8 ml/min. The injection volume was 10 µl. The analytical column was placed in column oven at 40°C. Mass spectrometric detector was equipped with an electrospray ionization (ESI) interface which was run in the positive ion mode and the interface heater was set at 550°C. The Multiple Reaction Monitoring (MRM) was used to monitor the transition of 409>238 for amlodipine and 264.2>233.3 for nortriptyline. The MS/MS parameters and nitrogen gas parameters were shown in Table 4 and Table 5.

Table 4 The MS/MS parameters for amlodipine and nortriptyline analyses.

parameter	Amlodipine (409>238)	Nortriptyline (264.2>233.3)
DP (declustering potential)	41	71
CE (collision energy)	15	23
CXP (collision cell exit potential)	14	14

Table 5 The nitrogen gas parameters of MS/MS for amlodipine and nortriptyline analyses .

parameter	value
CAD (collision gas)	5
CUR (curtain gas)	11
GS1 (ion source gas 1;nebulizer gas))	54
GS2 (ion source gas 2; heater gas)	64

Method validation

The validation method was done by following the Thailand guidelines for the conduct of bioavailability and bioequivalence studies and the US guidance for industrial bioanalytical method validation.

1. Selectivity

Six plasma blank samples were extracted and analyzed compared to the plasma sample containing 0.05 ng/ml of amlodipine and 1 ng/ml of nortriptyline. It would be selective if there was no interference peak at the same retention time of both amlodipine and nortriptyline.

2. Limit of quantification (LOQ)

Five plasma samples containing amlodipine at the low concentration of 0.05 ng/ml were extracted and analyzed. Then, the % accuracy and percentage of coefficient of variation (% CV) were calculated.

% accuracy = (measured concentration / spiked concentration) X 100

% CV = (SD/mean) X 100

3. Linearity / calibration curve

Plasma samples containing amlodipine at the concentrations of 0.05, 0.1, 0.5, 1, 5, 8, and 15 ng/ml were extracted and analyzed. The correlation between the ratio of amlodipine/nortriptyline (area under the curve) and concentration of amlodipine was plotted and the coefficient of determination (r^2) of the graph was calculated.

4. Accuracy

Five of each plasma samples containing amlodipine at the concentrations of 0.3, 3 and 10 ng/ml were determined on the same day (intra-day) and between days (inter-day) for five day. The % accuracy was calculated.

5. Precision

Five of each plasma samples containing amlodipine at the concentrations of 0.3, 3 and 10 ng/ml were determined on the same day (intra-day) and between days (inter-day) for five day. The % CV was then calculated.

6. Recovery of extraction

Five of each plasma samples containing amlodipine at the concentrations of 0.3, 3 and 10 ng/ml were extracted. Amlodipine in the samples were determined compared to that of standard solution. Then, % recovery of extraction was calculated.

% recovery = (peak area of sample/peak area of standard solution) X 100

7. Stability

7.1 Freeze-thaw stability

Three aliquots of plasma samples containing amlodipine at the concentrations of 0.3 and 10 ng/ml were kept at -80°C for 24 hours and then thawed at room temperature (cycle 1). After that, the samples were frozen and thawed for the next two cycles. After the third cycle, the samples were analyzed compared to the freshly prepared sample.

7.2 Long-term stability

Three aliquots of plasma samples containing amlodipine at the concentration of 0.3 and 10 ng/ml were kept at -80°C for two months. Amlodipine in samples were determined every week in the first month and every two week in the second month compared to that of the freshly prepared sample.

7.3 Short-term stability

Three aliquots of plasma samples containing amlodipine at the concentrations of 0.3 and 10 ng/ml were kept at -80°C for 24 hours and thawed at room temperature. The samples were analyzed when kept at room temperature for 3 and 6 hours compared to the freshly prepared sample.

7.4 Autosampler stability

Three aliquots of plasma samples containing amlodipine at the concentrations of 0.3 and 10 ng/ml were extracted and reconstituted, then kept in the autosampler. The samples were analyzed when kept in the autosampler for 3 hours compared to the freshly prepared sample.

7.5 Stock solution stability

Stock solutions of amlodipine and nortriptyline at the concentration of 1 and 10 ng/ml were kept at 4°C. The stock solutions were analyzed every week for one month compared to the freshly prepared solution.