CHAPTER V

CONCLUSION AND RECOMMENDATION

In this study, the extraction and analytical methods for amlodipine determination from plasma were developed. The extraction method was developed by using two techniques i.e. SPE and liquid-liquid partition. Using C2 SPE, the recoveries of amlodipine and nortriptyline (internal standard) were quite low. Moreover, the samples were not well cleaned up. There were interferences at the same retention time of both compounds when detecting with UV detector. To solve this problem, more steps of washing were taken. Even though the interferences were decreased but the lower recoveries of both compounds were obtained.

The liquid-liquid partition technique was applied to extract amlodipine and nortriptyline from plasma. Various solvents were used. The highest recovery of amlodipine (83.75%) was obtained when using the mixture of diethylether and n-heptane at the ratio of 80:20 as an extraction solvent. In contrast, the recovery of nortriptyline (4.85%) was very low. The new internal standard, sertraline, was therefore introduced. This was the first time that sertraline was used as internal standard in the analysis of amlodipine. Amlodipine and sertraline showed good recovery when they were extracted with the mixture of diethylether and n-heptane at the ratio of 70:30. Furthermore, there was no interference at the same retention times of both compounds when detected with UV detector. Therefore, this method was selected for amlodipine and sertraline extraction from plasma samples.

In bioequivalence study, the lowest level of amlodipine in plasma at least 0.5 ng/ml should be detected. So a very sensitive and selective analytical method was required. HPLC coupled to various detectors i.e. UV, fluorescence and MS/MS detectors were tested. Because of the simplicity of the technique, HPLC with UV detector was firstly used for determination of amlodipine in plasma in this study. Using HPLC with UV detector, amlodipine and sertraline were separated from other plasma components by

using C18 reverse phase column and detected with 240 nm of UV detector. The LOQ of amlodipine in plasma determination was 10 ng/ml which was still too high for our purpose.

HPLC with fluorescence detector was used in the next experiment. Amlodipine in plasma had to be derivetized with fluorescing agent after extraction because it did not have fluorescence property. NBD-Cl was selected to react with amlodipine and an internal standard, sertraline, in pH7 phosphate buffer at 80^{o}C for 25 minutes. The derivatization of NBD-amlodipine and NBD-sertraline were separated by using phenyl reversed phase column and detected by fluorescence at the excitation wavelength of 459 nm and emission wavelength of 528 nm. The results of this method were not satisfactory as many interferences was occurred in the chromatogram. To elute all peaks from the detection system, it took 55 minute-total run time which was too long for routine analysis. In addition, the LOQ value of amlodipine in plasma was about 7 ng/ml. Because of these problems and the complication of sample preparation, HPLC with fluorescence was not selected for the further study.

As HPLC with UV and fluorescence detectors did not give enough sensitivity for amlodipine in plasma determination, HPLC-MS/MS which has been known as the most sensitive and powerful technique was selected. Nortriptyline was reconsidered for using as an internal standard because sertraline could not completely be eluted from the detection system. The extraction method had to be reoptimized. Amlodipine and nortriptyline was extracted from plasma using liquid-liquid partition method. Plasma samples were basified with 0.01 M sodium hydroxide solution and extracted with the solvent mixture of 80:20 v/v diethylether and n-heptane. By this method, the recoveries of amlodipine and nortriptyline after extracted from plasma were more than 60%. Amlodipine and nortriptyine were determined using HPLC-MS/MS equipped with an ESI interface which was running in the positive mode. The MRM mode was used to monitor the transition of 409 to 238 for amlodipine and 264.2 to 233.3 for nortriptyline. The mobile phase for HPLC was a mixture of 55:45 v/v 0.01 M formic acid solution and acetonitrile. The flow rate was set at 0.8 ml/min. The separation was done through C18 reverse phase

analytical column placed in column oven at 40°C. The amount of 10 µl reconstituted sample was injected into the analytical column by autosampler. This method was a very selective method because there were no interferences in the chromatograms at the same retention time of amlodipine and nortriptyline. The LOQ of this method was 0.05 ng/ml of amlodipine in plasma. Comparing to other methods described in literature review, the HPLC-MS/MS method developed was the most selective and sensitive method. Therefore, it was selected for further study.

In the method validation, the calibration curve showed good linearity within the range of 0.05-15 ng/ml of amlodipine in plasma with r^2 of 0.9996. The LOQ was 0.05 ng/ml with intra-day and inter-day accuracy and precision in the range of Thailand guidelines for the conduct of bioavailability and bioequivalence studies and the US guidances for the industrial bioanalytical method validation requirement. The stability test showed that all samples were stable through two months at -80°C. After the samples were thawed, they were stable through six hours at room temperature. The samples showed no change when they were placed in the autosampler for three hours. Although the stock solutions were stable through one or two week, they should be freshly prepared to avoid the change of the concentration due to the evaporation of solvent.

In conclusion, the HPLC-MS/MS and liquid-liquid extraction methods reported in our study had a high selectivity and sensitivity. This technique could be used for determination of amlodipine concentrations in human plasma in pharmacokinetic and bioequivalence studies of amlodipine.