CHAPTER IV

RESULTS AND DISCUSSIONS

Amlodipine is a one of effective antihypertensive drugs. However, the price of innovative amlodipine is quite high. If a generic amlodipine can be launched to the market, patients will have an opportunity to use the lower price drugs. In order to assure that generic amlodipine has the same efficacy as the innovative one, the bioequivalence of the generic drug and the innovative one have to be studied. Generally, the bioequivalence study composes of clinical and analytical part. Clinical part involves with drug administration, evaluation and monitoring in volunteers. For the analytical part, it involves with analyzing amlodipine concentration in plasma, in order to conduct pharmacokinetic analyses and, subsequently, evaluate bioequivalence. The analytical methods with high extraction recovery, accuracy and sensitivity have to be developed and validated. In bioequivalence study of 10 mg amlodipine tablet, the drug concentration of at least ten times lower than C_{max} (5.9 $\pm$ 1.2 ng/ml) should be detected. Therefore, in this study the analytical method that could determine amlodipine in plasma in the level as low as 0.5 ng/ml had to be found.

Extraction method development

1. Solid phase extraction method

Extraction method of amlodipine using C2 SPE from plasma was previously described by Josefsson et al. [14]. In their study, C2 SPE cartridges were preconditioned with 2 ml acetonitrile, 1 ml water and 1ml pH 7 phosphate buffer. The cartridges were added with 1 ml of pH 7 phosphate buffer, UK 52.829 solution equivalent to 10 ng/ml and 1 ml of plasma sample under gentle reduced pressure. The cartridges were washed with 2 ml 20% acetonitrile in water and then 1 ml acetonitrile. Finally, the samples were eluted with 1 ml 2.5% ammonia solution in acetonitrile and the solvent was then evaporated. From their study, the recovery of amlodipine and UK 52.829 (internal standard) were

98 % and 95% respectively. The mechanism of extraction was probably due to the interactions of silanol residues of C2 SPE material with the basic amino group on the side chain of both amlodipine and UK 52.829. In our study, nortriptyline (Figure 4) instead of UK 52.829 was used as an internal standard. As nortriptyline also has a basic amino group on the side chain, it could be extracted by using the same technique as amlodipine and UK 52.829. Amlodipine and nortriptyline at the concentration of 300 ng/ml and 200 ng/ml, respectively, were extracted from plasma by using C2 SPE as described by Josefsson et al. The differences from Josefsson's study were: 1) the cartridges were washed with 2 ml of 10% acetonitrile in water and then 1.5 ml of acetonitrile 2) the samples were eluted with 2 ml of 4% ammonia solution in acetonitrile. From the detection with HPLC with UV detector at 210 nm, the recovery of amlodipine and nortriptyline were approximately 53 and 47%, which were quite low. Moreover, the amount of both drugs could not be determined precisely because there were some interference at the same retention time of amlodipine and nortriptyline. According to the result of our study, the % recoveries of drugs were lower than Josefsson's study. One of the reasons might be that the amount of C2 sorbent mass used in our study (500 mg) was more than that Josefsson used (100 mg). Chromatograms of plasma sample and plasma blank from C2 SPE extraction are shown in Figure 3. These interferences could be eliminated with more steps of washing, but the % recovery of drug and internal standards were consecutively decreased. As a result, this method was not used for the further study.

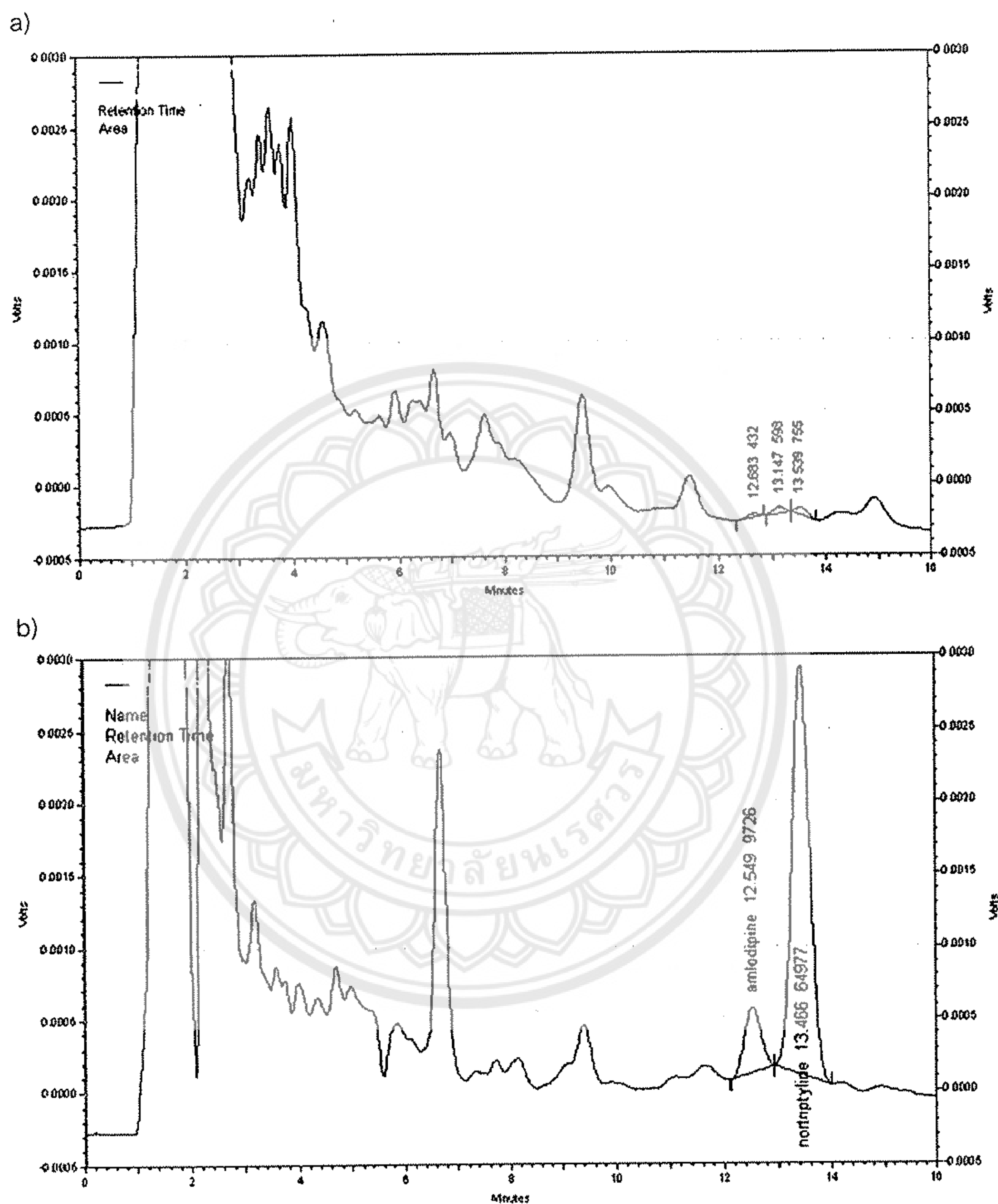

Figure 3 HPLC chromatogram of a) plasma blank, b) plasma sample containing 300 ng/ml of amlodipine and 200 ng/ml of nortriptyline obtained from C2 SPE detected at 210 nm

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2. Liquid-liquid partition method

From literature review, liquid-liquid partition was reported in many studies [8-10, 12, 15-16]. However, the method in this experiment was developed from that reported by Tatar and Atmaca [10] because it was a single step extraction and the same internal standard, nortriptyline, was used. In the study of Tatar and Atmaca [10], the amount of 0.1 ml nortriptyline solution (50 ng/ml) was added into 0.5 ml plasma samples. Then, they were basified with 0.5 ml of 0.1 N sodium hydroxide solution. The mixtures were extracted with the solvent mixture of n-heptane and isopropanol in the ratio of 99:1 and the solvent portion was dried. From their results, the recoveries of 0.25, 3 and 18 ng/ml amlodipine and 50 ng/ml nortriptyline were 72.00, 87.33, 93.56% and 95.52%, respectively.

In our study, the plasma samples containing 300 ng/ml of amlodipine without being basified were firstly extracted with the solvent of n-heptane and isopropanol in the ratio of 99:1 and 80:20. The results from HPLC analysis at 210 nm showed that there were many interferences in the chromatogram. The basified samples may give the better results because in basic condition, amlodipine would be unionized and could be extracted by that non-polar solvent mixture. The proper concentrations of sodium hydroxide solution were tested by using the concentrations of 0.1 and 0.5 M. Then, the samples were extracted with the solvent of n-heptane and isopropanol in the ratio of 99:1. The recoveries of 500 ng/ml amlodipine that basified with 0.1 and 0.5 M sodium hydroxide solution were 78% and 85%, respectively. However, when using the same method to extract of 300 ng/ml amlodipine and 50 ng/ml nortriptyline, the lower recoveries (64.04% and 73.62%, respectively) were obtained. The recoveries of both amlodipine and nortriptyline were also lower than the study of Tatar and Atmaca [10]. As a result, different solvents were used to extract amlodipine in plasma in order to get the higher recovery of the compound. At least 80% recovery of amlodipine was needed to improve the sensitivity of HPLC with UV detector. Amlodipine and nortiptyline were extracted with various solvents as shown in Table 6. The results of the extraction with diethylether and n-heptane in the ratio of 80:20 showed highest % recovery of

amlodipine (83.75% ± 2.46) but the % recovery of nortriptyline was very poor (4.85% ± 0.61). According to this result, the internal standard was changed from nortriptyline to sertraline. Sertraline (Figure 4) was used as an internal standard in the analysis of amlodipine for the first time. It was selected because its pKa (9.5) is close to pKa of amlodipine (8.6). It should be extracted from plasma and detected with the same method as amlodipine. Furthermore, sertraline has the basic amino group in the molecule that required for derivatization of HPLC with fluorescence detector. The % recoveries of amlodipine and sertraline after the extraction with several of solvents were shown in Table 7. The highest % recoveries of both amlodipine (89.64% ± 1.54) and sertraline (80.28% ± 5.89) were obtained when extracted with diethylether and n-heptane in the ratio of 70:30. Therefore, this solvent mixture was used for liquid-liquid extraction from plasma in the further studies.

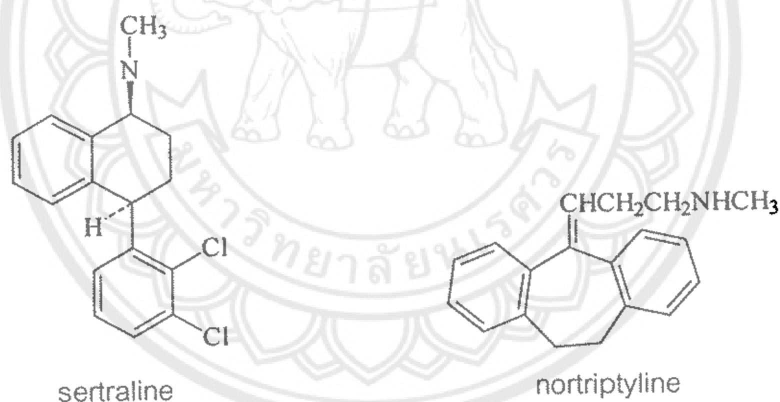

Figure 4 Structures of sertraline and nortriptyline

Table 6 The % recoveries of 300 ng/ml amlodipine and 50 ng/ml nortriptyline in plasma from the extraction with various solvents and detected using HPLC with UV detector at 210 nm.

Extraction solvent	Mean of % recovery* ± SD	
	amlodipine	nortriptyline
n-heptane:isopropanol (99:1) (n=4)	64.04 ± 1.24	73.62 ± 4.84
Dichloromethane (n=3)	70.25 ± 3.70	65.05 ± 3.86
Chloroform (n=6)	36.64 ± 1.20	38.34 ± 3.25
Diethylether (n=6)	81.74 ± 4.61	11.61 ± 4.83
n-heptane: isopropanol (95:5) (n=3)	60.65 ± 2.71	92.22 ± 12.52
n-heptane: isopropanol (90:10) (n=3)	43.31 ± 6.89	86.65 ± 11.19
n-heptane: isopropanol (85:15) (n=3)	26.23 ± 0.26	92.16 ± 11.87
n-heptane: isopropanol (80:20) (n=3)	29.64 ± 5.90	83.18 ± 1.96
diethyl ether: n-heptane (95:5) (n=3)	69.96 ± 1.19	10.86 ± 0.57
diethyl ether: n-heptane (90:10) (n=3)	75.84 ± 10.68	1.69 ± 0.20
diethyl ether: n-heptane (80:20) (n=3)	83.75 ± 2.46	4.85 ± 0.61

* % recovery = (area under curve of sample / area under curve of standard) X 100

Table 7 The % recoveries of 300 ng/ml amlodipine and 50 ng/ml sertraline in plasma from the extraction with various solvents and detected using HPLC with UV detector at 210 nm.

Extraction solvent	Mean of % recovery* ± SD	
	amlodipine	sertraline
diethyl ether:n-heptane 75:25 (n=3)	87.09 ± 4.06	79.04 ± 9.83
diethyl ether:n-heptane 70:30 (n=3)	89.64 ± 1.54	80.28 ± 5.89
diethyl ether:n-heptane 60:40 (n=4)	78.27 ± 4.95	69.54 ± 6.26
diethyl ether:n-heptane 50:50 (n=3)	68.29 ± 5.39	56.76 ± 2.93

* % recovery = (area under curve of sample / area under curve of standard) X 100

Analytical method development

1. HPLC with UV detector analysis

Generally, HPLC with UV detector might not be a perfect analytical method for pharmacokinetic studies of amlodipine because of low sensitivity of the detection technique. However, it was chosen in this study as it is relatively simple and can directly analyze amlodipine without any derivatization process. Furthermore, it can be used for amlodipine determination during the development of extraction method.

In the literatures, amlodipine was mostly separated on the C18 reverse phase column with many different parameters such as mobile phases, flow rates of mobile phase, the isocratic or gradient modes of elution [12-15]. These parameters would be optimized in order to get the well separating condition. In this study, C18 reverse phase column was selected for separating amlodipine from other compounds. Several buffering agents and solvents were tested as a mobile phase. Potassium dihydrogen phosphate buffer and acetonitrile showed the possibility to be a mobile phase. Then, the concentrations, the ratio of both agents and the final pH was optimized. As a result, the suitable mobile phase consisted of 0.1 M potassium dihydrogen phosphate and acetonitrile in the ratio of 2.15:1 and the final pH was 2.9. However, the ratio would be

slightly changed depending on the sample. In the same way, the optimum wavelength of UV detector was found. The maximum absorption wavelength of 0.1 ng/ml amlodipine was obtained by scanning with PDA detector apparatus coupled with HPLC. From scanning results, the maximum absorption wavelength of amlodipine was at 240 nm. However, the wavelength was changed if the samples were prepared with the different process. For example, amlodipine after extraction with C2 SPE was detected with 210 nm to avoid the interfering peaks. In this experiment, 240 nm was used for increasing the method sensitivity. From the analysis of amlodipine and sertraline, the chromatogram showed no interferences at the same retention time as both drugs (Figure 5). The LOQ which referred to the sensitivity of the method was 10 ng/ml. From the literature reviewed, there were two publications that showed the LOQ values. The first one was the study of Luksa et al. [12] which aimed at purification of amlodipine enantiomers from plasma. The LOQ was lower than 5 ng/ml when using 5 ml of plasma sample. The other one was studied by Patel et al. [13]. They tried to quantitate the six combinations of antihypertensive which amlodipine was included in tablets. LOQ was 25 ng/ml was reported. However, these two publications could not be compared to this experiment because of the different purpose and procedure. Although the sensitivity of HPLC with UV detector developed in this experiment was not sufficient for determination of amlodipine in plasma for bioequivalence study, it seems to be useful for other purposes such as determination of amlodipine in dosage form.

a)

b)

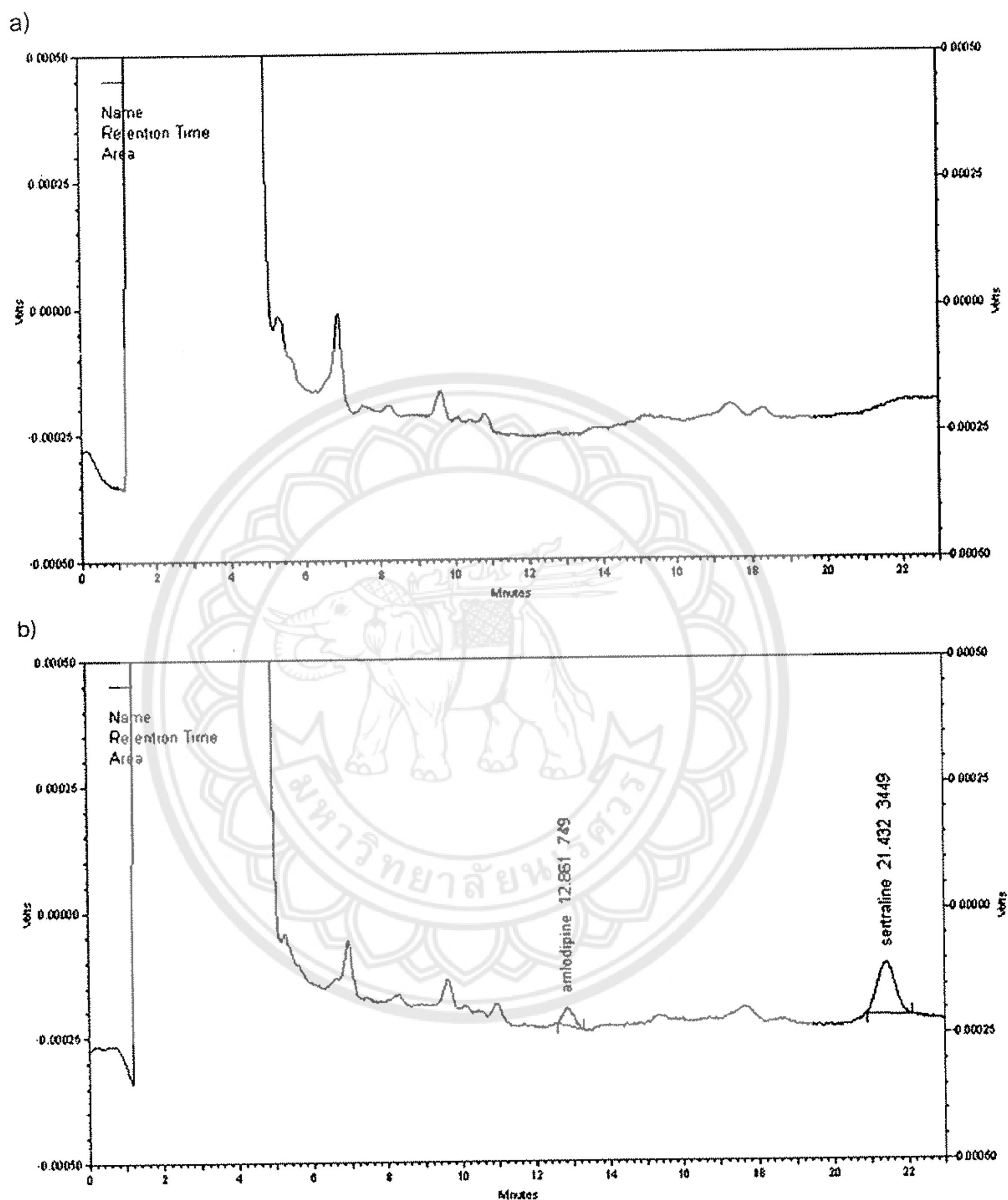

Figure 5 HPLC chromatograms of a) plasma blank, b) plasma sample containing 10 ng/ml of amlodipine and 200 ng/ml of sertraline obtained from liquid-liquid partition method detected at 240 nm

2. HPLC with fluorescence detector analysis

HPLC with fluorescence detector is one of the very selective and sensitive analytical methods. The use of this technique for amlodipine determination was previously reported by Tatar and Atmaca [10]. The LOQ value of 0.25 ng/ml of amlodipine in plasmawhich was suitable for our purpose was found. As amlodipine does not have the fluorescence property it needs to be derivatized with fluorescencing agent before the analysis. From the study of Tatar and Atmaca [10], NBD-Cl (Figure 6) which was a specific fluorescencing reagent for primary and secondary aliphatic amines was used to derivatize amlodipine and nortriptyline (internal standard). After extraction, amlodipine and nortriptyline were derivatized in pH 8.5 borate buffer at 70°C for 30 minutes. The derivative of NBD-amlodipine and NBD-nortriptyline were detected at the excitation wavelength of 459 nm and emission wavelength of 528 nm.

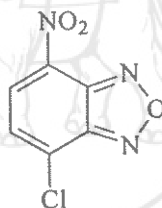

Figure 6 Structure of NBD-Cl

Using the same derivatization process, we found that the recovery of amlodipine was very poor. It might be due to the incomplete of reaction. To optimize the derivatization method, results of reaction between amlodipine and NBD-Cl in different of pH, incubation temperatures and incubation times were investigated. Two types of buffers, phosphate and borate buffers were tested at the same pH. The result showed that higher fluorescence intensity was obtained when the reaction was done in phosphate buffer. The influence of buffer medium's pH on the reaction of amlodipine and NBD-Cl was examined by varying pH of phosphate buffer from 5 to 12. The effect of pH on the derivatization of amlodipine and NBD-Cl is shown in Figure 7. The highest intensity was obtained when reaction was carried out at pH 7. To determine the optimum

temperature and time of incubation, the samples were incubated at 60, 70, 80 and 90°C. The samples at each temperature were analyzed after incubated for 10, 30, 45, 60 and 180 minutes. At 80°C, two extra sampling times i.e. at 35 and 40 minutes after incubation were added. The maximum intensity of NBD-amlodipine derivative was obtained when incubating the sample at 80°C for 30 minutes as shown in Figure 8. However, the intensity of the NBD-amlodipine derivative rapidly decreased after 30 minutes. The incubation condition at 80°C for 25 minutes was chosen. In conclusion, the extracted sample was derivatized in phosphate buffer at pH 7 and incubated at 80°C for 25 minutes. Before analysis, the derivatized samples were extracted with a solvent to eliminate the excess substances. Dichloromethane, butanol, ethyl acetate, diethylether and the mixture of diethylether and n-heptane (70:30 v/v) were tested. The cleaner samples with high recovery of fluorescing compounds were obtained when using ethylacetate. However, there was a peak at the same retention time of NBD-amlodipine derivative. Silica SPE which prepared by filling silica gel of 0.063-0.200 mm particle size into the pipette tip was used to clean up the samples after extracted with ethylacetate. As a result, the chromatograms of samples which passed and not passed silica SPE were not difference.

Sertraline was selected as an internal standard instead of nortriptyline because of low recovery of nortriptyline after sample extraction. According to derivatization, amlodipine and sertraline were changed to the derivatives. Their properties would be difference. Therefore, the HPLC condition using in HPLC with UV detector could not be used. In this experiment, the C8, C18 and phenyl reverse phase columns were tested for separating the derivatives from other compounds. Phenyl column showed the best separation when detecting at the wavelength previously described by Tatar and Atmaca [10]. Several mobile phases in isocratic elution mode tested did not give satisfactory results. There were many peaks around that of NBD-amlodipine derivative. For this reason, the gradient elution mode was applied as mentioned in Table 3. The chromatograms of plasma blank and plasma sample containing derivative of NBD-amlodipine and NBD-sertraline (Figure 9) showed many

interference peaks including a peak at the same retention time of NBD-amlodipine. Since there were some peaks belatedly eluted, the total run time was 55 minutes which was too long for the routine analysis. In addition, the LOQ of 7 ng/ml obtained was not sufficient for quantify the plasma concentration of amlodipine in bioequivalence study. Consequently, this method was not considered for the further study.

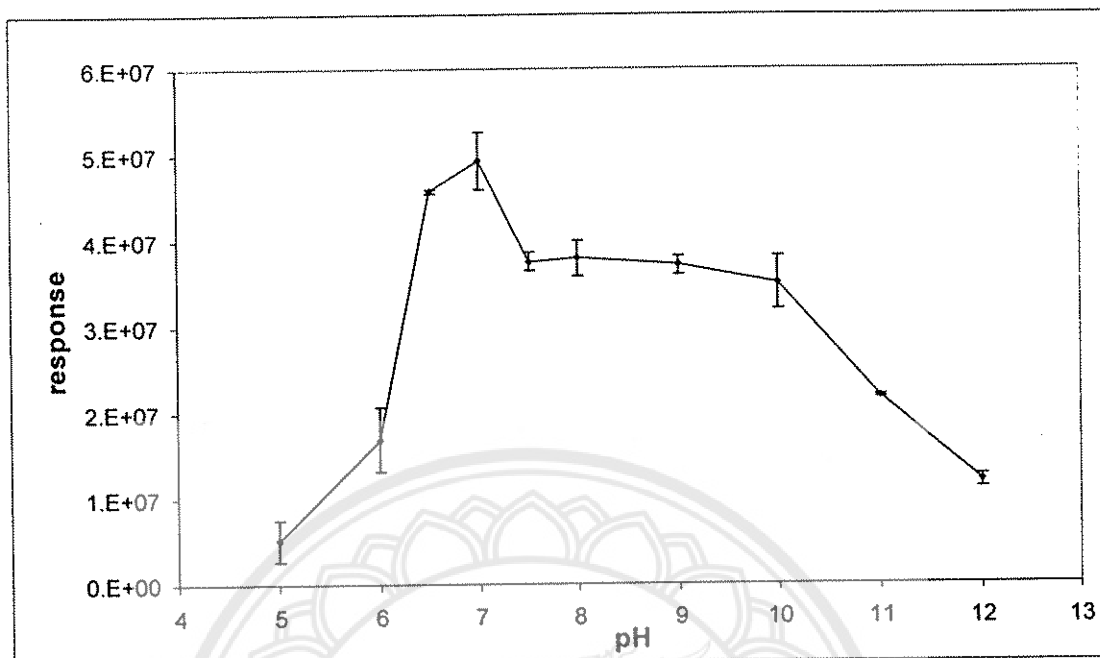

Figure 7 Effect of pH on the derivatization of amlodipine with NBD-Cl

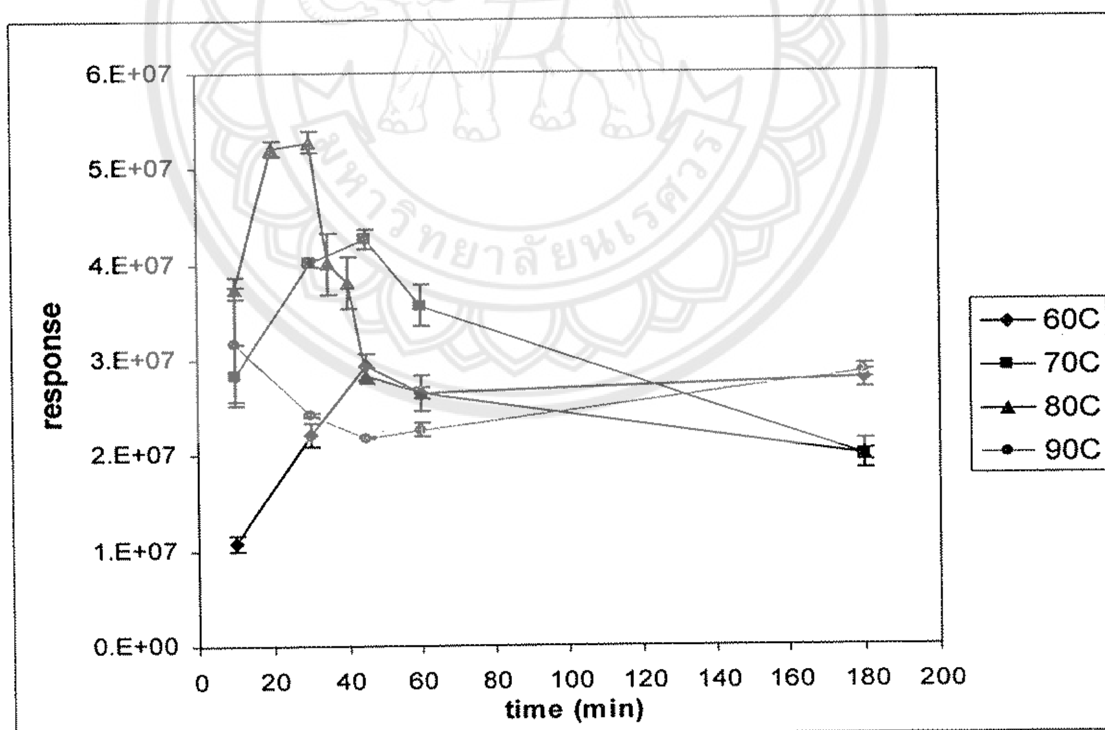

Figure 8 Effect of temperature and time on the derivatization of amlodipine with NBD-Cl

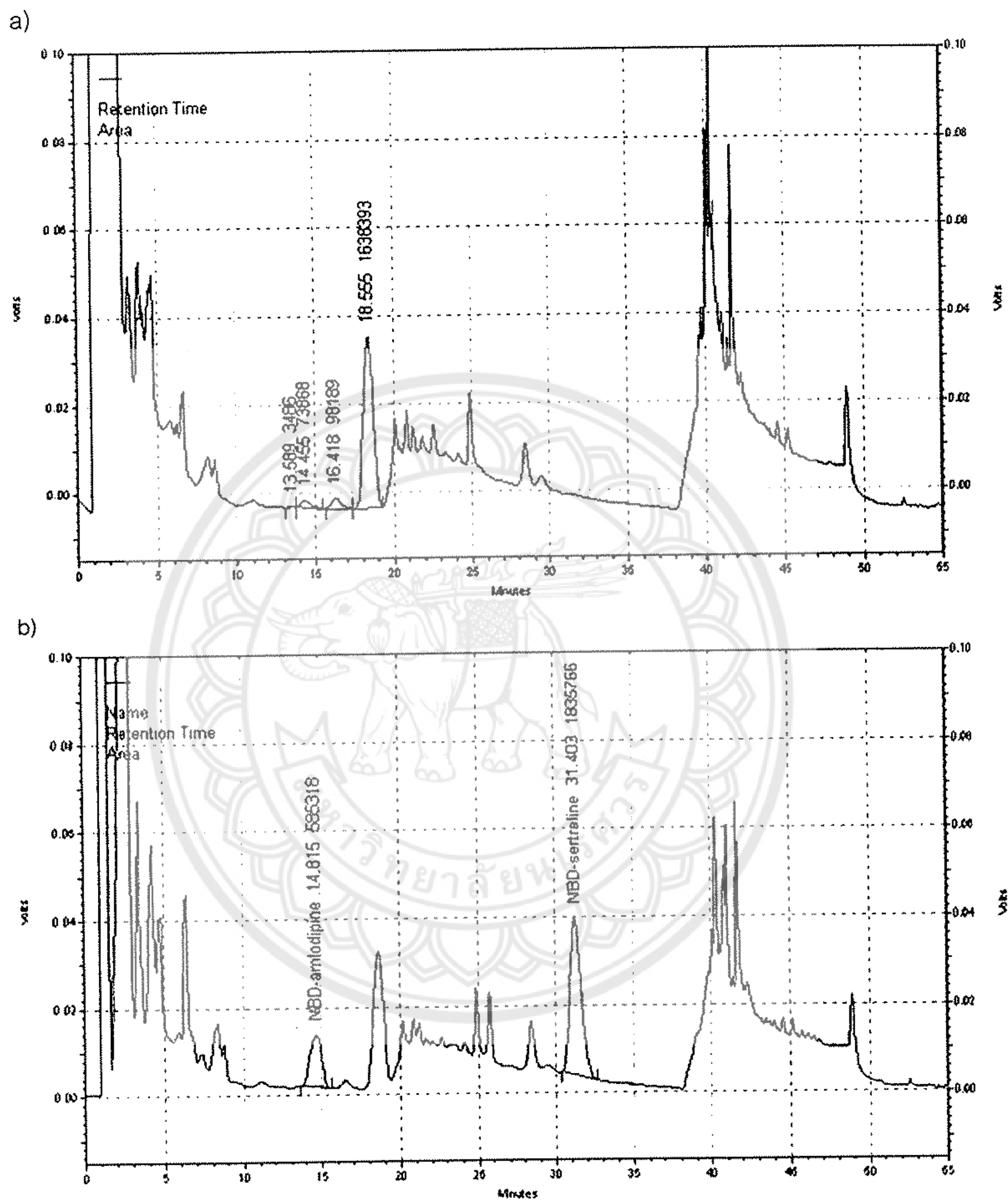

Figure 9 HPLC fluorescence chromatograms of a) plasma blank, b) plasma sample containing 10 ng/ml amlodipine and 50 ng/ml sertraline that were derivatized with NBD-Cl

3. HPLC with MS/MS detector analysis

As HPLC connected with UV and fluorescence detectors did not give the satisfactory sensitivity for amlodipine determination, HPLC-MS/MS which has been known as the most sensitive and powerful technique was chosen as a tool for the analysis. It was not selected as the first tool in this study because it was a costly method. In this study, the triple quadrupole MS/MS was used. It composed of two identical mass filter quadrupoles (Q1 and Q3) separated by a collision cell (Q2). A sample mixture after separated by HPLC was introduced into the electrospray ionization (ESI) source. The ESI generated the ionized molecules such as molecular ion of amlodipine and sertraline. In an MS /MS scan, Q1 would filter the ions according to their m/z and allow only the interested ions to enter Q2. The interested ions would be further fragmented in Q2 and the fragmented ions would then pass to Q3. Q3 was used to filter the fragmented ions of interest and then collected at the detector. The principle of MS/MS is illustrated in Figure 10.

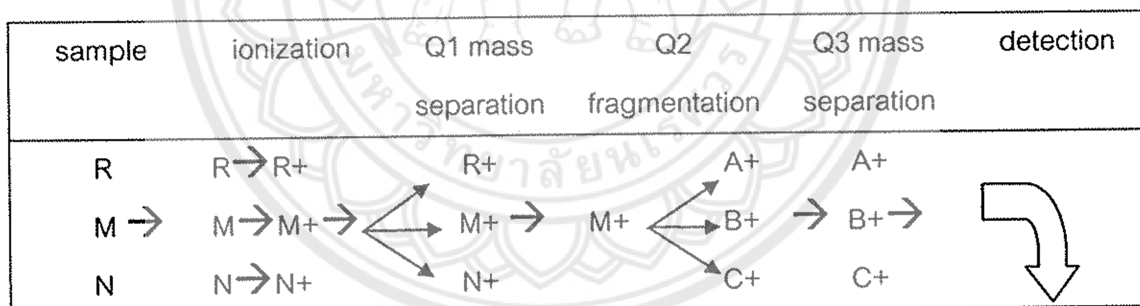

Figure 10 The procedure of fragmentation in a triple quadrupole MS/MS

Full scan positive mass spectra of amlodipine and sertraline showed the ionized molecular peak of m/z 409.1 and 306.0, respectively. The most abundant fragmented ions were at m/z of 238.0 and 158.9 for amlodipine and sertraline, respectively. These m/z were used as a monitoring parameter. Other MS/MS parameters required in the analysis were obtained using auto tuning process of the MS/MS apparatus. In the analysis, amlodipine and sertraline were separated from other compounds by using C18 reversed phase column, gradiently eluted with a mobile phase

consisting of formic acid solution and acetonitrile. The transitions of 409.1 to 238.0 for amlodipine and 306.0 to 158.9 for sertraline were monitored. The structure of m/z transitions of amlodipine and sertraline were shown in Figure 11.

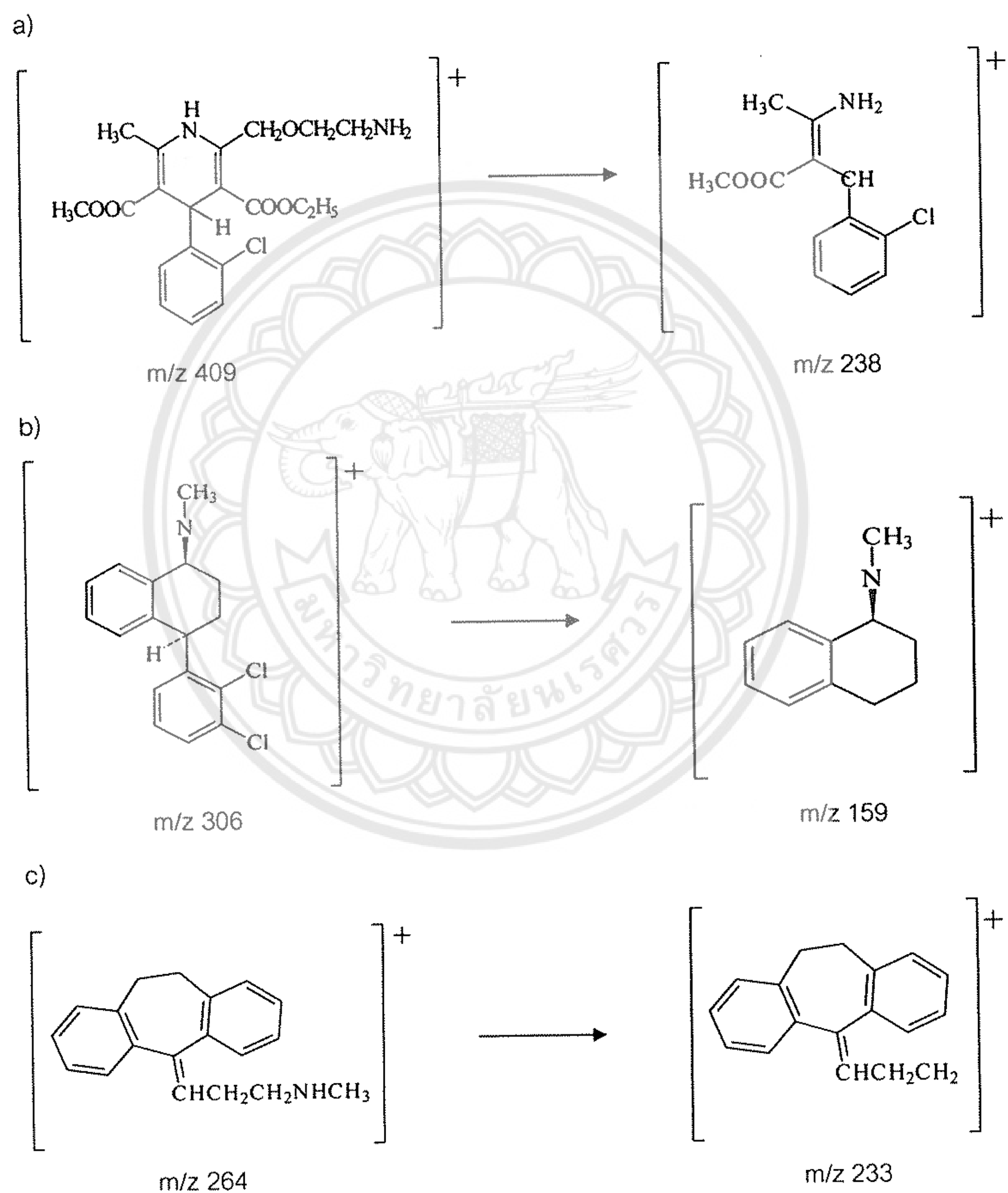

Figure 11 The structures of m/z transition of a) amlodipine, b) sertraline and c) nortriptyline

The results showed that sertraline could not completely be eluted from the detection system. Such a remaining peak would certainly interfere the analysis, changing mobile phase might solve this problem. Formic acid solution in mobile phase was replaced by acetic acid solution at the same ratio. When mobile phase was changed, amlodipine instead of sertraline, remained in the system and the sensitivity was lower than that from mobile phase containing formic acid. Therefore, nortriptyline which had been used as an internal standard in the previous experiment was reconsidered. Accordingly, the MS/MS parameters for an internal standard analysis were found. Nortriptyline showed the ionized molecular peak of m/z 264.2 and the most abundant fragmented ion was m/z of 233.3. So it was monitored at the transition of 264.2 to 233.3 (Figure 11). As mentioned before, nortriptyline had poor recovery of extraction, so the extraction method was modified by reducing the concentration of sodium hydroxide solution in basifying process from 0.5 M to 0.01 M. After modifying, the sample was extracted with diethylether and n-heptane in the ratio of 80:20. The recovery of 62.7-69.7% for amlodipine and 76.80% for nortriptyline were obtained from this method. According to these result, the % recovery of nortriptyline was higher than the previous study. One of the reasons might be that the concentration of nortriptyline in this study (1 ng/ml) was lower than the previous study (50 ng/ml). Analysis using HPLC-MS/MS showed no interference in the chromatogram and the LOQ of 0.05 ng/ml was found. The chromatograms of plasma blank and plasma sample contained 0.05 ng/ml amlodipine and 1 ng/ml nortriptyline are shown in Figure 12 and Figure 13, respectively. From literature, Carvalho et al. [16] developed the HPLC-MS/MS with ESI source using desipramine as an internal standard. The extraction method was quite similar to our method. The LOQ reported (0.1 ng/ml) was higher than that from our study. In Carvalho's study, HPLC eluent was split before enter to the MS. The molecular transition of amlodipine was the same as in our study (m/z 409.0 to 238.1). The retention time (3.4 min) was longer because of the lower flow rate. Zhong et al. [17] also developed the HPLC with ESI source. 4'-hydroxypropafenone was use as an internal standard. The molecular transition of amlodipine was 409 to 238. The LOQ reported was 0.4 ng/ml

which was higher than that from our study. The total run time was 3.7 min. It is obvious that our method gave the better sensitivity than the previous studies of Carvalho et al. and Zhong et al. From the sensitivity and selectivity found in this experiment, HPLC-MS/MS should be the best method to determine amlodipine in human plasma. This method was then selected for further study.

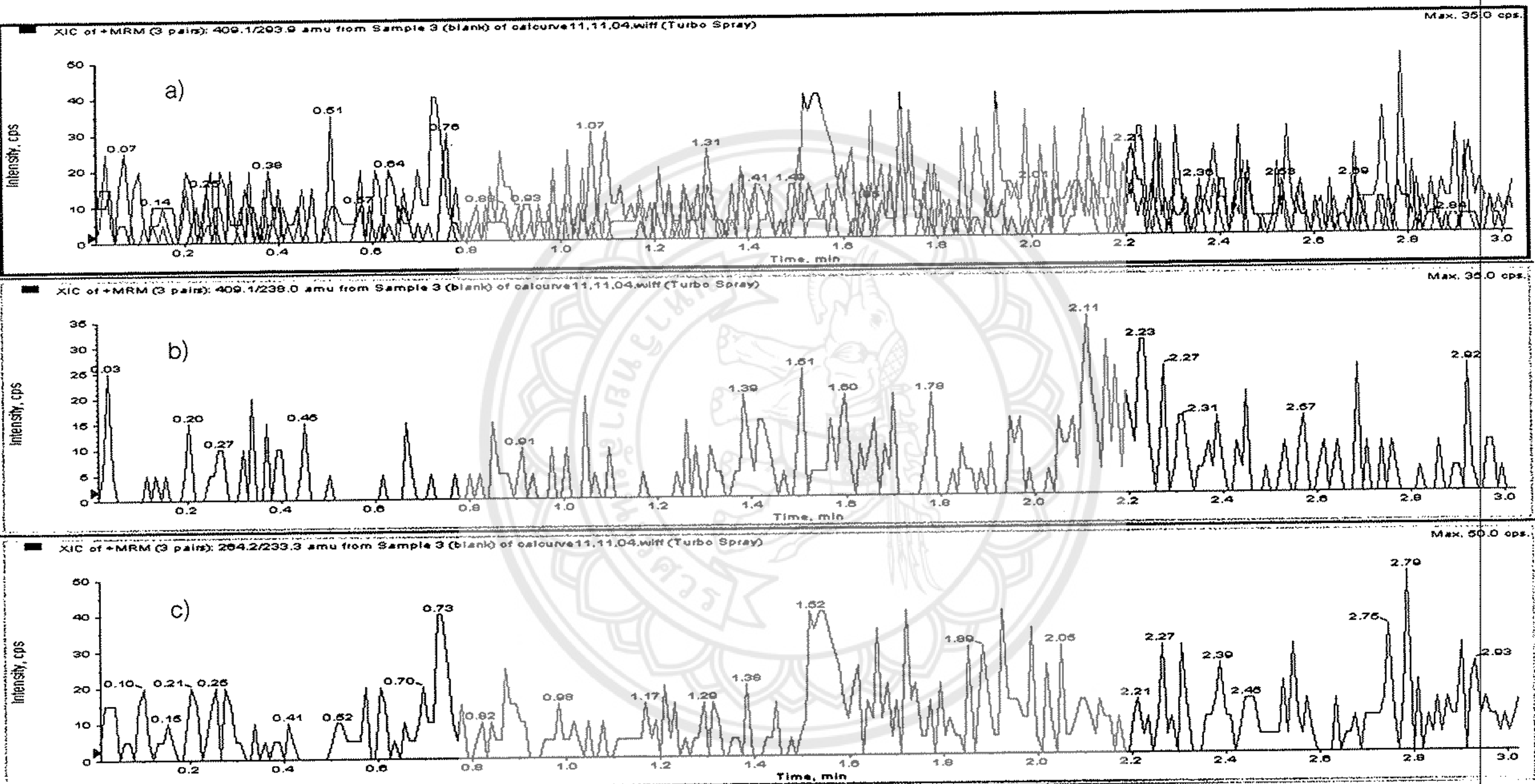

Figure 12 The MS/MS chromatograms of plasma blank for a) total ion count, b) amlodipine and c) nortriptyline

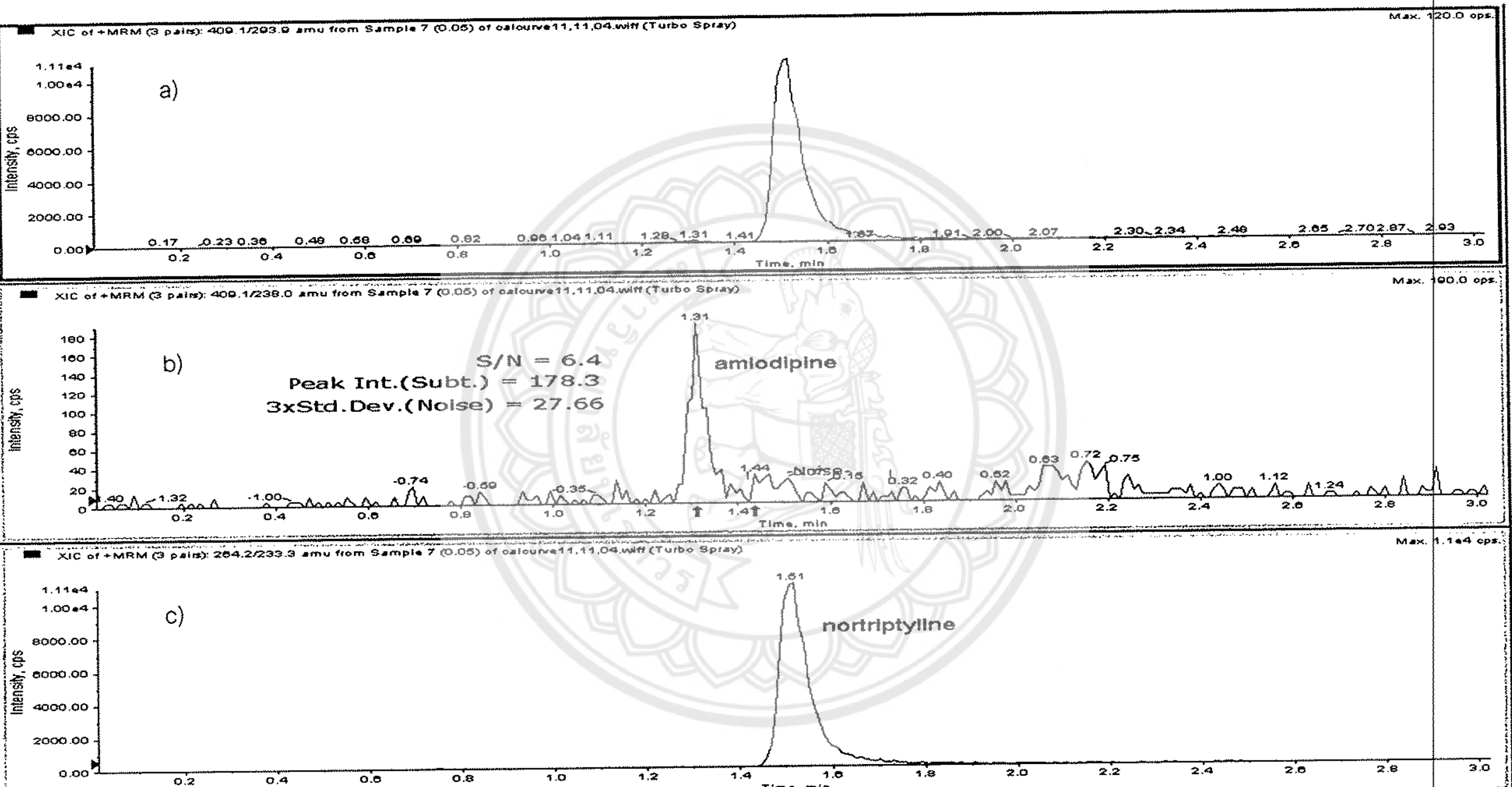

Figure 13 The MS/MS chromatograms of plasma sample for a) total ion count, b) 0.05 ng/ml amlodipine (409.1>238.0) and c) 1 ng/ml nortriptyline (264.2>233.3)

Method validation

As the purpose of method validation was to demonstrate the reliability of a method for quantitative determination, the HPLC-MS/MS method developed was validated for amlodipine in plasma determination determination. The method validation including determination of selectivity, accuracy, precision, recovery of extraction, calibration curve and stability of amlodipine in plasma was done by following the Thailand guidelines for the conduct of bioavailability and bioequivalence studies [19] and the US guidances for the industrial bioanalytical method validation [20].

1. Selectivity

According to HPLC-MS/MS mechanism, only selected ion was detected. Thus it was a very selective method for amlodipine in plasma analysis. Six plasma blanks were used for checking the selectivity of the method. They were extracted and analyzed comparing to the plasma extract containing 0.05 ng/ml amlodipine and 1 ng/ml nortriptyline (Figures 12 and Figure 13). None of the chromatograms showed interference at the same retention time of both compounds. As a result, the satisfactory selectivity was obtained.

2. Limit of quantification (LOQ)

LOQ is the lowest standard concentration on the calibration curve. At the LOQ concentration, the signal to noise ratio of analyte should be at least five (S/N = 5) with the accuracy of 80-120% and % CV not exceeds 20% [19, 20]. From our study, the LOQ was at the concentration of 0.05 ng/ml of amlodipine in plasma. The S/N of 6.4 is showed in Figure 13. This concentration showed the intra-day accuracy of 103.42% and intra-day precision (% CV) of 8.56%. The inter-day accuracy and precision were 101.17% and 5.98%, respectively. Since accuracy and precision were in the range of acceptance criteria, this concentration was accepted as LOQ.

3. Linearity / calibration curve

Calibration curve is a relationship between the ratios of area under the curve of amlodipine and nortriptyline and concentrations of amlodipine in plasma. In this study, the calibration curve was done at the concentrations of amlodipine in the range between 0.05 and 15 ng/ml. It shows good linearity with r^2 of 0.9996 as shown in Figure 14.

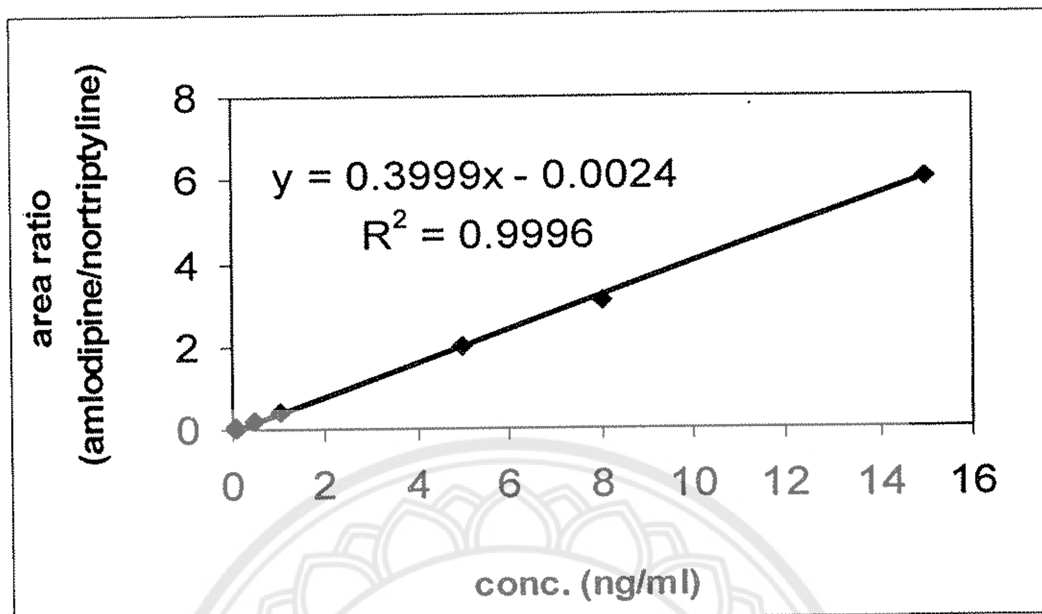

Figure 14 Calibration curve of amlodipine in plasma using HPLC-MS/MS

4. Accuracy

The accuracy of an analytical method described the closeness of measured concentration to the known concentration. The intra-day accuracy values of amlodipine determination at concentrations of 0.3, 3 and 10 ng/ml were 98.9, 95.3 and 103%, respectively. The inter-day accuracy of the same concentration were 100, 97.4 and 95.6%, respectively (Table 8). From both guidelines of Thailand guidelines for the conduct of bioavailability and bioequivalence studies and the US guidances for the industrial bioanalytical method validation, the % accuracy must be 85-115% [19, 20]. Therefore, the accuracy of this method compiled with both requirements.

5. Precision

The precision of an analytical method described the closeness of individual measurements of an analyte when the procedure was applied repeatedly to multiple aliquots of a single homogeneous volume of plasma. Precision in one day (intra-day) and in between days (inter-day) had to be determined. The precision was expressed in the value of % CV. The number of % CV should not exceeds ± 15% [19, 20]. The intra-day precision values of amlodipine determination at concentrations of 0.3, 3 and 10 ng/ml were 9.42, 8.92 and 6.16%, respectively. The inter-day precision of the same

concentration were 9.63, 4.37 and 5.64%, respectively (Table 8). As results, this method was precise for amlodipine determination according to both guidelines.

Table 8 Accuracy and precision of amlodipine in plasma from intra-day and inter-day experiments.

Concentration (ng/ml)	Intra-day (n=6)		Inter-day (n=5)	
	Mean of % accuracy*	Mean of % CV**	Mean of % accuracy*	Mean of % CV**
0.3	98.9	9.42	100	9.63
3	95.3	8.92	97.4	4.37
10	103	6.16	95.6	5.64

* acceptance criteria are 85-120%

**acceptance criteria are not exceed $\pm$ 15%

6. Recovery of extraction

The recovery of extraction indicated the extraction efficiency in the experiment. The recovery of extraction should be at least 50-60%[19]. The % recoveries of samples containing 0.3, 3 and 10 ng/ml of amlodipine were determined. The % recoveries were calculated in comparison with that of standard solution and the results are shown in Table 9. The recoveries of amlodipine and nortiptyline were not very high but quite consistent.

Table 9 %recoveries of amlodipine and nortriptyline after extraction with diethylether and n-heptane (80:20).

concentration	Mean of % recovery
amlodipine 0.3 ng/ml (n=8)	69.72 $\pm$ 4.88
amlodipine 3 ng/ml (n=5)	63.46 $\pm$ 5.12
amlodipine 10 ng/ml (n=8)	62.73 $\pm$ 4.53
nortriptyline 1 ng/ml (n=8)	76.80 $\pm$ 4.22

7. Stability

Drug stability in biological fluid depends on the storage condition as well as the chemical properties of the drug and the biological matrix. The stability testing was used to study the variation of analyte contained in plasma samples in the experiment. The tests i.e. freeze-thaw, long-term, short-term and autosampler stability tests were used to evaluate the stability of amlodipine in plasma. Moreover, the stability of amlodipine and nortriptyline stock solutions at the storage condition should be evaluated.

7.1 Freeze-thaw stability

The objective of this test was to check how freeze-thawing effected on the stability of the samples. Freeze-thaw experiment was done with plasma contained amlodipine at the concentrations of 0.3 and 10 ng/ml. After three freeze-thaw cycles, the amounts of amlodipine remaining in the samples were calculated as a percentage of amlodipine comparing to that before experiment. The results indicated that the samples were stable through three cycles of freeze-thaw cycles (Table 10).

Table 10 %remaining of 0.3 and 10 ng/ml amlodipine in plasma after three of freeze-thaw cycles.

Concentration (ng/ml)	Mean of % remaining*
0.3 (n=3)	91.18
10 (n=3)	108.01

*acceptance criteria are 85-115%

7.2 Long-term stability

The stability of samples at the storage temperature over the analysis period was checked. The stability evaluation should cover the time between the first day of first sample collection and the last day of sample analysis. In our study, the stability of plasma contained amlodipine at the concentration of 0.3 and 10 ng/ml kept at -80°C were checked for two months. From the results, the samples were stable through two months (Table 11).

Table 11 %remaining of 0.3 and 10 ng/ml amlodipine in plasma after long-term storage.

Concentration (ng/ml)	Mean of % remaining*					
	1 wk.	2 wk.	3 wk.	4 wk.	6 wk.	8 wk.
0.3 (n=3)	97.14	100	91.43	100	111.43	111.43
10 (n=3)	104.01	105.79	113.27	103.56	106.86	108.19

*acceptance criteria are 85-115%

7.3 Short-term stability

Short-term stability used for evaluating the stability of samples that were thawed and kept at room temperaturebefore the analysis. In our study, the plasma containing amlodipine at the concentration of 0.3 and 10 ng/ml were determined after thawed and kept at room temperature for 3 and 6 hours. The results show that after being thawed the samples were stable through six hours (Table 12).

Table 12 %remaining of 0.3 and 10 ng/ml amlodipine in plasma after short-term storage.

Concentration (ng/ml)	Mean of % remaining*	
	3 hr.	6 hr.
0.3 (n=3)	100	106.45
10 (n=3)	104.91	99.56

*acceptance criteria are 85-115%

7.4 Autosampler stability

The stability of extracted samples in autosampler should be determined over the expected run time of the batch size. In our study, the expected run time for one batch was approximately three hours. Therefore, the amlodipine stability in autosampler through three hours was checked. The results showed that, the samples were stable while staying in autosampler for three hours (Table 13).

Table 13 %remaining of 0.3 and 10 ng/ml amlodipine in plasma after storage in autosampler.

Concentration (ng/ml)	Mean of % remaining*		
	1 hr.	2 hr.	3 hr.
0.3 (n=3)	100	102.78	100
10 (n=3)	101.55	99.48	99.28

*acceptance criteria are 85-115%

7.5 Stock solution stability

The stability of stock solutions of amlodipine and nortriptyline kept at 4°C were checked through one month. The results are shown in Table 14. In Thailand guidelines for the conduct of bioavailability and bioequivalence studies [19], the change of drug content should not exceed ± 2%. From this study, the stock solutions of 1 and 10 ng/ml of amlodipine and nortriptyline were stable for one week. The results from weeks two and three did not reach the requirement. In general, the stock solutions would not be kept for more than one week. It should be freshly prepared to avoid the change of the concentration due to the evaporation of solvent.

Table 14 %remaining of stock solution of amlodipine and nortriptyline in stock solution kept at 4°C for 1-3 weeks

Concentration (ng/ml)	% remaining*		
	1 wk.	2 wk.	3 wk.
amlodipine 1 ng/ml	100.53	99.29	91.98
amlodipine 10 ng/ml	99.64	104.83	92.49
nortriptyline 1 ng/ml	98.84	98.84	108.22
nortriptyline 10 ng/ml	100.79	102.49	109.39

*acceptance criteria are 98-102%

From the results of validation, all parameters were within the range of acceptances criteria. It was confirmed that the HPLC-MS/MS and the extraction method could be used for determination of amlodipine in plasma.