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Development and validation of liquid chromatography - Tandem mass spectrometry method for determination of amlodipine in human plasma and its application

P. Pongnarin[1], W. Thanakosai[1], P.Kingkhangphloo[1], W. Chandranipapongse[1], S. Kongpatanakul[1], S. Chatsiricharoenkul[1].*
Department of Pharmacology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand

Abstract

A sensitive liquid chromatography tandem mass spectrometry method was developed to quantify amlodipine in human plasma. Alkalinized plasma spiked with desipramine, an internal standard, was extracted by liquid-liquid extraction and evaporated an organic part to dryness. The residue was reconstituted and injected into an Acquity Ultra Performance LC™, (Waters, Co., Ltd. USA) with C_{18} column. The isocratic elution of mobile phase was performed by 90% of acetonitrile and 10% of 10 mM ammonium acetate pH 4 at flow rate of 0.20 mL/min with 5 minutes of total run time. Mass spectrometric analysis was performed using a Quattro Premier XE mass spectrometer, (Micromass Technologies, UK) coupled with an electrospray ionization (ESI) source in the positive ion mode. The MRM transitions of m/z 409.17>238.19 and 409.17>294.09 were selected for amlodipine and 267.09>208.06 for desipramine. The retention times were 1.63 and 1.69 minutes for amlodipine and desipramine, respectively. The linearity of the method revealed a correlation coefficient of >0.998 within the concentration range of 0.05 - 20 ng/mL. This work has been fully validated according to the Guidance for Industry: Bioanalytical Method Validation (USFDA CDER, 2001, BP) with high degree of accuracy and precision. This method was applied to quantify amlodipine concentrations in human plasma samples in a bioequivalence study.

Keyword: Amlodipine, LC-MS/MS, Method development, Method Validation

1. INTRODUCTION

Amlodipine besylate is a dihydropyridine agent and classified as a third generation calcium channel blocker. It selectively inhibits calcium influx across cell membranes in cardiac and vascular smooth muscle. With a greater effect on vascular smooth muscle, low dose amlodipine reduces blood pressure by decreasing peripheral vascular resistance without interfering the contractility of myocardial tissue[1-5]. Amlodipine besylate is chemically described as (R,S)3-ethyl-5-methyl-2-(2-aminoethoxymethyl)-4-(2-chlorophenyl)-1,4-dihydro-6-methyl-3,5-pyridinedicarboxylate benzenesulphonate. Its empirical formula is $C_{20}H_{25}ClN_2O_5 \cdot C_6H_6O_3S$ Figure 1. The molecular weight of amlodipine besylate and amlodipine are 567.05 and 408.8 g/mol, respectively.

The determination of amlodipine level in biological samples has been reported with high performance liquid chromatography with ultraviolet (HPLC-UV)[6-9], and mass spectrometric detector[10-14]. Although several methods have been reported for the determination of amlodipine in plasma, these methods have low sensitivity and narrow range of linearity[10-14]. These are important parameters, especially when the determination is performed in clinical samples retrieved from subjects who took the drug at low dose.

We developed a highly sensitive liquid chromatography tandem mass spectrometry method for the determination of amlodipine in human plasma, using desipramine as an internal standard. This method had a wide linearity range of, 0.05 to 20 ng/mL, with a short analytical

***Corresponding author:** somruedee.cha@mahidol.ac.th

run time of 5 minutes. The method was fully validated according to the Guidance for Industry, Bioanalytical Methods Validation, U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (USFDA CDER, 2001, BP)[15]. This method met all requirements of the guidance with high degree of accuracy and precision. This method was used in a bioequivalence study of healthy Thai subjects.

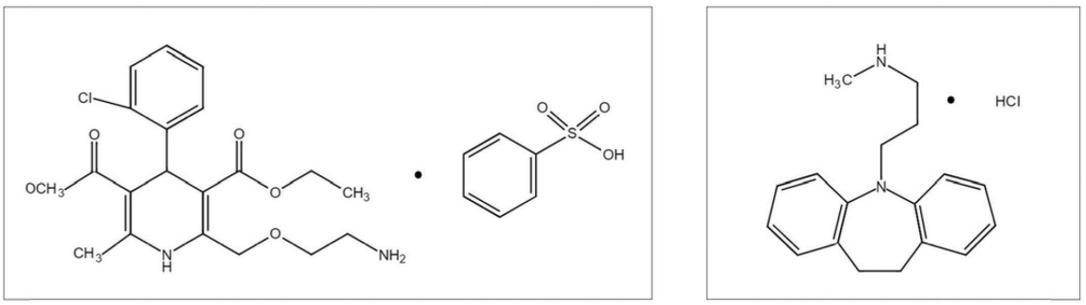

Figure 1. Chemical structures of amlodipine besylate(A) and desipramine HCl(B).

2. MATERIALS AND METHODS

2.1 Instrumentation

The high performance liquid chromatography with mass spectrometry system (LC-MS/MS) was performed on separation module of a Acquity Ultra Performance LC™, (Waters, Co., Ltd. USA) equipped with a Quattro Premier XE mass spectrometer, (Micromass Technologies, UK). The data management system was performed using Masslynx 4.1 SCN627, (Micromass Technologies, UK).

2.2 Chemicals and reagents

Reference substance of amlodipine besylate, (on the anhydrous basis; 99.8%) and desipramine hydrochloride (on the as is basis, 99.8%) were obtained from The United States Pharmacopeial Convention, Inc., USA. Type I water was prepared by a Milli Q system, Millipore Corporation (Massachusetts, USA). HPLC-grade of acetonitrile, methanol and propan-2-ol plus analytical reagent grade of methyl t-butyl ether, formic acid and hexane were purchased from Scharlau (Barcelona, Spain). Ammonia solution, analytical grade, was obtained from Merck, Darmstadt, Germany. Ammonium bicarbonate (≥ 99%) and ammonium acetate (≥ 98%), all analytical grades, were purchased from Sigma Aldrich Chemie (Gmbh, Germany). Drug-free human plasma was obtained from the Department of Transfusion Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand.

2.3 Standard solution preparation and Calibration curve

The USP reference standard; amlodipine besylate and desipramine HCl were accurately weighed. Amlodipine primary stock standard solutions were prepared in methanol for used as calibration standards (CS) and quality control (QC) with a final concentration of 1.82 and 0.88 µg/mL, respectively. Working standard solutions were prepared by dilution of stock standard solution with 50% methanol to a final concentration of a linearity analytical detection range of 0.05 - 20.00 ng/mL. The final concentrations of 11 different concentration levels of the CS were detailed as 0.05, 0.10, 0.50, 0.75, 1.00, 5.00, 7.50, 10.00, 15.00, 17.50, 20.00 ng/mL. Four different concentration levels of the QC samples, LLOQ, LQC, MQC and HQC were at 0.05, 0.15, 8 and 19 ng/mL, respectively. All standard solutions and sample preparation were prepared under light-protected condition and stored at -70 ± 10 °C.

2.4 Sample preparation

Sample extraction by liquid-liquid extraction technique (LLE) was performed. Then chromatographic separation of amlodipine and an internal standard was carried on a LC-MS/MS system. All samples were prepared under light-protected condition. The sample extraction procedures, described in the result, were applied to samples for calibration standards, quality control (QC) and clinical samples.

2.5 Method validation

Before using the method for quantitative bioanalysis, the method was fully validated according to the United States Food and Drug: Administration Guidance for Industry: Bioanaly tical Method Validation; USFDA 2001. Calibration standard (CS) samples and quality control (QC) samples were prepared from spiking amlodipine standard solution in matrix-based sample for accuracy, precision and stability study. Each calibration curve included 2 blank samples (plasma with and without internal standard) and 11 concentrations of CS samples. Set of 6 replicate QC samples at concentrations of 0.05 (LLOQ), 0.15 (LQC), 8 (MQC) and 19 (HQC) ng/mL were used in validation studies. For stability studies, set of 4 replicate QC samples at LQC and HQC were used. The CS and QC samples were prepared by spiked amlodipine standard solution into pooled plasma from six sources of normal blank plasma. The validation met all accepted criteria of aforementioned guidance.

3. RESULTS AND DISSCUSION

3.1 Method development : LC-MS/MS

An optimum chromatographic separation incorporating with positive electrospray ionization mass spectrometry revealed high sensitivity and reproducibility of typical peak shape and retention time. The reverse phase column, Luna® HST C18 column (50 mm x 3 mm, 2.5 µm) (Phenomenex Inc.,Torrance, CA) was used with proper column temperature of 30±5˚C. An isocratic mobile phase was acetonitrile: 10 mM Ammonium acetate pH 4.5, (90: 10, v/v) with a flow rate of 0.2 mL/min at 5 minutes of total run time. All samples were placed in an auto-sampler organizer at 10±5˚C. The syringe cleaning system was operated before and after sample injection using 200 µL of a weak wash solvent, Milli Q water plus 90% (v/v) acetonitrile, then followed by 600 µL of a strong wash solvent, 100% propan-2-ol. We use ammonium acetate to prevent ion suppression in the LC-MS/MS system. The LC-MS/MS system has been completely optimized for the best specific experimental condition. A solution containing amlodipine standard or desipramine was directly infused into the electrospray ionization source. Manually fine tuning of mass spectrometer was operated by 5 µL/min flow of 500 ng/mL amlodipine standard solution through a t-connector between the LC system and the mass spectrometer. The amlodipine molecule ion can be identified and improve the detection specificity. The parameters of mass spectrometer applied throughout the experiment were source temperature of 120˚C, desolvation temperature of 350˚C, cone gas flow rate at 30 L/Hr, desolvation gas flow rate at 700 L/Hr. Compound dependent parameters for the best abundant and specific daughter ions were set as follows; voltage of the source with positive electrospray ionization (ESI+), capillary voltage of 4.0 kV, cone voltage of 12.0 V and 13 V of the collision energy. The full scan spectra showed prominent and stable product ions fragmentation without adduct ions of all compounds Figure 2. The LC-MS/MS analyses were performed in multiple reaction monitoring (MRM) detection mode, providing a high selectivity for the quantification of amlodipine. The mass transition ion-pairs at m/z 409.17>238.19 and 409.17>294.09 amu for $[\text{amlodipine} + H]^+$ and at m/z 267.09>208.06 amu for $[\text{desipramine} + H]^+$ were used.

3.2 Method development : sample extraction

The critical step in LC-MS/MS bioanalysis is sample extraction because it can significantly impact MS result by enhance or suppress ionization of the interesting substance. In this method, proper sample extraction workflow was determined. Amlodipine chemical

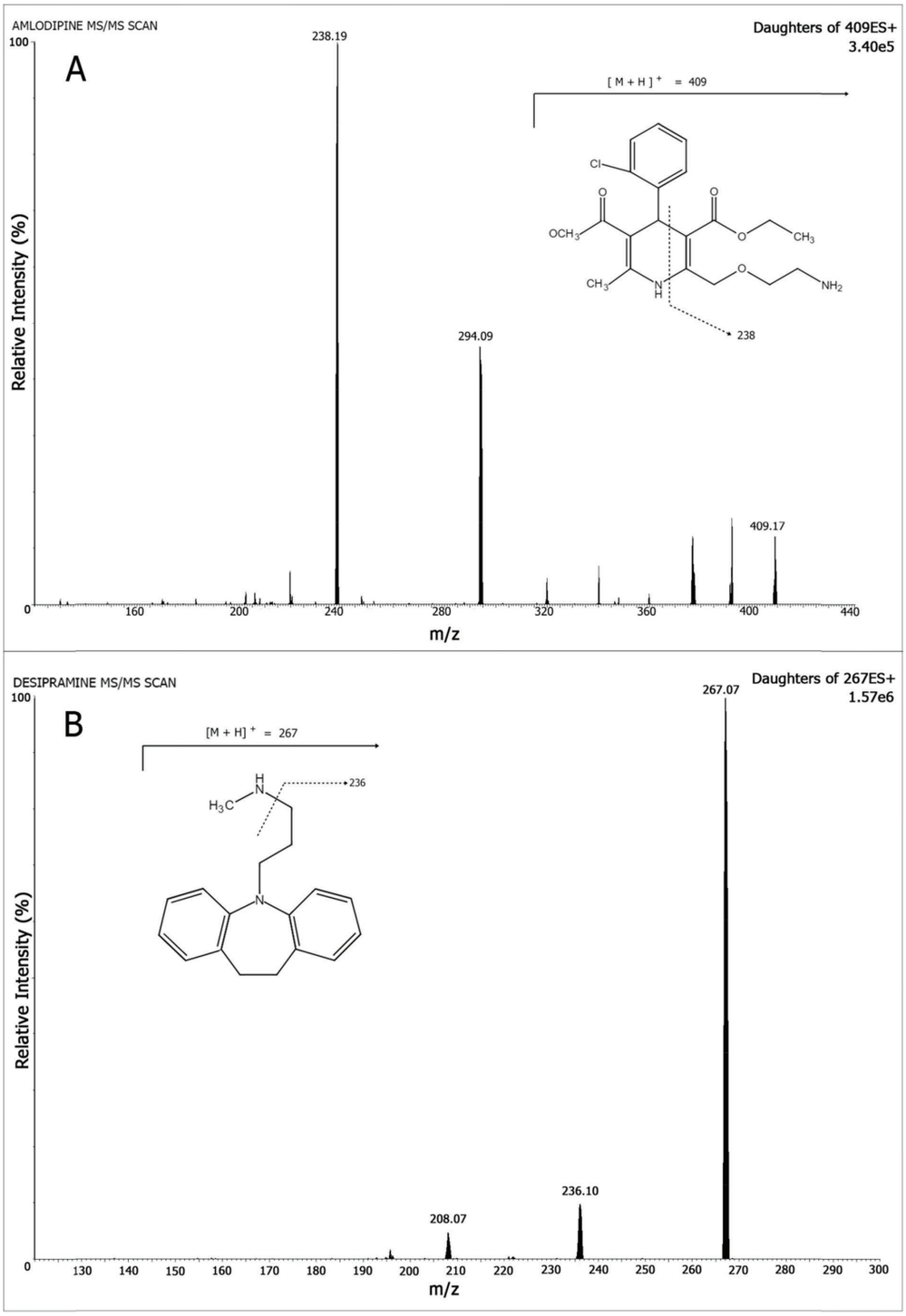

Figure 2. The product ions scan mass spectra and chemical structure of (A) Amlodipine and (B) Desipramine,(IS.).

formula was different from that of the prototype, nifedipine, by an addition of a basic amino side chain attached to the characteristic dihydropyridine ring. As the pK_a value is 8.6 and partition coefficient (octanol/water) value is 3.0, plasma samples were alkalinized because the pH above the pK_a value is important for quantitation of basic drugs in ESI+ LC-MS/MS[16]. We carefully investigated the use of various high pH modifiers in the sample treatment step, and also non-polar organic solvents in the extraction step. We found that an addition of 70 µL ammonium bicarbonate buffer (10 mM) at pH 10.0 into plasma sample and further extracted by a mixture of organic solvents, consisting of methyl-t-butyl ether and hexane, (80 : 20, v/v), resulted in satisfactory recovery and better peak shape of amlodipine and desipramine. Then the analytes were evaporated to dryness under a gentle stream of nitrogen gas at 30 ˚C. The residue was reconstituted and injected into the LC-MS/MS system. The representative LC-MS/MS chromatograms were shown in Figure 3 A-C with run time of 5 minutes. The retention times of amlodipine and desipramine were 1.63 and 1.69 minutes, respectively.

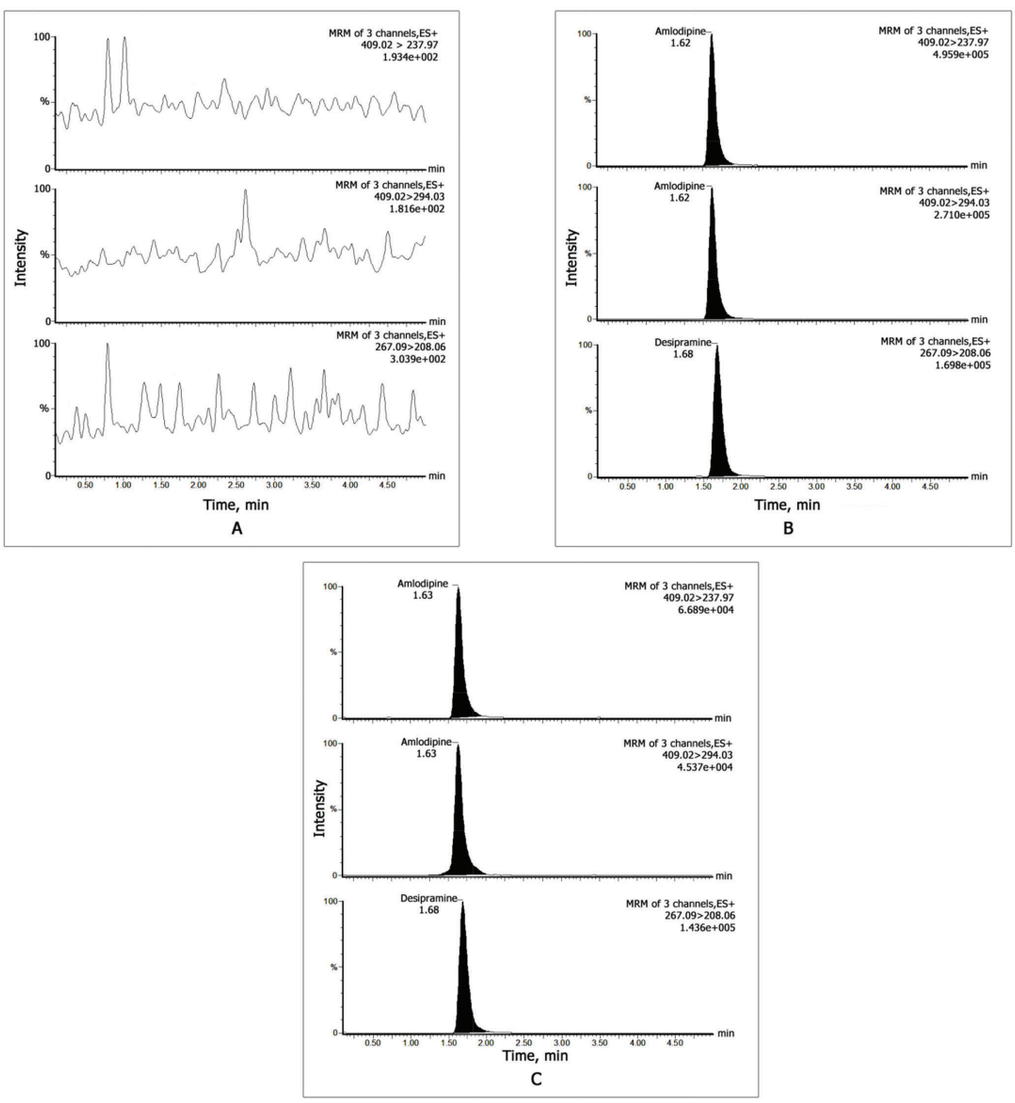

Figure 3. The representative LC-MS/MS chromatograms obtained from (A) blank human plasma sample, (B) Calibration standard sample at 15 ng/mL (C) clinical plasma sample at 48 h after an oral administration of 10 mg amlodipine in a fasting state. All of samples were processing by the developed extraction procedure and LC-MS/MS conditions.

3.3 Assay performance and validation

3.3.1 Linearity and sensitivity

This developed LC-MS/MS method has a detection limit value of 6 pg/mL and a lower limit of quantification (LLOQ) of 0.05 ng/mL. The eleven-point calibration curve was linear over the concentration range of 0.05-20 ng/mL. The best linear fit and least-squares residuals for the calibration curve were achieved with a 1/x weighing factor, resulted in a correlation coefficient of more than 0.995. The calibration curve was constructed by plotting the analyte to internal standard peak area ratio (y) against the analyte concentration (x), and was shown in Figure 4. The results of 3 sets of calibration curve were summarized in Table 1. The linearity of the present method was within the acceptance limit according to the USFDA guidance for bioanalytical method validation.

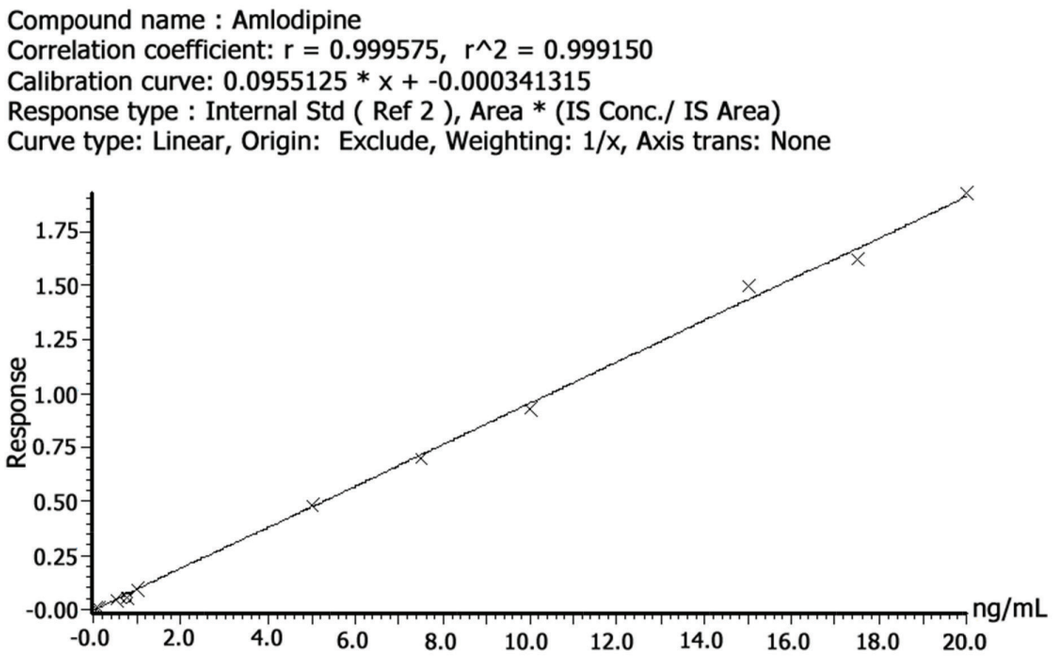

Figure 4. Calibration standard curve of amlodipine was constructed by plotting peak-area ratios of amlodipine plasma concentration to the IS. against nominal concentration, using weighted (1/x) least squares linear regression

Table 1. Precision and accuracy of amlodipine calibrations standards (CS) in human plasma obtained on the developed LC-MS/MS method.

CS Level (ng/mL)	1	2	3	4	5	6	7	8	9	10	11
Nominal Concentration	0.05	0.10	0.50	0.75	1.00	5.00	7.50	10.00	15.00	17.50	20.00
Measured Concentration[a]	0.05	0.10	0.51	0.68	0.95	4.92	7.51	9.90	15.16	18.16	19.44
% CV	4.20	13.98	0.18	3.54	6.46	6.31	1.66	1.82	3.05	6.13	4.26
% Accuracy	109.60	102.30	101.88	90.03	94.54	98.46	100.16	99.02	101.04	103.79	97.18

[a]Mean, n = 3 sets of calibration curve

3.3.2 Specificity and selectivity

Six different lots of human plasma were studied for the specificity. The representative LC-MS/MS chromatogram obtained from extracted plasma sample; human blank plasma (Figure 3(A)); calibration standard sample (Figure 3(B)) and clinical plasma sample at 48 h after an oral administration of 10 mg amlodipine in a fasting state (Figure 3(C)). The results demonstrated no interference at retention time from the endogenous plasma components and no cross-interference between amlodipine and IS. The developed method is selective and specific for amlodipine and IS.

3.3.3 Recovery of extraction and matrix effect

The recovery results showed highly consistent recovery coefficient in both analyte and IS. The mean absolute recovery of LQC, MQC and HQC are 82.45%, 89.17% and 88.21%, respectively for amlodipine and 100.07% for desipramine. The results suggested that the method is reproducible. Additionally, matrix effect was an important factor for evaluation because biological matrix could affect the signal of amlodipine in LC-MS/MS analysis. We noticed that there were a few differences between signals of the standards sample and the post-extracted spiked sample. The matrix factor values were 0.99, 1.04, 1.08 and 1.04 for LQC, MQC, HQC of amlodipine and IS, respectively. The results demonstrated no measurable matrix effect with % CV of 7.07, 4.81, 8.33 and 10.58 for LQC, MQC, HQC of amlodipine and IS, respectively.

3.3.4 Accuracy and precision

The within-run and between-run accuracy and precision of the analytical method were determined by six replicate analyses of amlodipine at four concentrations of the QC samples (LLOQ, LQC, MQC, and HQC) in three individual batches on three different days. The within-run for precision and accuracy ranged from 2.56 to 11.67% and from 80.40 to 117.80%, respectively. The between-run for precision and accuracy ranged from 5.97 to 12.70% and from 94.53 to 102.41%, respectively. All of precision and accuracy results were within the acceptance limit according to guidelines set by USFDA for bioanalytical method validation (Table 2). These data confirmed this newly developed method has precision, accuracy and reproducibility to determine amlodipine at level of 0.05 – 20 ng/mL in human plasma.

Table 2. Within-run and between-run precision and accuracy study for amlodipine quality control sample (QC).

	Nominal concentration (ng/mL)		Measured value (ng/mL) Mean ± SD	Accuracy		Precision	
				Within-run[b]	Between-run[c]	Within-run[b]	Between-run[c]
LLOQ	Day 1		0.05 ± 0.01	108.80		11.40	
	Day 2	0.05	0.05 ± 0.01	96.00	99.20	11.67	12.70
	Day 3		0.05 ± 0.00	92.60		9.29	
LQC	Day 1		0.14 ± 0.01	95.73		9.40	
	Day 2	0.15	0.14 ± 0.01	96.13	94.53	8.81	7.97
	Day 3		0.14 ± 0.01	91.73		5.38	
MQC	Day 1		7.91 ± 0.20	98.89		2.56	
	Day 2	8.00	7.98 ± 0.32	99.72	102.41	4.03	5.97
	Day 3		8.69 ± 0.47	108.63		5.40	
HQC	Day 1		18.73 ± 0.78	98.60		4.18	
	Day 2	19.00	20.31 ± 0.58	106.87	100.12	2.85	6.64
	Day 3		18.03 ± 1.11	94.91		6.15	

[b] Six replicates (n=6) at each concentration of QC level for within-run analysis.
[c] Three runs (n=18) at each concentration of QC level for between-run analysis.

3.3.5 Stability study

The stability of amlodipine in human plasma and stock standard solution were tested and summarized in table 3. Stability data were shown in the form of mean value obtained from 2 concentration of QC level (LQC and HQC, n = 3). Amlodipine was proved to be stable in human plasma at specified storage conditions for post-preparative stability, re-injection reproducibility, bench top stability, freeze-thaw stability (three cycle), long-term stability (100 days) with the percentage differences and precision (%CV) were within an acceptable range of ±15%. Accuracy of the observed mean concentration was within 85-115% of their respective nominal concentration. All of data were reflected situations of sample handling and analysis. The stability of amlodipine and desipramine in stock solution were evaluated for 30 days at -70 ± 10°C. We found that the stability results were within ±2% of their observed peak area at 30 days storage stock solution by respective peak area of freshly preparing stock solution. This indicated that amlodipine was stable in plasma during sample preparation process and storage conditions.

Table 3. Stability of amlodipine in different storage condition (n = 4)

Stability	Level	ng/mL	% CV	% Accuracy	% Change
Stock solution stability[d]					
Amlodipine					
6 hrs., 25 ± 2 °C	HQC	10000.00	0.41	101.67	1.67
30 days, -70 ± 10°C	HQC	10000.00	0.50	98.39	-1.61
Desipramine(IS.)					
6 hrs., 25 ± 2 °C	IS	10000.00	1.57	98.20	-1.80
30 days, -70 ± 10°C	IS	10000.00	4.33	98.20	-1.28
Post-preparative stability[e]					
24 hrs, 10 ± 5°C	LQC	0.15	3.15	103.87	0.26
(re-constitution samples)	HQC	19.00	5.93	102.21	-3.19
5 days –70 ± 10°C	LQC	0.15	13.15	92.80	-10.42
(dry samples)	HQC	19.00	5.28	96.07	-9.01
Re-injection reproducibility[e]	LQC	0.15	2.31	95.07	-2.06
24 hrs, 10 ± 5°C	HQC	19.00	1.57	107.48	-0.53
Bench top stability[e]	LQC	0.15	1.02	111.07	7.21
4 hrs., 25 ± 2°C	HQC	19.00	1.66	101.72	-3.66
Freeze-thaw stability[e](three cycle)	LQC	0.15	1.66	92.27	-10.94
24 hrs., –70 ± 10°C	HQC	19.00	5.83	107.02	1.36
Long-term stability[e]	LQC	0.15	8.04	103.60	0.00
100 days, –70 ± 10°C	HQC	19.00	3.76	101.94	-3.45

[d]Neat standard solution ; [e]QC samples

3.3.6 Application

The validated method has been successfully used to quantify amlodipine concentrations in human plasma samples after an administration of single oral dose of 10-mg-amlodipine in a bioequivalence study (Figure 5). The study was approved by the Institutional Review Board (IRB) of the Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand.

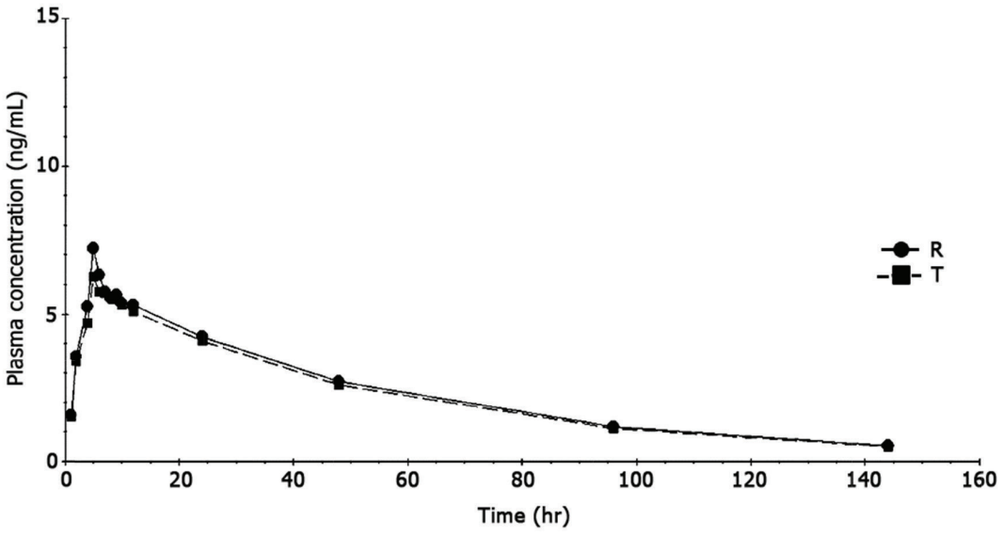

Figure 5. Representative data showing geometric mean of plasma concentration-time profiles of 28 healthy subjects after the administration of oral single dose of 10 mg of amlodipine

4. CONCLUSION

This newly developed LC-MS/MS method is highly sensitive, specific, and reproducible for quantification of amlodipine in human plasma with a wide linear dynamic range (0.05 to 20 ng/mL). It was validated and met all the requirements according to the USFDA standard guideline with high degree of accuracy and precision. In addition, the stability study indicated that amlodipine was stable in plasma during sample preparation process and storage conditions. It was also successfully applied in a bioequivalence study.

5. ACKNOWLEDGEMENTS

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REFERENCES

1. Benowitz NL. Antihypertensive Agents. In: Katzung BG, Masters SB, Trevor AJ. Editors. Basic & Clinical Pharmacology, 12th ed. New York: McGraw-Hill; 2012. p.183
2. Michel T, Hoffman BB. Treatment of myocardial ischemia and hypertension. In: Brunton LL, editor. Goodman & Gilman's pharmacological basis of therapeutics, 10th ed. New York: McGraw-Hill; 2011. p.776-777
3. Brunton LL, Parker KL, editor. Goodman & Gilman's manual of pharmacology and therapeutics. New York: McGraw-Hill; 2008. p.544-60.
4. Choi SM, Kim JE, Ahn BO, Kwon JW. Effects of Amlodipine Orotate on hypertension-related complications in spontaneously hypertensive rats. *Arzneimittel*

Forschung - Drug Research. 2006; 56(1): 12-17.

5. Craig CR, Stitzel RE. Modern Pharmacology with Clinical Applications, 6th ed . Philadelphia: Lippincott Williams & Wilkins; 2004. p.218-24.
6. Talele GS, Porwal PK. Development of validated bioanalytical HPLC-UV method for simultaneous estimation of Amlodipine and Atorvastatin in rat plasma. Indian J Pharm Sci. 2015; 77(6): 742–50.
7. Zarghi A, Foroutan SM, Shafaati A, Khoddam A. Validated HPLC method for determination of amlodipine in human plasma and its application to pharmacokinetic studies. Il Farmaco. 2005; 60(9): 789–92
8. Porwal PK, Talele GS, Development of validated HPLC-UV method for simultaneous determination of Metformin, Amlodipine, Gilbenclamide and Atorvastatin in human plasma and application to protein binding studies. Bulletin of Faculty of Pharmacy, Cairo University. 2017; 55: 129–39.
9. Basavaiah K, Chandrashekar U, Nagegowda P. Spectrophotometric and high performance liquid chromatographic determination of Amlodipine Besylate in pharmaceuticals. ScienceAsia. 2005; 31: 13-21.
10. Francis MP, Muralidharan S, Rajan S, Nagarajan, Suresh B. Simple and validated method for estimation of Amlodipine by LC-MS (ESI) using healthy Indian human volunteers: and evaluation of Pharmacokinetic parameters. Journal of Bioanalysis & Biomedicine. 2010; 2(3): 69-74.
11. Gonzalez O, Alonso RM, Ferreiros N, Weinmann W, Zimmermann R, Dresen S. Development of an LC-MS/MS method for the quantitation of 55 compounds prescribed in combine cardiovascular therapy. Journal of Chromatography B. 2011; 879: 243-52.
12. Ramani AV, Sengupta P, and Mullangi R. Development and validation of a highly sensitive and robust LC-ESI-MS/MS method for simultaneous quantitation of simvastatin acid, amlodipine and valsartan in human plasma: application to a clinical pharmacokinetic study. Biomedical Chromatography. 2009; 23: 615–22.
13. Carvalho M, Oliveira CH, Mendes GD, Sucupira M, Moraes ME, De Nucci G. Amlodipine bioequivalence study: quantification by liquid chromatography coupled to tandem mass spectrometry. Biopharmaceutics & Drug Disposition. 2001; 22(9): 383-90.
14. Ravi VB, Inamadugu JK, Pilli NR, Sreenivasulu V, Ponneri V. Simultaneous determination of telmisartan and amlodipine in human plasma by LC–MS/MS and its application in a human pharmacokinetic study. Journal of Pharmaceutical Analysis. 2012; 2(5): 319-26.
15. U.S. Department of Health and Human Services, Food and Drug Administration. Center for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CVM), Guidance for Industry Bioanalytical Method Validation, Rockville, Maryland. May 2001 BP.
16. Hendriks G, Uges DRA, Franke JP. pH adjustment of human blood plasma prior to bioanalytical sample preparation. Journal of Pharmaceutical and Biomedical Analysis. 2008;47: 126–33.