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Research article

Development and validation of liquid chromatography tandem mass spectrometrymethod for the quantification of manidipine in human plasma

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LC-MS/MS; Manidipine; Desipramine; Liquid-liquid extraction

ABSTRACT

A sensitive and selective liquid chromatography tandem mass spectrometric (LC-MS/MS) analytical method has been validated to quantify manidipine in human plasma using desipramine as an internal standard (IS). Liquid-liquid extraction (LLE) with a mixture of methyl-*t*-butyl ether and hexane (4:1, v/v) was used for sample preparation. The separation of analytes and internal standard was performed on a C_{18} column (3 mm x 50 .mm, particle size 2.5 µm) with gradient elution of (A) 0.05% formic acid and (B) acetonitrile. The mass spectrometry method was performed employing positive electrospray ionization (ESI) operating in multiple reaction monitoring (MRM) mode, monitoring the transitions of m/z 610.98 > 166.95 for manidipine and m/z 266.95 > 235.94 for the IS. The total analytical run time was 6 min. The calibration curve was linear over manidipine concentrations ranging from 0.1 ng/mL to 20 ng/mL in plasma with a correlation coefficient (r^2) of 0.995 or better. The lower limit of quantification (LLOQ) and limit of detection (LOD) for manidipine were 0.1 ng/mL and 0.0125 ng/mL, respectively. Accuracy and precision were within the acceptance criteria of the United States (US) Food and Drug Administration (FDA) guidelines. The rapid and highly sensitive LC-MS/MS method has been developed and successfully applied to a bioequivalence study of manidipine in Thai healthy volunteers after oral administration.

1. INTRODUCTION

Hypertension is a major risk factor for cardiovascular disease and mortality worldwide. Uncontrolled high blood pressure, not only increases health problem and risk of cardiovascular disease but also put burden on public sector's healthcare expenditure. Manidipine is a third-generation dihydropyridine calcium blocking agent. It inhibits the calcium influx through L-type calcium channels in peripheral vascular and coronary smooth muscle cells, and found to be efficacious in the treatment of hypertensive patients. Currently, many generic manidipine products have been released and generic substitution is common. The reliable and sensitive bioanalytical method for manidipine determination in human plasma is required in order to conduct bioequivalence studies of generic products[1].

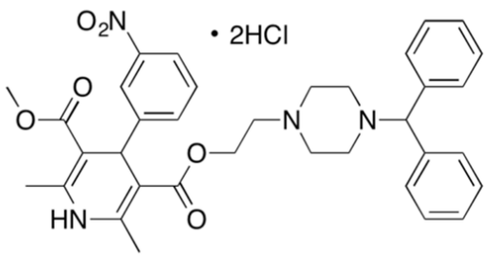
Several bioanalytical methods for determination of manidipine have been used include HPLC with UV detection[2-6], HPLC with electrochemical detection[7], and LC-MS/MS[8-11]. However, these methods have high quantification limit, long run-time, and require large plasma volume[2-10]. Although Park et al. developed a sensitive LC-MS/MS method with a LLOQ of 0.07 ng/mL, this method requires laborious solid-phase extraction which is expensive and time consuming [11].

The present study aimed to develop a sensitive, specific, rapid and economical bioanalytical method for the quantification of manidipine in human plasma. The method has been developed and fully validated following USFDA guidance for bioanalytical method validation[12]. The validated method of this study found to be superior to previous reports in terms of the lower limit of quantification, plasma sample volume, and total analytical run time in comparison to previous reports[2-10]. And this method was successfully applied to support a bioequivalence study of manidipine hydrochloride 20 mg in Thai healthy volunteers.

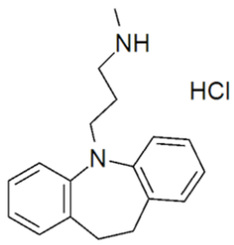
2. MATERIALS AND METHODS

2.1. Chemical and reagents

Manidipine dihydrochloride (Purity 99.22%) was obtained from Dong in Chemical Co., Ltd., KOREA using desipramine hydrochloride (Purity 99.8%) as internal standard (IS) from the United State Pharmacopieal Convention, Inc., USA. Their structures were presented in Figure 1.



Manidipine dihydrochloride



Desipramine hydrochloride

Figure 1. Chemical structure of manidipine dihydrochloride and desipramine hydrochloride

Other reagent such as HPLC grade of acetonitrile, analytical reagent grade of acetic acid and ammonia solution were purchased from Merck, Darmstadt, Germany. HPLC grade of methanol and propan-2-ol, analytical reagent grade of hexane and methyl-*t*-butyl ether were purchased from Scharlau, Barcelona, Spain. Type I water or Milli Q water was obtained from water purification system, Millipore Corporation, USA. Drug free human plasma was obtained from the Department of Transfusion Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand.

2.2. Instrumentations

The LC-MS/MS system consisted of Acquity Ultra Performance LC™ from Waters Co., Ltd. (USA) and mass analyzer Quattro Micro™ (Micromass Technologies, UK). The chromatographic separation was performed on the Luna HST C_{18} column (3 mm x 50 mm, particle size 2.5 µm) with OPTI-GUARD column from Phenomenex Ltd., USA. The system control and data analysis were performed with MassLynx software version 4.1 SCN627 (Micromass Technologies, UK).

2.3. Liquid chromatographic and mass spectrometric conditions

Acquisitions of MS parameters such as temperature, gas flow rate, voltage, etc., were optimized in manual tune mode. Manually tuning of MS was operated by direct injection of manidipine and IS working solution at the concentration of 500 ng/mL and flow rate of 10 mL/min into the

ESI source of the mass spectrometer. The multiple reaction monitoring (MRM) transitions in both positive and negative ESI mode were tested. The results showed that the ESI operated in positive mode gave more intense response for manidipine and IS. The optimized parameter of mass spectrometry for the best abundant and specific daughter ions were set as follows: source temperature at 120°C, desolvation temperature at 350°C, cone gas flow rate at 30 L/Hr and desolvation gas flow rate at 550 L/Hr. In addition the capillary voltage was set at 1 kV, cone voltage at 40 V for manidipine and 30 V for IS. The collision energies for the manidipine and IS fragmentation were set to 25 eV and 16 eV, respectively.

For the LC conditions, various mobile phase compositions and different analytical columns were evaluated and optimized through several trials to achieve the best separation, symmetrical peak shape and short total analytical run time. Based on literature review, the analyte was separated in acetonitrile, methanol and water[7,13-14]. The the mixture of (A) Milli Q water and (B) acetonitrile was chosen for shorter run time and better response. Formic acid and ammonium acetate with different pH range, ratios were tried as the modifiers. Formic acid was selected since it was easily miscible in Milli Q water and improved peak symmetry and ionization efficiency for manidipine and IS. Several chromatographic conditions were attempted using Luna HST C_{18} column (3 mm x 50 mm, particle size 2.5 µm) and Kinetex Hilic column (2.10 mm x 50 mm, particle size 1.7 µm). Separation was carried out on Luna HST C18 column which produced better peak shape and response, even at the LLOQ. For the gradient elution, two solvents were used: (A) 0.05% formic acid and (B) acetonitrile. The mobile phase composition was held initially at 40% (B) for 0.5 min and then changed during the linear gradient elution from 40% (B) to 85% (B) run up to 2.00 min and held to 2.20 min. The mobile phase was changed from 85% (B) to 100% (B) run up to 4.50 min and held to 4.80 min. The mobile phase was then returned to 40% (B) run up to 5.50 min and held to complete run time of 6 min. The sample injection volume was 10 mL. The flow rate of mobile phase was adjusted to 0.20 mL/min. The column and auto-sampler were maintained at 30±5°C and 5±2°C, respectively.

2.4. Sample preparations

The 20 µL of IS working solution (desipramine; 250 ng/mL) was mixed with 200 µL of manidipine spiked plasma and 1 mL of methyl-*t*-butyl ether and hexane, 4:1 (v/v). The mixture was vortexed for 10 min and centrifuged for 15 min at 10,000 rpm. After centrifugation, 800 µL of supernatant was transferred to evaporate under nitrogen stream at 30 °C until dry. The residue was reconstituted with 100 µL of acetonitrile and Milli Q water, 1:1 (v/v). The reconstituted sample was mixed and centrifuged at 10,000 rpm for 10 min. Finally, the supernatant was transferred to a vial and injected (10 µL) into the LC-MS/MS system for the analysis. All samples were protected from light during these processes.

2.5. Preparation of stock and working solutions

The stock solutions of manidipine and desipramine (IS) were prepared by separately dissolving in 100% methanol at concentration of 800 mg/mL and 600 mg/mL (calculated for the free base), respectively. The working solutions for manidipine were prepared from stock solutions by serial dilution with 50% methanol to provide working solution concentrations ranged from 2-400 ng/mL. These diluted working solutions were used to prepare the calibration curve and quality control samples. The working solutions for IS were prepared by diluting stock solution in 50% methanol to obtain the concentration of 250 ng/mL. All stock and working solutions were prepared and protected from light then stored in -70±10 °C freezer.

2.6. Preparation of calibration curves and quality control samples

A ten-point standard calibration curve ranged of 0.1 to 20.0 ng/mL for manidipine was prepared by spiking the drug free human plasma with manidipine working solution.

Quality control samples were also prepared in a similar manner with final concentration of 0.1 ng/mL for LLOQ, 0.3 ng/mL for LQC, 9 ng/mL for MQC and 18 ng/mL for HQC.

2.7. Method validation

The LC-MS/MS method was validated

in accordance to the industry guidance, Bioanalytical Methods Validation, U.S. Department of Health and Human Service, Food and Drug Administration, Center for Drug Evaluation and Research (USFDA CDER, 2001, BP)[12].

2.7.1. Specificity and selectivity

Six different sources of drug free human plasma were screened for interference of analyte and IS. Subsequently, 6 sources of drug free human plasma with no interference were selected and pooled for full method validation.

2.7.2. Limit of detection (LOD) and Lower limit of quantification (LLOQ)

According to the FDA's guidance for bioanalytical method validation, LOD and LLOQ were determined from the signal-to-noise ratio (S/N) of the analyte peak. The analyte signal to noise ratio (S/N) should be at least 3 times and 5 times for the LOD and the LLOQ, respectively.

2.7.3. Linearity

Three independent calibration curves were evaluated to validate the linearity of the method. Each calibration curve consisted of ten non-zero concentrations of manidipine range of 0.1 to 20.0 ng/mL. Blank samples (plasma without analyte and IS) and zero sample (plasma with IS) were also analyzed, but were not used to construct the calibration curve. The correlation coefficient (r^2) of 0.995 or better was desirable for all calibration curves.

2.7.4. Accuracy and precision

Intra-day accuracy and precision were determined by analyzing six replicates of QC samples at four concentrations (LLOQ, LQC, MQC and HQC) within a batch. Inter-day accuracy and precision were determined by analyzing eighteen replicates at four concentrations from three analytical runs on two consecutive days.

2.7.5. Recovery of extraction

The extraction recovery of manidipine was measured by analyzing six replicates of QC samples at LQC, MQC and HQC, whereas IS was measured at a concentration of 25 ng/mL. Recoveries of manidipina and IS were determined by comparing the peak areas of plasma spiked standards before extraction (pre-extraction) to the peak areas of plasma spiked standards after extraction (post-extraction).

2.7.6. Stability

Stability tests were performed to evaluate the manidipine stability in stock solutions and plasma samples under different conditions. The stock solution stability of manidipine (10,000 ng/mL) and IS (1,000 ng/mL) were performed on solutions stored at room temperature (25±2 °C) for 6 h and at −70±10 °C for 35 days. Stability tests in plasma were performed at LQC, MQC and HQC levels using three replicates at each level. To evaluate short-term stability, QC samples were frozen, thawed and placed at room temperature for 6 h. Freeze and thaw stability was evaluated after three cycles from −70±10 °C to room temperature. Post-preparative stability was evaluated after storing the reconstituted samples in the autosampler at 5±2 °C for 18 h and dry samples at -70 ±10 °C for 5 days. Long term stability was evaluated after storing QC samples at -70±10°C for 314 days. The stability was calculated by comparing the test results for stability samples at three concentrations with freshly prepared samples.

2.7.7. Anticoagulant effect

The effects of two different types of anticoagulants were tested, lithium heparin which used in clinical sample and citrate-phosphate-dextrose (CPD) which used for method validation. The anticoagulant effect was assessed by analyzing six replicate QC samples at LQC, MQC and HQC for each anticoagulant.

2.7.8. Matrix effect

Matrix effect was evaluated in six different sources of drug free human plasma. Each source was analyzed in tripicate at three manidipine concentrations (LQC, MQC and HQC) and 25 ng/mL for IS. Matrix factor (MF) of manidipine and IS was determined by comparing the peak areas of plasma spiked standards after extraction (post-extraction) to the peak areas of neat standard solutions (un-extraction). Subsequently, IS-normalized MF

was calculated by dividing the matrix factor of manidipine by the matrix factor of IS.

3. RESULTS AND DISCUSSION

3.1. Method development

For sample preparation, liquid-liquid extraction (LLE) with different proportions of methyl-*t*-butyl ether and hexane were studied. The methyl-*t*-butyl ether: hexane mixture in a 4:1 (v/v) proportion gave the highest recovery with good peak shape of both manidipine and IS. The plasma volume required for the present method was 200 µL which was less than the previous studies[2-3,6-8].

The mass spectrometry method was performed employing positive electrospray ionization (ESI) operating in multiple reaction monitoring (MRM) mode. The precursor ion scan spectra for manidipine and IS were dominated by protonated molecules [M+H]+ m/z 610.98 and 266.95, respectively. The MRM acquisitions were performed using the transition 610.98 > 166.95 for manidipine and 266.95 > 235.94 for IS (Figure 2). The collision energies and the cone voltages were optimized to achieve the highest sensitivities. The collision energies were 25 and 16 eV for manidipine and IS, respectively. The cone voltages were applied at 40 and 30 V for manidipine and IS, respectively.

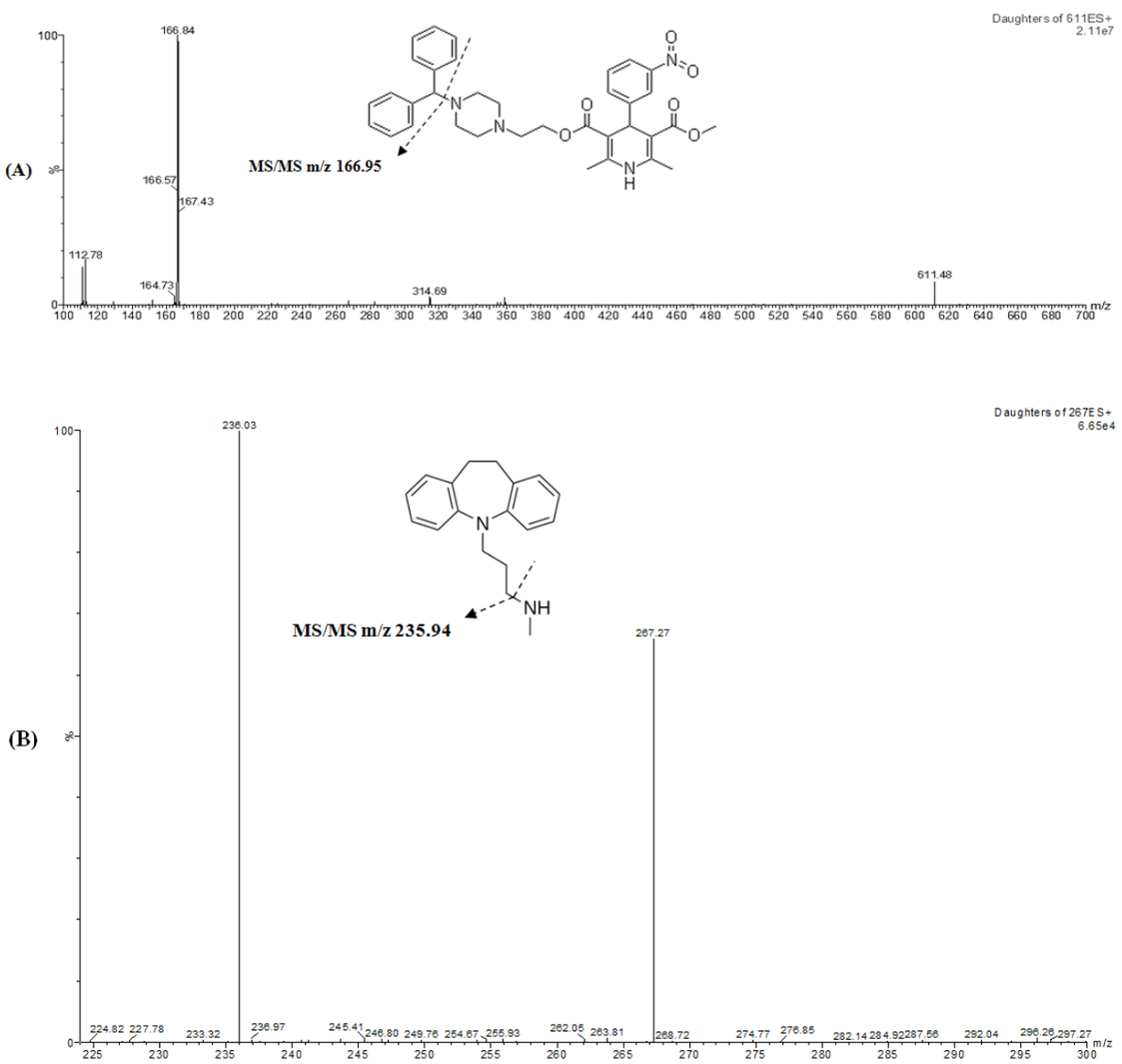


Figure 2. Product ion MS/MS spectra of manidipine (A) and desipramine (B)

In reversed phase liquid chromatography, a good separation and symmetric peak shapes were obtained by using mobile phase consisting of 0.05% formic acid and acetonitrile on Luna HST C18 column (3 mm x 50 mm, particle size 2.5 µm) with gradient elution. Moreover, this method demonstrated a shorter total run time (6 min)[3,5,9] and required smaller injection volume [2-7,9] when compared with the previous study.

3.2. Method validation

3.2.1. Specificity and selectivity

The specificity and selectivity of the intended method were established by screening the drug free human plasma from six different sources. No interfering peak was observed in the chromatograms at the retention time of manidipine and IS. Figures 3A–3C demonstrate the specificity and selectivity of the method with the chromatograms of drug free human plasma, LLOQ samples and clinical plasma samples, respectively.

3.2.2. Limit of detection (LOD) and lower limit of 0quantification (LLOQ)

The LOD of manidipine was found to be 0.0125 ng/mL. The LLOQ was 0.1 ng/mL, which offers high sensitivity enough for manidipine determination in human plasma.

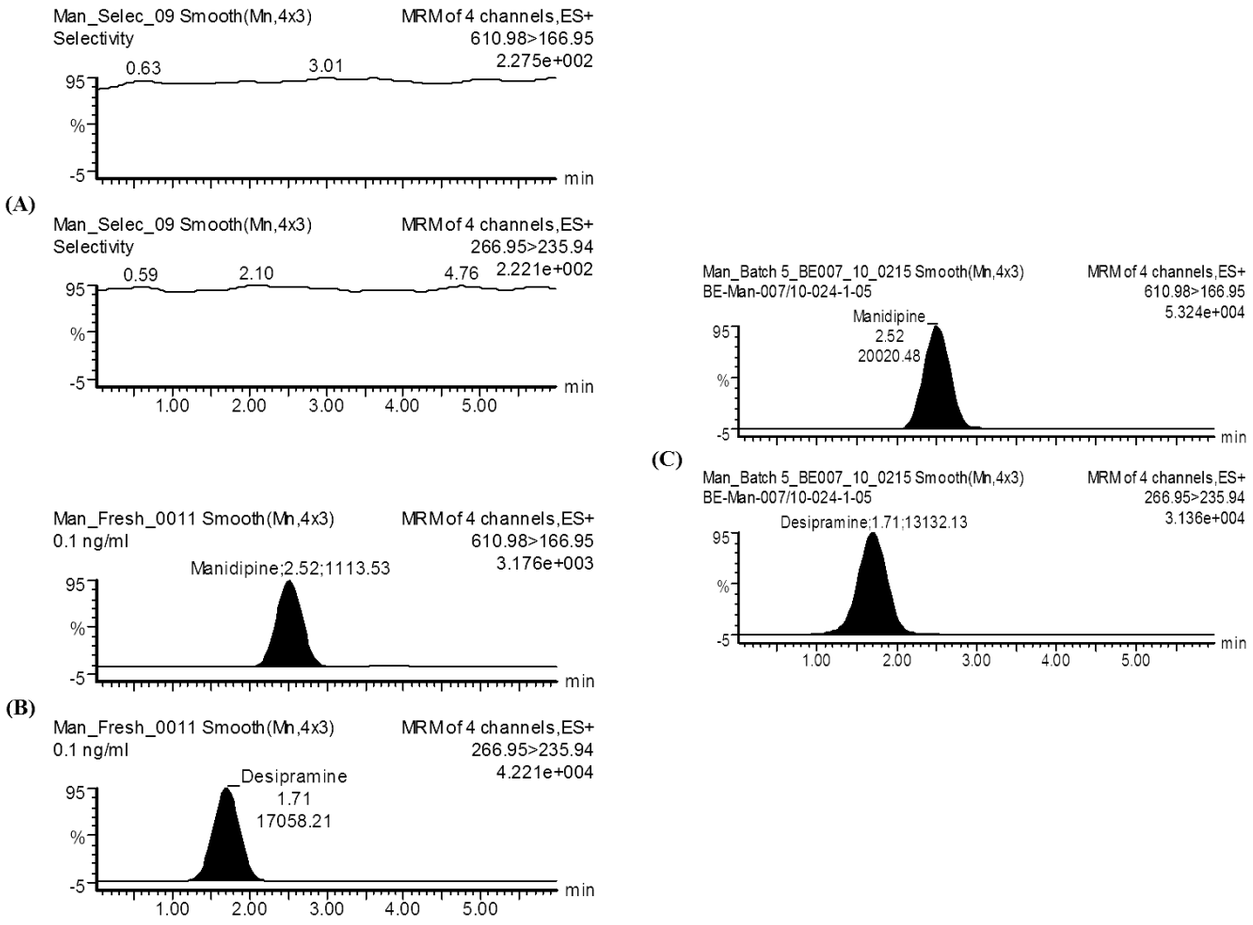


Figure 3. The chromatogram of extracted drug free human plasma (A), LLOQ sample at 0.1 ng/mL (B) and clinical plasma sample at 1.5 h after an oral administration of 20 mg manidipine hydrocloride tablets (C)

3.2.3. Linearity

As shown in Figure 4, the calibration curve exhibited good linearity within the range 0.1-20 ng/mL. The equation model was obtained by weighted least squares linear regression analysis with a weighting factor of 1/x (x = concentration). The correlation coefficient (r^2) was found to be ≥ 0.995. The results of the three sets of calibration curve are presented in Table 1. The mean back-calculated concentrations were meeting acceptance criteria for accuracy and precision.

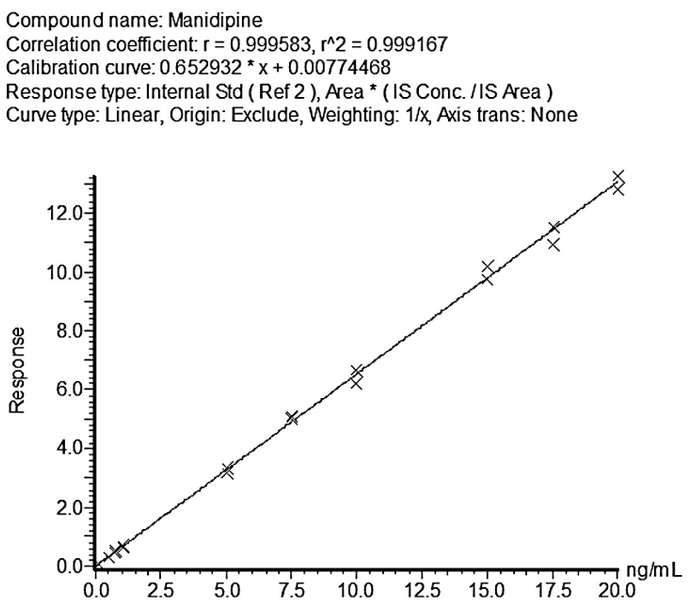


Figure 4. The calibration curve of manidipine

Table 1. The accuracy and precision of calibration curve for manidipine in human plasma.

Nominal concentration (ng/mL)	0.1	0.5	0.75	1	5	7.5	10	15	17.5	20
Mean (n=3)	0.1046	0.5026	0.7321	0.9768	4.9219	7.6581	9.9540	14.9153	17.0364	20.5483
S.D. (±)	0.0108	0.0255	0.0568	0.0435	0.2578	0.1319	0.2511	0.4071	1.3815	0.5984
Accuracy (%)	104.60	100.52	97.61	97.68	98.44	102.11	99.54	99.44	97.35	102.74
CV (%)	10.33	5.07	7.76	4.45	5.24	1.72	2.52	2.73	8.11	2.91

n = number of replicate

3.2.4. Accuracy and precision

The intra-day and inter-day precision and accuracy data are summarized in Table 2 and Table 3, respectively. The intra-day and inter-day precision, expressed as %CV were not exceed 20% for LLOQ and 15% for other concentrations. The intra-day and inter-day accuracy deviation values were within 20% of norminal concentration for LLOQ and within 15% for other concentrations. The results revealed satisfactory precision and accuracy of the present method.

3.2.5. Recovery of extraction

The recovery of manidipine at LQC, MQC and HQC were 90.19%, 83.26% and 81.52%, respectively. The recovery of IS was 96.17%. These results ensured that the extraction was efficient and reproducible.

Table 2. The intra-day accuracy and precision of manidipine in human plasma.

Nominal concentration (ng/mL)	LLOQ 0.1	LQC 0.3	MQC 9	HQC 18
Batch 1				
Mean (n=6)	0.1044	0.3181	9.3839	19.2235
S.D. (±)	0.0110	0.0064	0.3897	0.8336
Accuracy (%)	104.40	106.03	104.27	106.80
CV (%)	10.54	2.01	4.15	4.34
Batch 2				
Mean (n=6)	0.1081	0.3036	9.0212	17.3845
S.D. (±)	0.0021	0.0120	0.1364	0.2281
Accuracy (%)	108.10	101.20	100.24	96.58
CV (%)	1.94	3.95	1.51	1.31
Batch 3				
Mean (n=6)	0.0941	0.3060	9.4660	17.8520
S.D. (±)	0.0104	0.0378	0.2855	0.7328
Accuracy (%)	94.10	102.00	105.18	99.18
CV (%)	11.05	12.35	3.02	4.10

n = number of replicate

Table 3. The inter-day accuracy and precision of manidipine in human plasma.

Nominal concentration (ng/mL)	LLOQ 0.1	LQC 0.3	MQC 9	HQC 18
Mean (n=18)	0.1022	0.3092	9.2904	18.1534
S.D. (±)	0.0103	0.0227	0.3371	1.0112
Accuracy (%)	102.20	103.07	103.23	100.85
CV (%)	10.08	7.34	3.63	5.57

n = number of replicate

3.2.6. Stability

Stock solutions stability were tested and summarized in Table 4. No significant difference was found for both manidipine and IS after storage at 25±2ºC for 6 h or -70 ±10 °C for 35 days.

The stability studies of manidipine in human plasma were conducted and summarized in table 5. Manidipine was stable in human plasma at 25±2ºC for 6 h, -70±10 °C for 314 days and after three cycles of freeze and thaw. The extracted samples of manidipine were stable in auto-sampler at 5±2 °C for 18 h and in freezer at -70 ±10 °C for 5 days. Accuracy of mean concentrations was within 85-115% of their nominal concentrations and % variation were all within ±15%.

Table 4. The Stock solution stability of manidipine and desipramine.

Conditions	Nominal concentration (ng/mL)	Peak Areas Mean (n=3)	S.D. (±)	Variation (%)
Stock solution stability at 25±2 ºC for 6 h	Manidipine (10000 ng/mL)	32428110	2163717.9	1.27
	Desipramine (1000 ng/mL)	326858.53	6121.1421	1.50
Stock solution stability at -70 ±10 °C for 35 days	Manidipine (10000 ng/mL)	31869946	955318.20	0.48
	Desipramine (1000 ng/mL)	326894.65	2535.1448	1.49

n = number of replicate

Table 5. The stability of manidipine in human plasma.

Conditions	Nominal concentration (ng/mL)	Mean (n=3)	S.D. (±)	Accuracy (%)	Variation (%)
Short-term stability at 25±2 ºC for 6 h	LQC (0.3)	0.3159	0.0041	105.30	1.77
	MQC (9)	8.7997	0.1119	97.77	8.95
	HQC (18)	18.6988	0.3243	103.88	2.12
Freeze and thaw stability at -70±10 °C for 3 cycles	LQC (0.3)	0.2815	0.0071	93.83	9.31
	MQC (9)	8.5046	0.3235	94.50	12.00
	HQC (18)	17.7571	0.1606	98.65	7.05
Long-term stability at -70±10 °C for 314 days	LQC (0.3)	0.2641	0.0073	88.03	14.92
	MQC (9)	8.5514	0.0692	95.02	11.51
	HQC (18)	17.4379	1.2489	96.88	8.72
Post-preparative stability at 5±2 °C for 18 h	LQC (0.3)	0.2913	0.0097	97.10	6.15
	MQC (9)	9.7539	0.2093	108.38	0.93
	HQC (18)	19.2750	0.8604	107.08	0.90
Post-preparative stability at -70 ±10 °C for 5 days	LQC (0.3)	0.3039	0.0128	101.30	2.09
	MQC (9)	9.1822	0.0853	102.02	4.99
	HQC (18)	17.7253	0.4229	98.47	7.22

n = number of replicate

3.2.7. Anticoagulant effect

Among LQC, MQC, and HQC level, the accuracy of CPD and lithium heparin were ranged from 88.98-94.29% and 98.08-107.24%, respectively. While the %CV of CPD and lithium heparin were not exceed 15%. The results indicated that there was no anticoagulant effect in this study.

3.2.8. Matrix effect

For manidipine, the matrix factor calculated at LQC, MQC and HQC were in range of 0.85-0.92 with %CV ranged from 3.41-4.71%. The matrix factor of IS was 0.98, with %CV of 3.06%. The IS normalised MF ranged from 0.87-0.94 with %CV ranged from 4.26-5.56%. This results indicated that there was negligible ionization suppression or enhancement from the plasma matrix in this study.

3.2.9. Application

The validated method was successfully applied to support a single dose bioequivalence study of 20 mg manidipine hydrochloride tablets in 38 healthy Thai volunteers (Figure 5). The study protocol was approved by Institutional Review Board of Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand.

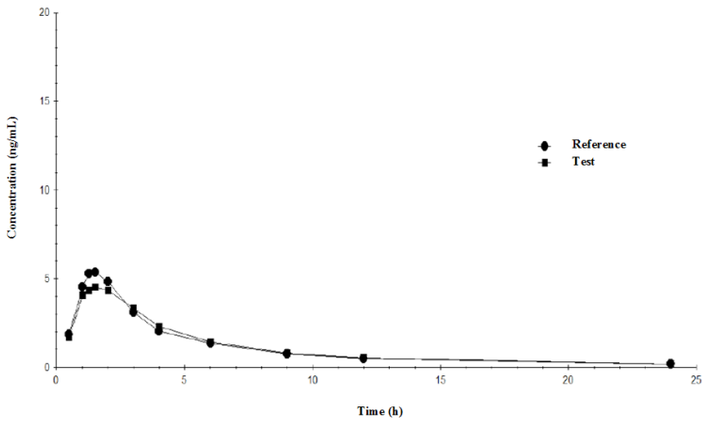


Figure 5. Mean plasma concentration-time profile of manidipine after oral administration of single dose of 20 mg manidipine hydrochloride tablets in 38 Thai healthy volunteers

4. CONCLUSIONS

In the present study, a simple, sensitive, specific and rapid LC-MS/MS method for the determination of manidipine in human plasma was successfully developed. We found that the lower limit of quantification, plasma sample volume and analytical run time are superior to those obtained from the previous reports. The method was fully validated and met all the requirements according to the USFDA guidance. It was successfully applied to the bioequivalence study of 20 mg manidipine hydrochloride in healthy volunteers.

5. ACKNOWLEDGEMENTS

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Conflict of interest (If any)

There is no potential conflict of interest.

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Ethical approval

The Institutional Review Board of Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand approved the study protocol. (COA no. Si650/2010)

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