



Original Article

Determination of amlodipine in human plasma by electrospray ionization LC-MS/MS method: validation and its stability studies

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Received 18 April 2007; Accepted 4 July 2008

Abstract

A sensitive and specific high-performance liquid chromatography combined with electrospray ionization (ESI) tandem mass spectrometry (LC-MS/MS) method, operating in the positive ionization mode, for quantifying of amlodipine in human plasma using tizanidine as internal standard (I.S.) was developed and validated. The analyte and I.S. were extracted by simple one step liquid/liquid extraction with a mixture of diethylether/dichloromethane (70/30, v/v). The chromatographic separation was performed on a C₁₈ analytical column under isocratic conditions using a mixture of 10mM ammonium formate/methanol/acetonitrile (30/50/20, v/v/v) as mobile phase at a flow rate of 1.0 mL/min. Total chromatographic run time was 5.0 min. Detection was performed on a API 2000 QTRAP quadrupole linear ion trap mass spectrometer via turbo ion spray ionization. Quantitation was performed using multiple reaction monitoring (MRM) mode to study parent → product ion transitions of m/z 409.4 → 238.1 for amlodipine and m/z 254.2 → 44.1 for I.S., respectively. The validation and stability studies were performed according to the Thai FDA guidance for assessment of bioequivalence study in Thailand. The results were within the accepted criteria as stated in the aforementioned guidance. Linearity in plasma was obtained over the concentration range 0.3-15.0 ng/mL, with a coefficient of determination (r^2) of 0.9993. Lower limit of quantification (LLOQ) was identifiable and reproducible at 0.3 ng/mL. The within- and between-run precision values were below 10% and the accuracy was ranged from 94.87 to 102.44% at all three quality controls samples levels. The analyte was found to be stable in plasma samples under three freeze-thaw cycles, long-term storage (3 months at -20°C), short-term storage (4 hours at room temperature), post-preparative and stock-solution stability. The robust and rapid LC-MS/MS method has been successfully applied for routine assay to support bioequivalence or pharmacokinetics studies of amlodipine administered as a single oral dose (10 mg tablet) to Thai healthy volunteers.

Keywords: amlodipine, LC-MS/MS, human plasma, validation, stability study

1. Introduction

Amlodipine is a calcium-channel blocking agent of the basic dihydropyridine derivative, inhibits the calcium influx through slow channels in peripheral vascular and coronary smooth muscle cells, and thus is useful in the

management of hypertension and angina pectoris (Reid *et al.*, 1988; Murdoch, and Heel, 1991; Haria, and Wagstaff, 1995). Amlodipine is well tolerated and does not appear to cause some of the undesirable effects often associated with other cardiovascular agents. However, it has low plasma concentration because after oral administration, amlodipine has a long elimination half-life of 40-50 h due to the large volume of distribution (21 L/kg) and usually is rapidly oxidized enzymatically to pyridine metabolites (Abernethy, 1989; Kelly and O'Malley, 1992; Meredith and Elliott,

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1992). Therefore, it needs a very sensitive and specific analytical method for determination of the level of unchanged amlodipine in human plasma. Different methods (thin-layer chromatography, gas chromatography, high performance liquid chromatography, liquid chromatography-mass spectrometry [LC-MS]) have been used for determination of amlodipine in plasma. (Beresford *et al.*, 1987; Pandya *et al.*, 1995; Vincent *et al.*, 2000; Tatar and Atmaca, 2000; Bahrami, and Mirzaeei, 2004; Park *et al.*, 2004; Zarghi *et al.*, 2005). However, to meet clinical needs these published methods have limited sensitivity and are unable to quantify amlodipine with good accuracy at low drug concentration in plasma. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was therefore selected in order to improve the selectivity and sensitivity of the determination method for amlodipine (Massaroti *et al.*, 2005; Nirogi *et al.*, 2006; Bhatt *et al.*, 2007; Ma *et al.*, 2007). These reported the use of LC-MS/MS with a triple-stage quadrupole mass detector for amlodipine quantitation, by the way ion-trap mass spectrometer detector enables MS-MS at an affordable price compared with triple quadrupole mass system. Therefore, the aim of this study was to determine the low concentration of amlodipine in human plasma by the high selectivity and sensitivity of an ion-trap mass detector functioning in multiple reaction monitoring (MRM) mode with electrospray ionization (ESI) interface using tizanidine as internal standard (I.S.). Furthermore, this present method was fully validated and its stability studied to ensure the proper quantification of amlodipine in human plasma down to the concentration limit of 0.3 ng/mL. The validation and stability studies protocols were performed according to the current protocol recommended in the standard Thai Food and Drug Administration (FDA) guidance for assessment of bioequivalence study in Thailand (<http://wwwapp1.fda.moph.go.th/drug>, 2006). At the same time, it was expected that this method would be efficient in analyzing plasma samples obtained for pharmacokinetic and/or bioequivalence studies after orally therapeutic doses of amlodipine.

2. Experimental

2.1 Chemicals and reagents

Amlodipine besylate and tizanidine hydrochloride (I.S.) (Figure 1) were produced from Cadia Pharma, Ltd. (Chennai, India). Acetonitrile (HPLC grade), ammonium formate, dichloromethane and diethylether were obtained from Thomas Baker (Mumbai, India). Methanol (HPLC grade) was from Bristsys-tech (Mumbai, India). The water was purified using a Milli-Q system (Milford, MA, USA).

2.2 Liquid chromatographic and mass spectrometric conditions

The 1100 Series HPLC system (Agilent Technologies, India) is equipped with a G1311A quaternary pump, a

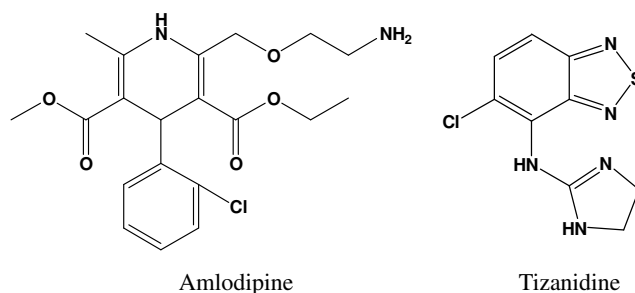


Figure 1. Chemical structure of the amlodipine (A) and tizanidine (I.S.) (B)

G1379A vacuum degasser, a G1329A autosampler equipped with a G1330B thermostat and a G1316A column oven. The chromatographic separation was carried out on Phenomenex (Torrance, CA, USA) Luna C₁₈ column (150×4.6 mm, 5 μm) with a C₁₈ guard column. The isocratic mobile phase composition was a mixture of 10 mM ammonium formate/methanol/acetonitrile (30/50/20, v/v/v), which was pumped at a flow rate of 1.0 mL/min with a split ratio of 20:80. The auto sampler temperature was set at 4°C and the injection volume was 50 μL. The column oven temperature was maintained at 30°C.

Mass spectrometric analysis was performed using API 2000 QTRAP quadrupole linear ion trap mass spectrometer (AB/MDS Sciex, Mumbai, India) equipped with turbo ion spray (electrospray) ionization source in the positive ion mode, heated to 400°C, and its ion spray voltage (ISV) set to 5500 V. Quantitation was performed using multiple reaction monitoring (MRM) mode to study parent → product ion transitions of m/z 409.4 → 238.1 for amlodipine and m/z 254.2 → 44.1 for I.S., respectively. The compound-dependent optimal MS parameters obtained were as follows; declustering potential (DP): 19 V, collision energy (CE): 16 V, cell exit potential (CXP): 17 V for amlodipine and DP: 50 V, CE: 46 V, CXP: 6 V for I.S. Nitrogen was used as curtain gas and collision gas which was set at 8 and 7 psi, respectively. Quadrupole 1 and quadrupole 3 were maintained at unit resolution and dwell time was set at 0.5 s.

2.3 Preparation of Standards and quality control samples

The standard stock solutions of amlodipine and I.S. were prepared by dissolving their accurately weighted compounds in methanol-water (50:50, v/v) to give the solution with a final concentration of 1.0 mg/mL. These solutions were then serially diluted with methanol-water (50:50, v/v) to provide working standard solutions of 1500 and 250 ng/mL for amlodipine and I.S., respectively. All the solutions were stored at 4°C and were brought to room temperature before use.

Blank human blood was collected with heparin from healthy, drug-free volunteers. After centrifugation, blank plasma was collected and stored at -20°C until used. Calibration standards were prepared by spiking drug-free human

plasma with working solution to provide concentrations of 0.3, 0.5, 0.8, 1.0, 3.0, 6.0, 8.0, 10.0 and 15.0 ng/mL. Standards were prepared daily in the amounts required for the assay. Quality control (QC) samples were prepared using drug-free human plasma to obtain three different concentrations of 0.9 (low QC sample; QCL), 5.0 (medium QC sample; QCM) and 12.0 ng/mL (high QC sample; QCH). Quality controls were prepared daily in the amounts required for the assay. The standards and quality controls were extracted on each analysis day along with the unknown samples. All solutions were kept at -20°C.

2.4 Plasma sample preparation

All frozen subject samples, calibration standards and quality control samples were thawed at room temperature and homogenized with a vortex mixer. To a 500 µL of aliquot of plasma in 10 mL clean glass test tubes, 20 µL of internal standard working standard (250 ng/mL) was added. The samples were vortex-mixed for about 30 s and 3.5 mL of extraction solvent (the mixture of diethylether/dichloromethane: 70/30, v/v) was added. The mixture was then shaken for 20 min with mechanical shaker. After centrifugation at 3500 rpm (1350 g) for 5 min, the upper organic layer was removed and transferred into another clean glass tube where it was evaporated to completed dryness at 40°C under a nitrogen stream. Samples were reconstituted with 150 µL of mobile phase then vortexed for 30 s ready for direct injection into the LC-MS/MS system.

2.5 Method validation

Method validation includes all of the procedures required to demonstrate that a method to quantify the concentration of amlodipine in plasma is reliable for the intended application (<http://wwwapp1.fda.moph.go.th/drug>, 2006).

2.5.1 Selectivity and specificity

These tests were performed by screening six different batches of blank human plasma. Each blank sample was tested for interference using the proposed extraction procedure and chromatographic/spectrometric conditions and compared with those obtained with an aqueous solution of the analyte at a lower limit of quantification (LLOQ) concentration.

2.5.2 Lower limit of quantification (LLOQ)

The LLOQ is defined as the lowest concentration on the calibration curve at which an acceptable accuracy within $\pm 20\%$ and a precision less than 20% can be obtained.

2.5.3 Linearity and standard calibration curve

Calibration curves (three replicate analyses) were

prepared using a set of 9 calibrations points, prepared in human blank plasma in the 0.3-15.0 ng/ml range, and stored frozen. The aliquots were not thawed until needed for use, and need stored under the same conditions as the study samples. The linearity of each calibration curves was determined by plotting the peak area ratios (y) of amlodipine to internal standard versus the amlodipine concentrations (x) in spiked plasma samples. The acceptance criterion for coefficient of determination (r^2) was 0.99 or more. Back-calculated concentration, slope, intercept and r^2 values were calculated using linear regression analysis. The calculated concentration should meet the following criteria: lower than 20% deviation from nominal concentration at the LLOQ level and lower than 15% deviation of standards other than LLOQ from nominal concentrations.

2.5.4 Accuracy and precision

Within-run and between-run precision and accuracy were evaluated at three quality control samples concentrations (QCL, QCM and QCH). Within- and between-run assay precision were determined as coefficient of variation (CV), and within- and between-run assay accuracies were expressed as percentages of theoretical concentration, as accuracy (%) = (found concentration / theoretical concentration) \times 100. Within-run assays involved five replicates per run and between-run assays were performed on 3 separate runs.

The following criteria were met in order to approve the within- and between-run precision and accuracy; precision: for each concentration level, coefficient of variation (CV) not exceeding 15%, accuracy: mean value of samples at each concentration level, within 85-115% of the actual value.

2.5.5 Recovery (Extraction efficiency)

Three set of six quality control (QC) samples, at low (QCL), medium (QCM) and high concentration (QCH) level were processed according to the method of plasma sample preparation previously described but without addition of I.S. The extracts were spiked with internal standard at a concentration matching the final concentration of the processed samples. Eighteen blank human plasma samples were processed without addition of amlodipine and internal standard. The extracts were spiked with concentrations of amlodipine and internal standard matching the final concentration of processed samples. Their responses represented 100% recovery.

Six blank human plasma samples were processed with addition of internal standard according the method previously described in plasma sample preparation. The extracts were spiked with amlodipine at a final concentration matching a processed sample at medium concentration level. The extraction yield was calculated by dividing the mean peak area ratio of the extracted samples by the corresponding

mean peak area ratio of the spiked post-extracts. The recovery of the analyte need not be 100%, but the extent of recovery of an analyte should be consistent, precise and reproducible.

2.6 Stability

The stability procedures evaluate the stability of the amlodipine in the human plasma under distinct timing and temperature conditions. The stability of the analyte and internal standard in stock solutions was also evaluated.

2.6.1 Freeze and thaw stability

Six replicates human plasma samples at two quality control samples concentrations; QCL and QCH were subjected to three freeze-thaw cycles of -20°C during 24 h. After the completion of third cycle, these samples were processed and analyzed comparing with fresh samples, and quantified with a standard set of calibration samples. The samples qualified the test if the deviation was within $\pm 15\%$.

2.6.2 Long-term stability

Three replicates human plasma samples at two quality control concentrations (low and high) were stored at -20°C for 3 months covered by the bioequivalence study, i.e., from the first day of volunteer sample collection up to the last day of samples analysis. These samples were processed and quantified using a fresh set of calibration samples. The stability samples were bracketed by freshly prepared quality control samples, one each at two concentrations (low and high). These QC samples were prepared with an independent stock solution. The samples qualified the test if the deviation was within $\pm 15\%$.

2.6.3 Short-term stability

To evaluate the short-term stability, five replicates human plasma samples at two quality control concentration levels (low and high) were frozen, thawed and left to stand at room temperature for 4 hours (which exceeds the expected duration that samples could be maintained at room temperature after thawing until they are analyzed), and then processed according to the plasma sample preparation as previously described. The samples were quantified with a set of calibration samples that had been processed immediately after thawing. The samples qualified the test if the deviation was within $\pm 15\%$.

2.6.4 Stability of post-preparation samples

Five replicates of human plasma samples at two quality control concentration levels (low and high) were processed together with a set of calibrators. The post-extracted samples were left to stand at auto-sampler tem-

perature (4°C) for 60 h before injection into the LC-MS/MS system and the concentration obtained were compared with the nominal values. The samples qualified the test if the deviation was within $\pm 15\%$.

2.6.5 Stock-solution stability

The stability of the amlodipine and I.S. stock solutions was performed by comparison of results from a solution kept for about 60 days at 4°C and from a solution that was extemporaneously prepared. The stability of amlodipine and I.S. stock solutions kept 6 hours on benchtop (at room temperature, in the presence of ambient light) was also tested.

For this purpose, six replicates of a pure solution of amlodipine at $10\ \mu\text{g}/\text{mL}$ in the methanol-water (50:50, v/v) solvent were prepared from each stock solution and injected in the LC-MS/MS system. The same test was performed with the stock solutions of I.S. Results were compared to fresh prepared solutions at corresponding concentration. The samples qualified the test if the deviation was within $\pm 15\%$.

3. Results and Discussion

3.1 Mass spectrometry

After amlodipine and I.S. were directly injected into the mass spectrometer along with the mobile phase with a positive ion interface, the full scan spectrum was dominated by protonated molecules $[\text{M}+\text{H}]^{+}$ m/z 409.4 and 254.2 for amlodipine and I.S., and the major fragment ions observed in each product spectrum were at m/z 238.1 and 44.1, respectively. Figure 2 shows the product ion mass spectra of amlodipine (A) and I.S. (B).

3.2 Method validation

3.2.1 Selectivity and specificity

The specificity and selectivity of the method were investigated by comparing the chromatograms of six different batches of blank human plasma with the peak response of amlodipine at LLOQ ($0.3\ \text{ng}/\text{mL}$). The product ion chromatograms extracted from plasma are depicted in Figure 3. As shown, the chromatogram retention times for amlodipine and I.S. were about 4.0 and 2.1 min, respectively, and there was no interference from endogenous substances observed at the retention time of the analytes. The total LC-MS/MS analysis time was 5.0 min per sample.

3.2.2 Lower limit of quantification

Intra-day and inter-day precision and accuracy were evaluated at LLOQ level within the studied range by repeated determination ($N=5$) in three different runs. The total variance and coefficient of variation were calculated directly from all the experimental measurements at LLOQ concentration ($0.3\ \text{ng}/\text{mL}$).

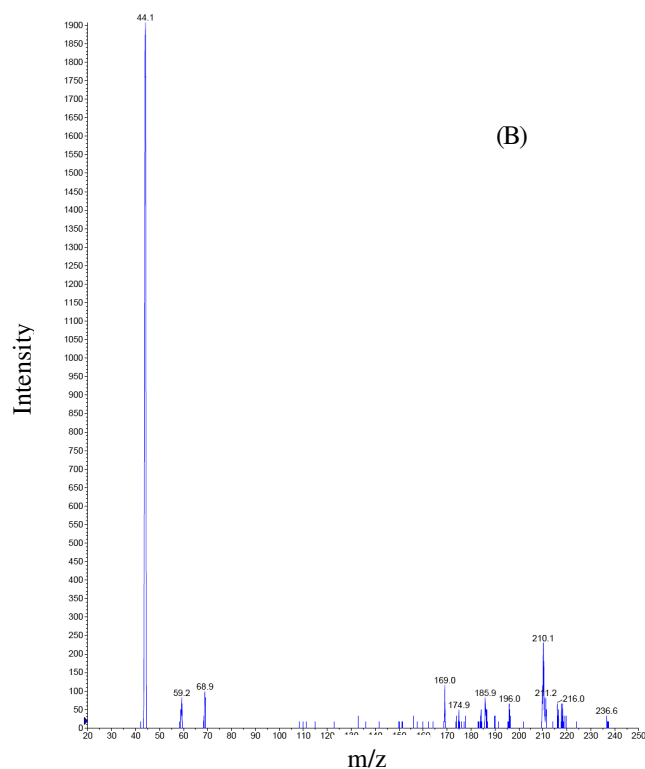
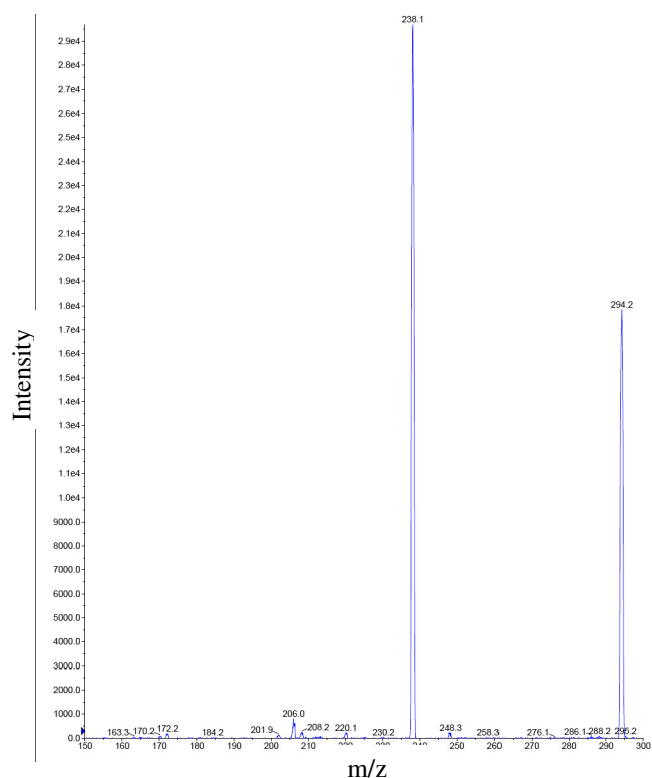


Figure 2. Product ion mass spectra of amlodipine (A) and I.S. (B)

At the lower limit of quantification, the QC samples presented the intra-day coefficient of variation (precision) between 3.4 to 4.8% and accuracies ranged from 92.0 to 97.7%. The inter-day coefficients of variation and accuracy were 6.7 and 95.7%, respectively. This was the lowest con-

centration of analyte that can be measured with both a coefficient of variation and accuracy of <20%, indicating that the use of the lowest QC level as LLOQ is justified. As shown in Figure 3, there was no endogenous peak at the retention time of amlodipine and I.S., and the analytes response were five times more than that of drug free (blank) extracted plasma. Moreover, this LLOQ is sufficient for pharmacokinetics studies.

3.2.3 Linearity and calibration curves

The linearity regression of the peak ratios versus concentrations were fitted over the concentration range of 0.30-15.0 ng/mL. The calibration curve exhibited good linearity ($r^2 = 0.9993$) and the curve showed good back-calculated precision and accuracy. The linear regression equation of the calibration curve was $y = 0.0718x - 0.0058$. The back-calculated concentration of a calibration samples were within the limits of acceptance; $\pm 20\%$ at the LLOQ and $\pm 15\%$ for the other concentration levels. The results of three representative calibration curves for amlodipine LC-MS/MS determination are given in Table 1.

3.2.4 Accuracy and precision

Precisions, which are represented as %CV at each concentration, were always $\pm 15\%$ in all quality controls samples concentration. Accuracies were within 85-115%. Within-run precision ranged from 2.26 to 4.35%, with accuracy ranging from 94.87 to 102.44%, while between-run precision ranged from 5.55 to 9.51%, with accuracy ranging from 97.99 to 100.97% (Table 2). Thus, the results obtained were reproducible and satisfied the criteria for acceptance of precision and accuracy.

3.2.5 Recovery

The mean absolute extraction (recovery) of amlodipine, determined at three different concentrations (low, medium and high QC samples), were $63.8 \pm 5.4\%$, $58.8 \pm 4.1\%$, and $63.2 \pm 5.3\%$ ($n=6$), respectively. The mean recovery for I.S. was investigated as $94.6 \pm 3.8\%$ ($n=6$). The results showed that the recoveries of amlodipine and internal standard were consistent, precise and reproducible, which has proved to be satisfied in bioanalysis.

3.3 Assessment of stability

The results of stability tests obtained were well within the acceptable limit. Furthermore, they revealed that no significant degradation occurred during the chromatography, extraction and sample storage of amlodipine plasma samples. Different stability experiments in plasma and the values for the precision and accuracy, expressed as percentage relative error (%RE) are shown in Table 3. The findings from these stability tests indicated that storage of amlodipine's plasma

Table 1. Calibration curve validation data for amlodipine LC-MS/MS determination

Nominal concentration (ng/mL)	Back concentration (ng/mL)			mean (ng/mL)	SD	%CV	RE%
	1	2	3				
0.3	0.311	0.334	0.297	0.314	0.02	5.95	4.67
0.5	0.515	0.448	0.525	0.496	0.04	8.44	-0.80
0.8	0.754	0.699	0.747	0.733	0.03	4.08	-8.33
1	0.898	0.983	0.986	0.956	0.05	5.23	-4.43
3	2.750	2.980	3.400	3.043	0.33	10.83	1.44
6	5.920	5.990	5.490	5.800	0.27	4.67	-3.33
8	8.570	8.020	8.020	8.203	0.32	3.87	2.54
10	11.000	10.500	10.200	10.567	0.40	3.82	5.67
15	17.100	16.400	14.500	16.000	1.35	8.41	6.67

Table 2. Within- and between-run precision and accuracy of the method for determination of amlodipine in human plasma

Level	Concentration added (ng/mL)	Within-run			Between-run		
		Concentration found (mean± S.D.) (ng/mL) ^a	CV (%)	Accuracy (%)	Concentration found (mean± S.D.) (ng/mL) ^b	CV (%)	Accuracy (%)
QCL	0.9	0.85 ± 0.02	2.26	94.87	0.88 ± 0.22	6.60	97.99
QCM	5.0	5.12 ± 0.22	4.35	102.44	5.05 ± 0.48	9.51	100.97
QCH	12.0	12.08 ± 0.40	3.33	100.67	11.99 ± 0.66	5.55	99.89

^aMean of five replicates observations at each concentration.

^bMean of 15 replicates observations over three different analytical runs.

Table 3. Stability results of amlodipine in plasma samples

Stability	Nominal concentration (ng/mL)	Concentration found (ng/mL)	CV (%)	RE (%)
Three freeze-thaw cycles	0.90	0.79	12.92	-11.91
	12.00	11.50	5.19	-4.44
Long-term	0.90	0.87	10.30	-3.41
	12.00	12.40	5.04	3.33
Short-term	0.90	0.93	4.79	3.53
	12.00	12.20	8.44	1.33
Post-preparation	0.90	0.84	5.34	-6.67
	12.00	12.10	10.10	0.50

samples is adequate and no-stability-related problems would be expected during the samples routine analysis for the bioequivalence or pharmacokinetic studies.

Stock solutions stability of amlodipine and internal standard (I.S.) in refrigerator and room temperature were tested and summarized as follows: no significant difference was determined for both amlodipine and I.S. after 60 days at 4°C (difference was -0.98 and 0.96% for amlodipine and I.S., respectively). No significant difference was found for amlodipine and I.S. after 6 h at room temperature (difference for amlodipine was -5.64% and difference for I.S. was 6.02%).

3.4 Application

The validated method has been successfully used to quantify amlodipine concentration in human plasma after the administration of a single 10 mg oral dose of amlodipine tablet in a bioequivalence study conducted in four Thai healthy male volunteers. The analyses were accomplished in accordance with the Thai FDA bioequivalence studies. The representative LC-MS/MS chromatograms resulting from the analysis of subject plasma sample 8 h after the administration of 10 mg oral single dose of amlodipine are presented in Figure 3. The mean plasma concentration-time profile of

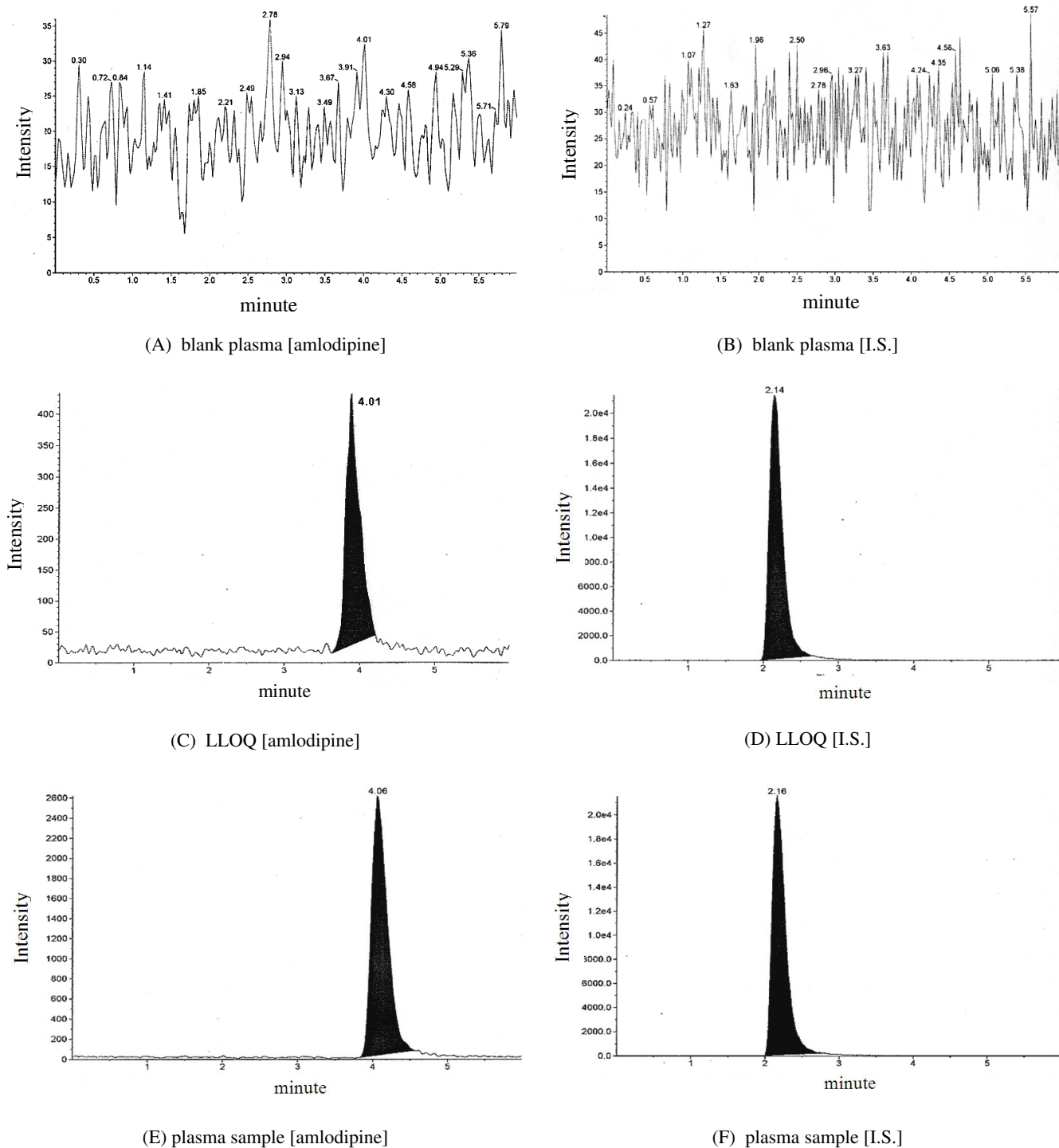


Figure 3. LC-MS/MS chromatograms of blank plasma, LLOQ and plasma sample from a volunteer 8 h after oral administration of amlodipine 10 mg- tablet for amlodipine (A, C and E) and I.S. (B, D and F), respectively.

four volunteers are showed in Figure 4.

4. Conclusion

A sensitive, selective, accurate, precise and rapid electro spray LC-MS/MS method for the determination of

amlodipine in human plasma is described. The method was successfully validated; stability studied and was found to meet the entire requirement of current Thai FDA guidelines. It was shown that this method has high sensitivity and specificity, and is capable of support for pharmacokinetic assays, such as in bioequivalence studies.

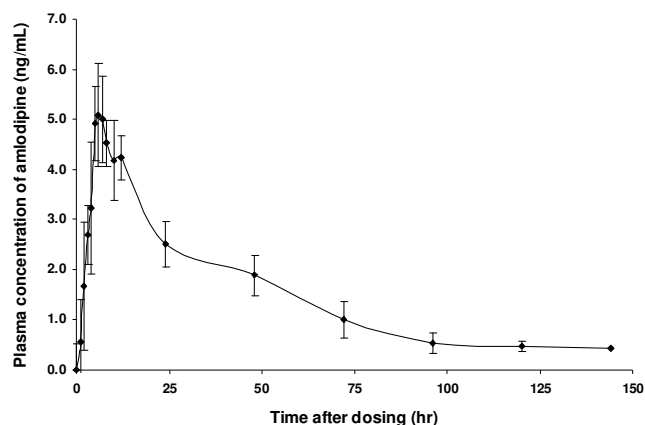


Figure 4. Mean plasma concentration-time profile of four healthy volunteers after 10-mg oral dose administration of amlodipine tablet. The error bars represent \pm standard deviation.

Acknowledgements

This study was sponsored in part by GIS Pharma Limited Partnership, Bangkok and Unique Pharmaceutical Laboratories, Mumbai, India. The authors gratefully acknowledge Pharmaceutical Laboratory Service Center, Faculty of Pharmaceutical Sciences, Prince of Songkla University, for providing necessary facilities to carry out this work.

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