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Original Article

QUANTITATION OF AMLODIPINE IN HUMAN PLASMA BY LCMS/MS ASSAY

SYED N. ALVI, RAJAA F. HUSSEIN, SALEH AL-DGITHER, MUHAMMAD M. HAMMAMI

Clinical Studies and Empirical Ethics Department, King Faisal Specialist Hospital and Research Center, P O Box # 3354, MBC-03, Riyadh 11211, Kingdom of Saudi Arabia
Email: muhammad@kfshrc.edu.sa

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ABSTRACT

Objective: To develop and validate a simple, precise, and rapid liquid chromatographic-tandem mass spectrometric (LC-MS/MS) method for quantification of amlodipine in human plasma.

Methods: Chromatographic analysis was performed on Atlantis dC18 column (2.1 x 100 mm, 3 µm) with a mobile phase consisting of acetonitrile and 10 mM formic acid (80:20, v: v) that was delivered at a flow rate of 0.3 ml/min. The eluents were monitored using electrospray ionization in the positive ion mode set at transition 409 → 238.4 and 254.3 → 43.9 for amlodipine and tizanidine hydrochloride (IS), respectively. The method was validated for linearity, accuracy, precision, and recovery as per US-FDA guidelines.

Results: The retention times of amlodipine and tizanidine (IS) were 1.26 and 1.22 respectively. The relationship between amlodipine concentration and peak height ratio of amlodipine to the IS was linear ($R^2 \geq 0.9868$) in the range of 0.2–20 ng/ml, and the intra-and inter-day coefficient of variations and bias were ≤14.4% and ≤13.6% and ≤13.7% and ≤11.2%, respectively.

Conclusion: The proposed method is simple, precise, and accurate for rapid measurement of amlodipine level using 0.5 ml human plasma. Further, the assay was successfully applied to determine amlodipine level in human plasma samples obtained from a healthy volunteer.

Keywords: Amlodipine, Tizanidine, Human plasma, LC-MS/MS

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INTRODUCTION

Amlodipine (CAS: 88150-42-9), a derivative of dihydropyridine, is widely used in the treatment of hypertension. It lowers blood pressure by inhibiting the influx of calcium ions [1]. Its absolute bioavailability is in the range of 60-65%, and it has a peak plasma concentration of 6-12 ng/ml within 8-10 h after the ingestion of a 10 mg therapeutic dosage [2, 3].

Various analytical methods have been reported for quantification of amlodipine in human plasma. They include thin-layer chromatography (TLC) [4], gas chromatography equipped with electron capture detection (GC-ECD) [5, 6], high-performance thin-layer chromatography (HP-TLC) [7], high performance liquid chromatography (HPLC) with ultra-violet (UV) detection [8, 9], fluorimeteric detection [10, 11]or electrochemical detection [12], and liquid chromatography-tandem mass spectrometry (LCMS-MS) [13-15]. In general, HPLC with UV detection is the preferred method for quantification of analytes that have strong absorbance in the UV range. Because amlodipine has low absorbance in UV range, most reported assays used either pre-column derivatization with 4-chloro-7-nitrobenzofurazan or LCMS/MS. There is limited data on the stability of amlodipine in processed and unprocessed human plasma [9, 14].

The present manuscript describes a precise and rapid LCMS/MS assay for quantitative determination of amlodipine in human plasma using tizanidine as an internal standard. The method involves simple liquid/liquid extraction, using 500 µl human plasma. The validated method was used to determine the stability of amlodipine under various clinical laboratory conditions, particularly in unprocessed human plasma samples for more than one year and has been successfully used to determine amlodipine level in human plasma samples obtained from a healthy volunteer.

MATERIALS AND METHODS

Chemicals and reagents

All chemicals were of analytical grade unless stated otherwise. Amlodipine USP reference standard and tizanidine hydrochloride (IS) were purchased from Sigma-Aldrich Co., St. Louis, MO, USA. Acetonitrile, methanol, dichloromethane, formic acid and *tert.* butyl methyl ether (HPLC grade) were purchased from Fisher Scientific, NJ. USA. Water for HPLC was prepared by reverse osmosis and further purified by using synergy water purification system (Millipore, Bedford, MA, USA). Drug-free human plasma was obtained from the blood bank of King Faisal Specialist Hospital and Research Centre (KFSHRC) Riyadh, Saudi Arabia. The study was approved by the Research Ethics Committee of KFSHRC, under Research Advisory Council (RAC# 2101100).

Instrument and chromatographic conditions

LC-MS/MS analysis was performed on Waters Alliance HPLC 2695 Separation module, consisting of quaternary pump, autosampler, column thermostat, and Micromass Quattro micro API bench-top triple quadruple mass spectrometer, interfaced with Z-spray electrospray ionization probe. Data acquisition and analysis were performed using MassLynx 4.0 software with Quan Lynx program (Waters Associates Inc, Milford, MA, USA).

Analysis was performed on a reversed phase Atlantis dC18 (2.1 X 100 mm, 3 µm) column equipped with Symmetry C_{18} (3.9 x 20 mm, 5 µm) guard column. The mobile phase, composed of acetonitrile and 10 mM formic acid (80:20, v: v), was filtered through a 0.22 µm membrane filter (Millipore Corporation, Bedford, MA, USA), degassed, and delivered at a flow rate of 0.3 ml/min. MassLynx software working under Microsoft Window XP professional environment was used to control the instruments, data acquisition, peak integration, peak smoothing, and signal-to-noise ratio measurements. The electrospray ionization source was operated in the positive-ion mode at a capillary voltage of 4.0 kV and a cone voltage of 10 V. Nitrogen was used as nebulizing and desolvation gas at a flow rate of 60 and 600 L/hr, respectively. Argon was used as the collision gas at a pressure of 1.28 x 10^{-3} mbar. The optimum collision energy for amlodipine and tizanidine hydrochloride (internal standard, IS) was 25 eV. The ion source and the desolvation temperatures were maintained at 105 and 350 ˚C, respectively. The product ion transitions response were recorded at m/z 409.8

238.4 for amlodipine and 254.3→ 43.9 for the IS. The assay was used to measure amlodipine stability in processed and unprocessed samples.

Preparation of standard and control samples

Amlodipine and the IS stock solutions were prepared in methanol (1.0 µg/ml). Calibration standards at nine different concentrations (0.2-20 ng/ml) and quality controls at four concentrations: (0.2, 0.6, 10, and 18 ng/ml) were prepared in human plasma. IS working solution was prepared in methanol (30 ng/ml). Standard and control solutions were vortexed for one minute, and 500 µl aliquots were transferred into 7 ml glass culture tubes and stored at-20°C until used.

Preparation of samples

100 µl of the IS working solution was added to each 500 µl plasma sample, calibration standard, or quality control samples in a 7 ml glass culture tubes and vortexed for 30 seconds. 4.0 ml extraction solvent mixture of *tert*. butyl methyl ether and dichloromethane (7:3,v: v) was added to each tube, vortexed for one minute, and centrifuged at 6000 rpm for 10 min at room temperature. The clear supernatant layer was transferred to a clean borosilicate culture tube and dried under gentle steam of nitrogen at 40°C. The residue was reconstituted in 100 µl mixture of methanol and water mixture (1:1, v; v), and 10 µl of the clear solution was injected into the LC-MS/MS system.

Stability studies

Two QC samples (0.6 and 18 ng/ml) were used for stability studies. Five aliquots of each sample were extracted and immediately analyzed (baseline). Five aliquots were allowed to stand on the bench-top for 24 h at room temperature before being processed and analyzed, five aliquots were stored at-20 ˚C for 68 w before being processed and analysis, and five aliquots were processed and stored at room temperature for 24 h or at-20 ˚C for 48 h before analysis. Fifteen aliquots were stored at −20 °C for 24 h. They were then left to completely thaw unassisted at room temperature. Five aliquots of each sample were analyzed and the rest stored at to-20 ˚C for another 24 h. The cycle was repeated three times.

RESULTS AND DISCUSSION

Optimization of LC and MS/MS conditions

Fig. 1 depicts the chemical structure of amlodipine and tizanidine, the internal standard (IS) used in the study. The mass spectrometric conditions were optimized by infusing a standard solution of the amlodipine and the IS with a syringe pump. Precursor and product spectra of amlodipine and IS are shown in fig. 2. The product ion transitions response were quantitatively measured as peak height at m/z 409.8→ 238.4 for amlodipine and 254.3 → 43.9 for the IS. The chromatographic conditions were optimized using mobile phase composed of 10 mM formic acid and acetonitrile (20:80, v: v) at flow rate 0.3 ml/min. Data were recorded in multiple reaction monitoring (MRM) mode.

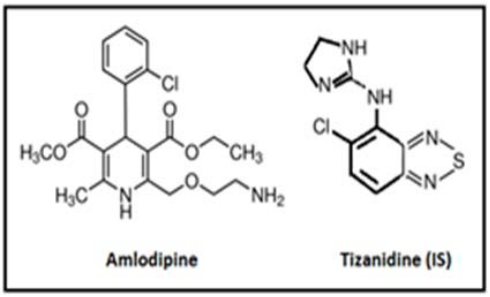

Fig. 1: Chemical structures of amlodipine and tizanidine (IS)

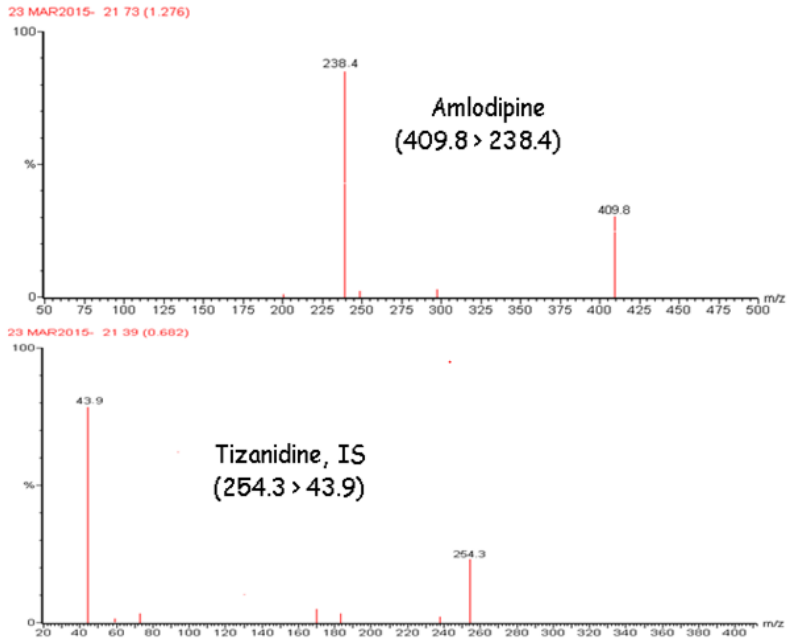

Fig. 2: Product ion spectrum of amlodipine and tizanidine (IS)

Effect of matrix

Generally, electrospray ionization is the most applicable method for bioanalysis. However, it is much more susceptible to matrix effects compared to atmospheric chemical ionization [16]. In present study, matrix effect was evaluated by comparing the peak response obtained from spiked plasma samples after sample preparation with the peak response obtained from direct injection of corresponding concentrations of amlodipine. No significant difference in response was observed.

Method validation

The method was validated according to standard procedures described in the US Food and Drug Administration (FDA) bioanalytical method validation guidance [17]. The validation

parameter included: specificity, recovery, linearity, accuracy, precision and stability.

Specificity

In order to evaluate specificity, we screened six batches of blank plasma and eight frequently used medications (aspirin, acetaminophen, ascorbic acid, ibuprofen, caffeine, nicotinic acid, omeprazole, and ranitidine) for potential interference. No interference was found by plasma components, and none of the drugs co-eluted with amlodipine or the IS. Fig. 3 depicts a representative chromatogram of drug free human plasma that was used in preparation of calibration curve and quality control samples. Fig. 4 depicts LCMS/MS chromatograms of plasma spiked with 30 ng/ml IS and amlodipine at three concentrations (0.6, 10 and 18 ng/ml), respectively.

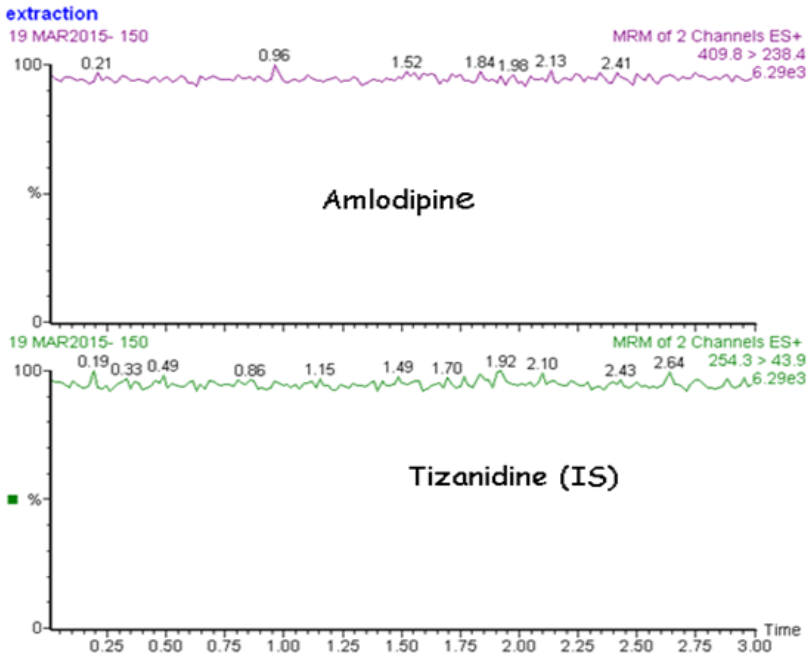

Fig. 3: MRM chromatogram of blank human plasma used in preparation of standard and quality control samples

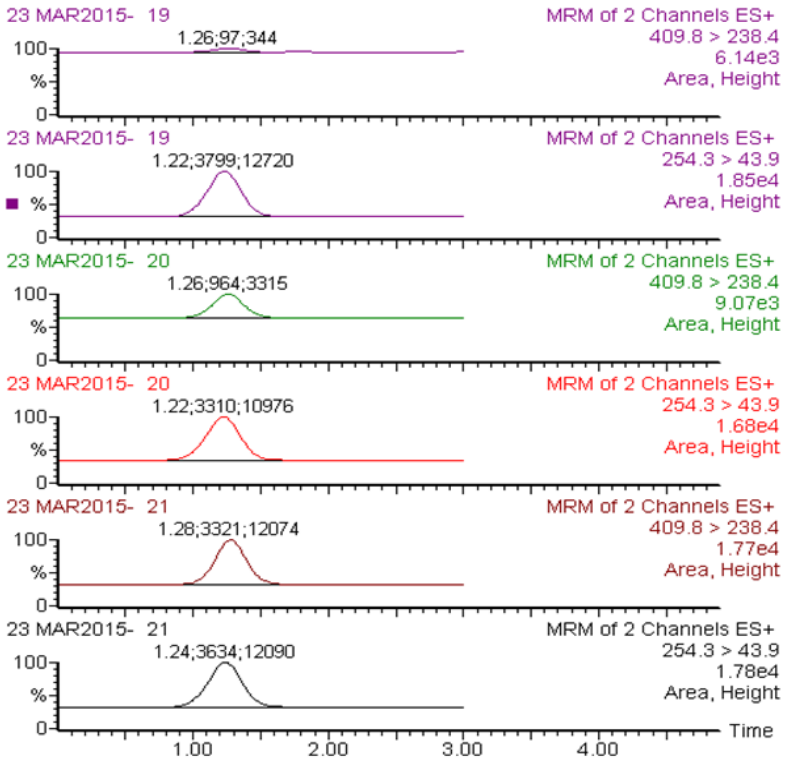

Fig. 4: MRM chromatograms of quality control plasma samples spiked with tizanidine (IS, 30 ng/ml) and amlodipine at three concentrations 0.6, 10.0 and 18 ng/ml

Recovery

Recovery of amlodipine was assessed by comparing analyses' peak heights obtained from spiked plasma and mobile phase samples, using five replicates of four concentrations (0.2, 0.6, 10 and 18 ng/ml) for amlodipine and 30 ng/ml for IS. Recoveries were in range of 62-79 % for amlodipine and 95 % for the IS. The results are presented in table 1.

Table 1: Extraction recovery of amlodipine and tizanidine (IS)

Amlodipine	Human plasma, *Mean (SD)	Mobile phase, *Mean (SD)	†Recovery (%)
0.2 (ng/ml)	133 (17)	216 (40)	62
0.6 (ng/ml)	390 (44)	573 (37)	68
10 (ng/ml)	6857 (785)	8632 (506)	79
18 (ng/ml)	9758 (990)	12873 (792)	76
IS (30 ng/ml)	17874 (1283)	18896 (1154)	95

*Mean peak height of 5 replicate. †Mean peak height of amlodipine in human plasma divided by mean peak height in mobile phase X 100. SD, standard deviation.

Linearity and limit of quantification

Linearity of the assay was evaluated by analyzing a series of standards at nine different concentrations over the range of 0.2–20 ng/ml. Corresponding peak height ratios and concentrations were subjected to regression analysis. The mean equation obtained from ten standard curves was y= 0.0457-0.0022x, with r^2 (SD) = 0.9868 (0.011). The detection and quantification limits were as 0.1 ng/ml and 0.2 ng/ml, respectively.

Accuracy and precision

Accuracy and precision were determined by measuring levels of amlodipine in four spiked human plasma samples (0.2, 0.6, 10 and 18 ng/ml). The intra-and inter-day imprecision and bias were determined over three different days. Intra-day (n=10) imprecision and bias were ≤ 14.4% and±13.7%, respectively. Inter-day (n=20) imprecision and bias were ≤13.6% and ≤11.2%, respectively (table 2).

Table 2: Intra-and inter-run precision and accuracy of amlodipine assay

Nominal level (µg/ml)	Intra-day (n=10) measured level			Inter-day (n=20) measured level		
	Mean (SD)	CV (%)	Bias (%)	Mean (SD)	CV (%)	Bias (%)
0.2	0.22 (0.03)	14.2	9.8	0.21 (0.03)	12.7	5.3
0.6	0.64 (0.09)	13.7	5.9	0.66 (0.08)	11.6	9.2
10	10.54 (1.52)	14.4	5.4	10.07 (1.37)	13.6	0.7
18	20.47 (2.14)	10.5	13.7	20.01 (2.35)	11.7	11.2

SD, standard deviation. CV, coefficient of variation = standard deviation divided by mean measured concentration x 100. Bias = measured level-nominal level divided by nominal level x 100.

Stability

It is necessary to perform stability studies of the analyte and IS to determine the range of appropriate conditions and times of storage. In pharmacokinetic and/or bioequivalence investigations, documentation of long-term stability of the analyte is a pre-requisite. There may be a long elapsed time between start of sampling and end of analysis. The availability of stability studies for extended period is essential for study planning and results interpretation. Previous studies did not address stability of amlodipine in human plasma beyond three months.

In the present study, amlodipine and IS stability in processed and unprocessed plasma samples was investigated. Amlodipine in processed samples (0.6 and 18 ng/ml) was found to be stable for 24 h at room temperature≥(93%) and 48 h at-20 ˚C (≥ 92%); in unprocessed plasma samples was stable for at least 24 h at room temperature (≥93%), sixty eight weeks at-20 ˚C or below (≥91 %), and after three freeze-and thaw cycles (≥88%). The data are summarized in table 3. Further, no significant change in chromatographic behavior of amlodipine or the IS was observed under any of the above conditions.

Table 3: Stability of amlodipine in different conditions

Storage condition	Nominal level ng/ml	Measured level mean±SD ng/ml	Stability (%)
Baseline (0h)	0.6	0.60 (0.05)	
	18	18.09 (1.21)	
Processed samples			
24 h (RT)	0.6	0.57 (0.16)	95
	18	16.87 (2.35)	93
48 h (-20 ˚C)	0.6	0.55 (0.09)	92
	18	18.44 (2.71)	102
Unprocessed samples			
24 h (RT)	0.6	0.56 (0.04)	93
	18	18.44 (1.17)	102
68 w (-20 ˚C)	0.6	0.55 (0.02)	91
	18	16.93 (1.40)	93
FT Cycle-1	0.6	0.56 (0.08)	94
(-20 ˚C)	18	17.55 (0.78)	97
FT Cycle-2	0.6	0.57 (0.12)	95
(-20 ˚C)	18	17.42 (2.67)	96
FT Cycle-3	0.6	0.54 (0.06)	89
(-20 ˚C)	18	15.94 (2.02)	88

Stability (%) = mean measured level at the indicated time divided by mean measured level at base line X 100 (n=5). RT, room temperature (22 ˚C). FT, Freeze-thaw cycle; samples were frozen at-20 ˚C and thaw at RT.

Application to volunteer samples

Fig. 5 depicts an overlay chromatogram of samples collected from a healthy volunteer before and 6.0 h after the ingestion of a single dose of 10 mg amlodipine. Measured levels of amlodipine were zero and 5.6 ng/ml, respectively.

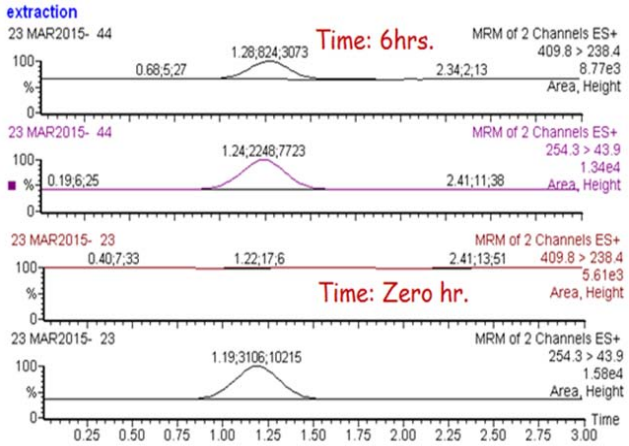

Fig. 5: MRM chromatograms of plasma samples obtained from a healthy volunteer before and 6 h after single 10 mg oral amlodipine dose

CONCLUSION

The described LC-MS/MS method is simple, precise, and accurate for rapid measurement of amlodipine level using 0.5 ml human plasma. The assay was used to study amlodipine stability under various condition encountered in the clinical laboratory. Further, the assay was successfully applied to determine amlodipine level in human plasma samples obtained from a healthy volunteer.

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CONFLICT OF INTRESTS

Declared none

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