Research Article

QUANTITATIVE DETERMINATION OF AMLODIPINE IN HUMAN PLASMA BY ULTRA PERFORMANCE LIQUID CHROMATOGRAPHY- ELECTRO SPRAY IONIZATION MASS SPECTROMETRY: APPLICATION TO A CLINICAL PHARMACOKINETIC STUDY

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ABSTRACT

A fast and sensitive ultra-performance liquid chromatography-mass spectrometry (UPLC-MS/MS) method was developed for the determination of amlodipine in human plasma, with Ondansetron Hydrochloride dihydrate as the internal standard. The separation was carried out on an ACQUITY (C18 100 x 2.1 mm, i.d 1.7μm) column, with 10mM Ammonium acetate Buffer solution (pH=4.5 with formic acid) and Methanol (20:80) as the mobile phase under isocratic conditions at a flow rate of 0.2 mL/min. The detection was performed on a triple quadrupole tandem mass spectrometer by multiple reaction monitoring (MRM) mode via electrospray ionization (ESI) source. The standard curves were linear (r2 > 0.99) over the concentration range of 0.20-20 ng/mL. The intra- and inter-day precision (R.S.D.) values were below 15%. The method is validated for the estimation of Amlodipine in human plasma over concentration range of 0.20 to 20.0 ng/mL. Expected recoveries were observed. Limit of detection of the methods is 0.1 ng/mL and Limit of quantitation is 0.20 ng/mL that shows that the developed method has adequate sensitivity. The method was successfully applied to the pharmacokinetic study of Amlodipine tablets in healthy male volunteers after oral administration..

Keyword: Amlodipine; Human Plasma; UPLC-MS/MS; Solid Phase Extraction.

INTRODUCTION

Amlodipine (am-LOE-di-peen) is a calcium channel blocker used to treat angina (chest pain) and high blood pressure, which is used as an anti-hypertensive and anti-anginal agent. It has a long elimination half-life and large volume of distribution. It has been reported that low plasma concentrations are achieved after oral administration of amlodipine (Murdoch, 1991 & Yuanyuan 2007), thus, a sensitive and specific analytical method is needed for determination of amlodipine in human plasma. Several analytical methods with UV detection for amlodipine in biological samples have been reported, however the sensitivity of these methods (Klinkenberg, 2003) is inadequate for pharmacokinetic studies and therapeutic drug monitoring due to low absorbance of the drug. However, to meet clinical needs these published methods have limited sensitivity and are unable to quantify amlodipine with good accuracy at low drug concentrations in plasma. Ultra Performance Liquid Chromatography-tandem mass spectrometry (UPLC-MS/ MS) was therefore selected in order to improve the selectivity and sensitivity of the determination method for amlodipine. Even though the reported LC-MS/MS methods have very high sensitivity and specificity than this method, most of them used liquid-liquid extraction for sample preparation from plasma (Addepalli, 2009; Sarkar, 2008; Nirogi 2006; Massaroti, 2005; Feng, 2002; Carvalho, 2001; Yasuda, 1996), only few methods tried the solid phase extraction procedure (Yuanyuan, 2007 & Bhatt, 2007), one with gradient elution technique (Yuanyuan, 2007) and other with isocratic elution technique (Bhatt, 2007). On the basis of this previous work, solid phase extraction method in which plasma samples could be applied to UPLC-MS/MS with sophisticated sample pre-treatment was developed. The method enables precise and accurate quantitation of amlodipine in small sample volumes over a wide concentration range with sufficient sensitivity for routine pharmacokinetic and therapeutic drug monitoring studies. 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 Ondansetron as internal standard (I.S.). Furthermore, this present method was fully validated to ensure the proper quantification of amlodipine in human plasma down to the concentration limit of 0.2 ng/mL in 1mL of plasma. 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.

MATERIALS AND METHODS

Chemicals, reagents and standards

Acetonitrile (HPLC grade), Methanol (HPLC grade), and Formic acid (AR grade) were obtained from Ranbaxy Fine Chemicals (Mumbai, India).The water was purified using Milli-Q system (Milford, MA, USA), Ammonium acetate Buffer Solution, Ammonia and working standards of Amlodipine were used.

Amlodipine standard stock solution (1 mg/mL): Amlodipine working standard equivalent to about 10.0 mg of Amlodipine weighed and transferred in to a 10 mL volumetric flask, to which about 5mL of methanol was added, and was sonicated to dissolve the material completely and volume made up to with methanol and was mixed.

Amlodipine standard solutions (spiking solutions): The following concentrations of amlodipine in methanol: water-(50:50, v/v) for Calibration Curve - 4.0, 8.0, 20.0, 40.0, 80.0, 160.0, 240.0, 320.0, 400.0 μg/mL and for Quality Control – 12.0, 120.0, 280.0 μg/mL were prepared.Internal standard solution: Ondansetron Hydrochloride dihydrate equivalent to 10mg of ondansetron weighed and transferred into a 100 mL volumetric flask, to which about 70 mL of Methanol was added. Then this solutionwas sonicated to dissolve the material completely and volume made up with Methanol and was vortexed. From the above solution 0.1 mL was pipetted into a 10 mL volumetric flask and volume made up with methanol to get 1 μg/mL concentration. From this solution, 1.25 mL solution was pipetted into a 50 mL volumetric flask and volume made up with water to get 25 ng/mL concentration.

Apparatus and operating conditions

The chromatography was performed on an ACQUITYTM UPLC system (Waters Corp, USA) with cooling autosampler and column oven enabling temperature control of the analytical column. An ACQUITY C18 column (100 mm x 2.1 mm, 1.7 μm, USA) was employed. The column temperature was maintained at 45 °C and chromatographic separations were achieved with isocratic elution using a mobile phase composed of 10mM Ammonium acetate buffer solution (pH 4.5) with Formic acid and 100% Methanol (20:80). The flow rate was set at 0.2 mL/min. The autosampler was conditioned at 4 °C and the injection volume was 15μL using partial loop mode for sample injection.

Triple-quadrupole tandem mass spectrometric detection was carried out on a Micromass® Quattro premier Mass spectrometer with an electrospray ionization (ESI) interface, Triple state Quadrupole analyzer and Photo multiplier detector system. The ESI source was operated in positive ionization mode. Quantification was performed using multiple reaction monitoring (MRM) of the transitions of m/z 409 > 238 for Amlodipine and m/z 293 > 170 for ondansetron (I.S.), respectively, with a scan time of 0.050 (sec) per transition. The optimal MS parameters obtained were as follows: capillary 3.50 kV, cone 15 kV, source temperature 120 °C and desolvation temperature 350 °C. Nitrogen was used as the desolvation and cone gas with a flow rate of 800 and 100 (l/h), respectively. Argon was used as the collision gas flow 0.30 (mL/min). All data collected in centroid mode were processed using MassLynx™ 4.0 software.

Plasma sample Extraction procedure

50µl of internal standard solution was admixed with 1.0mL of sample in a vial and vortexed for 1 minute. Oasis HLB Extraction cartridges were placed on vacuum manifold vacuum was set to 5 Hg pressure. Adding 1mL methanol to each cartridge and draining it out by applying vacuum conditioned the cartridges. Equilibration was done by adding 2mL water to each cartridge and draining it out by applying vacuum. 1.0 mL of pre-mixed sample was loaded to each cartridge and drained out by applying vacuum. Each cartridge was first washed with 1 mL of 0.1% Ammonia in water and then washed with 1mL of 5% methanol. The vacuum was released, manifold cover removed and waste fluids were discarded. Elution was done by adding 0.8 mL of 0.1% Ammonia in methanol solution the eluates were collected in suitable vessels and about 15µL was injected into the UPLC-MS/MS system for analysis.
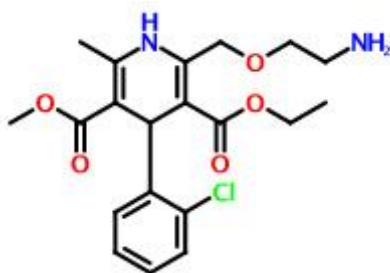
Figure 1: Structure of Amlodipine.

Pharmacokinetic study

The method was applied to determine the plasma concentrations of amlodipine from a clinical trial in which 8 healthy adult male volunteers received tablets (containing 10 mg amlodipine of test and reference formulations) after overnight fasting in a randomized, two way, two period, complete cross-over design In each dosing session, volunteers received either the reference formulation of Norvasc® 10 mg (amlodipine) tablets or test formulation of amlodipine tablets as a single dose, only on the study day, as per the randomization code at a fixed time. Washout period (in between the period) during the study was 7 days. Drinking water was not allowed from 1 h pre-dosing to 2 h post-dosing except while administering the dose with 240 mL of water. Standard breakfast was provided 4 h after dosing with an instruction to complete the entire breakfast within 30 min. A uniform low fat meal, consisting of caffeine-free, xanthine-free, and grape fruit-free foods and beverages was served at scheduled times (8 and 12 h after drug administration) during the in-house portion of the study. The pharmacokinetic study was approved by the Local Ethics Committee and all volunteers gave their signed informed consent to participate in the study according to the principles of the Declaration of Helsinki. Blood samples were collected 0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 6.0, 8.0, 12.0, 18.0, 24.0, 48.0, 72.0, 96.0 and 120 h post-dosing. Through an intra-venous cannula, blood samples (5 mL) were collected via disposable syringes in pre-treated centrifugal tubes. The total number of blood draws during the study was 34 and the total volume of blood drawn including 15 mL of screening and 25 mL discarded blood prior to venous canula collections did not

exceed 160 mL. The pre-dose blood sample was collected within a period of 1 h before dosing and post-dose samples were collected within 2 min of the scheduled time. The number of samples for each subject in each period was 17, therefore for 8 subjects and two periods a total of 272 samples were collected. The withdrawn samples were centrifuged at 4000 rpm for 10 min to separate plasma. They were transferred into labeled airtight containers and stored at deep freeze condition at −70°C until analysis. The total duration of sample storage until completion of analysis was 23 days.

RESULTS AND DISCUSSION

Mass spectrometry

When amlodipine and ondansetron were injected directly into the mass spectrometer along with the mobile phase with a positive ion interface, the full scan spectrum was dominated by protonated molecules [M+H]+ m/z 409 (Figure 2) and 293 for amlodipine and Odansetron, and the major fragment ions observed in each product spectrum were at m/z 238 (Figure 3) and 170, respectively. The analysis temperature, nebulizer gas, and ESI temperature were investigated to optimize the specificity and sensitivity of m/z 238 and m/z 170 ions detection.
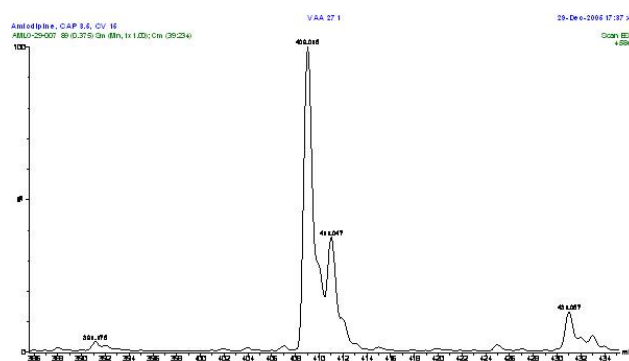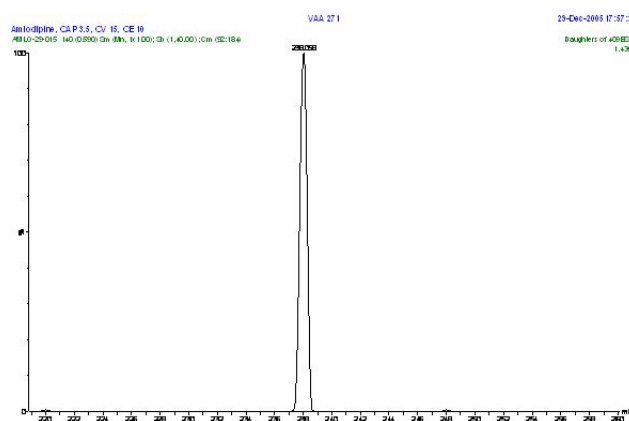
Figure 2:Parent Ion Scan of Amlodipine.

Figure 3 :Daughter Ion Scans of Amlodipine.

Chromatography

Chromatographic separation was performed by isocratic elution using a mobile phase composed of 10mM Ammonium acetate buffer solution (pH 4.5) with Formic acid and 100% methanol (20:80). The flow rate was set at 0.2 mL/min. The auto-sampler was conditioned at 4 °C and the injection volume was 15µl using partial loop mode for sample injection. The presence of a small amount of formic acid in the mobile phase improved the detection of the analytes in positive ion mode of the LC-MS/MS and, consequently, improved the sensitivity. The use of small particles of stationary phase allowed UPLC to push the limits of both peak capacity (due to higher efficiency) and speed of analysis (due to higher linear velocities) without compromising resolution. Two channels were used for recording, channel 1 for amlodipine with a retention time of 1.42 min, and channel 2 for the I.S. with a retention time of 1.62 min. As

shown in Figure 4 (Aqueous MQC of amlodipine and internal standard and Figure 5 (Plasma MQC of amlodipine and internal standard),

Both amlodipine and I.S. were well separated with excellent peak shapes, and no interfering peaks were observed in the blank plasma (Figure 6) and in all samples tested. The very narrow chromatographic peaks with a peak width about 6 s, produced by UPLCTM resulted in an increase in the chromatographic efficiency and sensitivity. Both amlodipine and I.S. were rapidly eluted with retention times less than 2 min, and the total run time was just 2.2 min per sample, and this met the requirement for a high sample throughput.
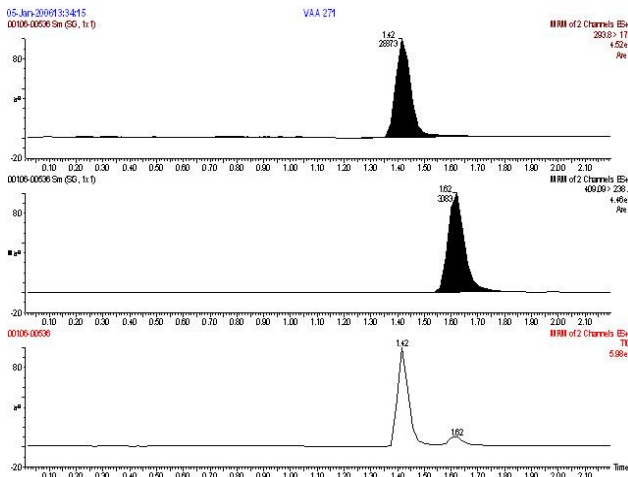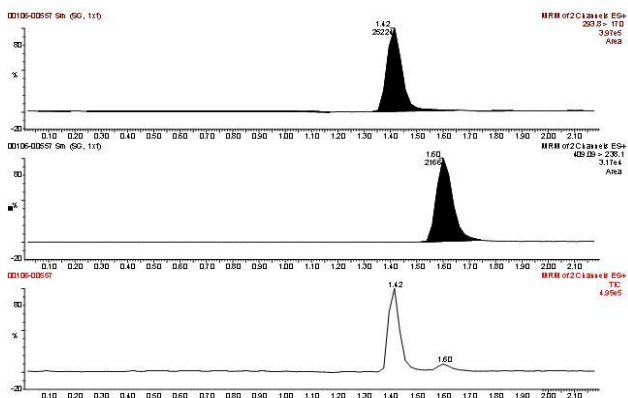
Figure 4: Aqueous MQC of Amlodipine and IS.

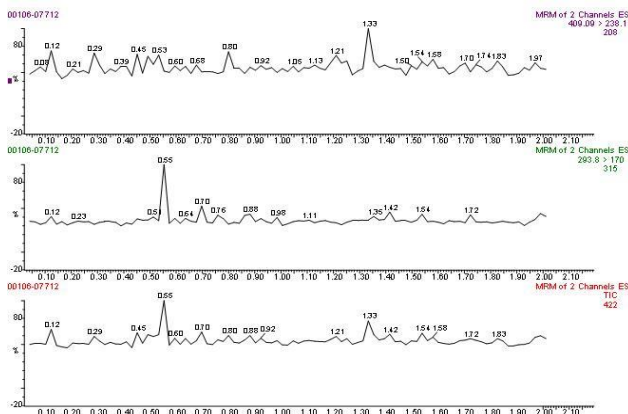**Figure 5: Plasma MQC of Amlodipine and IS.**

Figure 6: Chromatogram of Blank Plasma

METHOD VALIDATION

Selectivity

The six blank plasma samples obtained from six different volunteers were analyzed and the chromatograms were recorded. These chromatograms were compared with the chromatograms obtained from standard solutions. Each chromatogram was tested for interference. The combination of the sample preparation procedure and chromatography provided an assay, which is free from significant interfering endogenous plasma components at the retention times of amlodipine and the internal standard. Endogenous interferences were not detected at the retention time of amlodipine and internal standard.

Matrix effect

The possibility of a matrix effect caused by ionization competition between the analytes and co-eluents exists when using MS/MS for analysis. Matrix effect measured in six different lots of same matrix to ensure that precision, selectivity and sensitivity is not affected by different lots of matrix. Process and analyze three sets of LQC and HQC samples using six different lot of matrix. Calculated % bias was found to be LQC and HQC 2.35%, 3.95% respectively, which meant no significant matrix effect for amlodipine and I.S in this method. Thus, ion suppression or enhancement from the plasma matrix was negligible for this method.

Linearity and Range

The different concentrations of standard solutions were prepared to contain 0.2 to 20.0 ng/mL of amlodipine and 25.0 ng/mL of internal standard. These solutions were analyzed and the peak areas and response factors were calculated. The calibration curve (Figure 7) was plotted using response factor Vs concentration of the standard solutions. The standard curve fitting is determined by applying the simplest model that adequately describes the concentration-response relationship using appropriate weighting and statistical tests for goodness of fit. The calibration curve was constructed on six different days over a two weeks period to determine the variability of the slopes and intercepts. The lowest standard on the calibration curve was 0.2 ng/mL.

The results indicated little inter-day variability of slopes and intercepts, as well as good linearity (r^2 >0.99) over the concentration range studied, which indicates good precision and linearity of the method.

Compound name: Amlodipine
Correlation coefficient: r = 0.999838, r^2 = 0.999676
Calibration curve: 0.0171835 * x + 0.00135536
Response type: Internal Std (Ref 2), Area * (IS Conc. / IS Area)
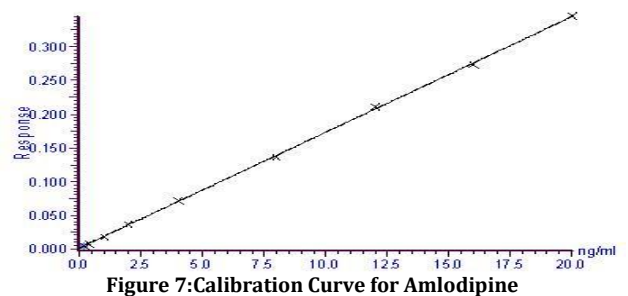Curve type: Linear, Origin : Exclude, Weighting: 1/x, Axis trans : None

Figure 7:Calibration Curve for Amlodipine

Accuracy

Accuracy of the method was determined by relative and absolute recovery experiments. The absolute recovery of amlodipine was determined by comparing the response factor of the drug obtained from the plasma with response factor obtained by the direct injection of amlodipine in mobile phase at three different levels. Recovery studies were carried out for three levels at six times and the % recovery, mean, standard deviation and % CV was calculated and is presented in table 1. An analysis of the results shows that the % CV of absolute and the relative recovery values are less than 15.00% thus establishing that the developed method is accurate and reliable.

Table 1: Method Validation Results.

S.No	Parameter	Acceptance Criteria	Results
1	Intra batch accuracy	Nominal concentration: 85-115%	LQC:89.96 HQC:90.60 MQC:103.86
2	Inter batch accuracy	Nominal concentration: 85-115%	LQC:89.86 HQC:90.69 MQC:103.86
3	Intra batch precision	% C.V: 15%	LQC:2.59 HQC:2.37 MQC:3.83
4	Inter batch precision	% C.V: 15%	LQC:3.82 HQC:3.65 MQC:5.35
5	Recovery of sample	% C.V: 20%	3.9
6	Recovery of internal standard.	% C.V: 20%	4.4
7	Freeze and thaw cycle at -70^{0}C	Mean % change after 2 cycles $\pm$15%	LQC: 8.01 HQC: 4.01
8	Long term stability in matrix	Mean % change after 40 days. $\pm$15%	LQC: 9.01 MQC:10.08 HQC:10.05
9	Bench top stability	Mean % change after 24 h $\pm$15%	LQC: 10.10 HQC:11.09

Precision

The precision of the method was determined by intraday precision and interday precision studies. The intraday precision was evaluated by analysis of blank plasma sample containing amlodipine at three different concentrations of LQC, MQC and HQC using nine replicate determinations for three occasions. The interday precision was similarly evaluated over two week period. The mean concentration, Mean % bias, standard deviation and % CV were calculated and is presented in table 1. The results of the precision studies reveal that the developed method is precise.

Recovery

Recovery for amlodipine and internal standard is performed by comparing the analytical results area of extracted samples at three different concentrations of LQC, MQC and HQC with unextracted standards results area that represents recovery of the amlodipine and internal standard. Recovery studies were carried out for three levels at five replicates and the %recovery, mean, standard deviation and % CV was calculated and is presented in table1 (amlodipine & internal standard). An analysis of the results shows that the % CV of amlodipine and internal standard recovery values are less than 15.00% thus establishing that the developed method is accurate and reliable.

STABILITY STUDIES

Stock solution stability

Height calibration standard of amlodipine and internal standard stock solutions were prepared and appropriately diluted to determine initial areas, after 8 hours at room temperature and for 1 day at 2-80 °C. The next day areas of stability samples and freshly prepared samples were compared to determine % change over stability of two days period. The results indicated for stock solution of amlodipine and internal standard stable at room temperature for 24 h and 2-80 °C for 48 h.

Bench top stability

Bench top stability of amlodipine in matrix is determined at LQC and HQC levels. Freshly prepared five replicates of samples were kept at room temperature for 24 h. After processed and analyzed along with calibration samples concentration was calculated to determine by % change. It was found to be stable in matrix for at least 30 h at room temperature with % change of 10.01% and 11% at LQC and HQC samples respectively.

Freeze and thaw stability

Freeze and thaw stability of amlodipine was determined after three freeze and thaw cycles at LQC and HQC levels. Stability in matrix after 2 freeze and thaw cycle at -70 °C. Concentrations were calculated to determine mean % change of after 2 cycles for LQC and HQC was found 8.01 and 4.01, respectively.

Long-term stability

Stability at -70 °C was determined by freezing five aliquots each of the LQC, MQC and HQC of amlodipine samples under the same conditions as that of the study samples. The storage time in a long term stability evaluation should exceed the time between the date of first sample collection and the date of last sample analysis.

Concentrations were calculated to determine % change after 40 days for LQC, MQC and HQC was found 9.01, 10.08 and 10.05 respectively.

Application of the method to a pharmacokinetic study in healthy volunteers

This validated analytical method was used to support the estimation of amlodipine in human plasma and evaluate the pharmacokinetic variables after a single oral dose in 8 healthy male human volunteers in a two way, two period, and complete cross-over design. A non-compartmental pharmacokinetic method was employed to estimate the pharmacokinetic parameters of clarithromycin. The time to peak plasma concentration (T_{max}) and the peak concentration (C_{max}) were obtained directly from the plasma clarithromycin concentrations. The $AUC_{0-\infty}$ and half-life ($t_{1/2}$) were determined by using Win-Nonlin Standard (version 3.0). An analysis of variance (ANOVA) was performed on the pharmacokinetic parameters C_{max} and $AUC_{0-\infty}$ using general linear models (GLM) procedures, in which sources of variation were sequence, subjects within sequence, period, and preparation. The 90% confidence intervals of the Test/Reference ratios for C_{max} and $AUC_{0-\infty}$ (log transformed) were determined (Figure 8).

- Oral administration of the Test product and Reference product in the fasting state exhibited measurable amlodipine blood levels in all the volunteers from 0.50 h onwards. Measurable amlodipine blood levels were noticed in all subjects up to 120.00 h.

- The mean peak plasma concentration *i.e.*, C_{max} for amlodipine after administration of the Test and Reference product was 3.447 $\pm$ 1.124 and 3.467 $\pm$ 1.374 ng /mL, respectively.

- The time to peak concentration *i.e.,* T_{max} for amlodipine after administration of the Test and Reference product was 7.250 ± 2.5 and 6.625 ± 1.5 h, respectively.

- The area under the plasma concentration-time curve *i.e.,* AUC_{0-120} for amlodipine after administration of the Test and Reference product was 164.683 ± 74.633 and 164.303 ± 77.496 ng.h/mL, respectively.

- The elimination rate constant (k_{eli}) for amlodipine after administration of the Test and Reference product was 0.020 ± 0.001 and 0.020 ± 0.001 h^{-1}, respectively.

- The elimination half-life ($t_{1/2}$) for amlodipine after administration of the Test and Reference product was 34.74 ± 4.63 and 35.78 ± 3.18 h.

- The area under the plasma concentration-time curve for infinitive time ($AUC_{0-\infty}$) for amlodipine after administration of the Test and Reference product was 177.92 ± 72.87 and 177.93 ± 78.54 ng.h/mL respectively.
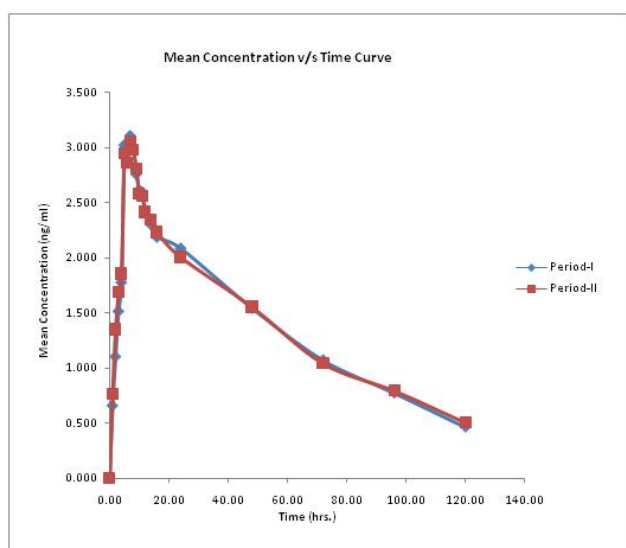
Figure 8: Mean concentration (ng/mL) v/s Time(h) Curve at every hour of each volunteer.

CONCLUSION

A selective, sensitive, and accurate UPLC–MS/MS method for quantification of amlodipine in human plasma was developed and validated. Compared with the published methods, the sharp peaks (high efficiency), short run time and reduced ion suppression produced by using special UPLC column are of particular advantage when coupled to electrospray mass spectrometer. The limit of quantification was 0.2 ng/mL for plasma amlodipine analysis. The method was successfully applied to the pharmacokinetic study of amlodipine tablets in healthy volunteers. The geometric mean and the 90% confidence interval (CI) Test /Reference rations were 101.2 (92.9–110.2%) for AUC_{last}, 99.6 (91.5-108.4%) for AUC_{0-inf} and 98.5 (89.0-109.1%) for C_{max}. Since the 90% CI for AUC_{last}, AUC_{0-inf} and C_{max} ratios were within in the 80-125% interval proposed by the US FDA, it was concluded that amlodipine 10 mg tablet (test formulation) was bioequivalent to Norvasc® 10 mg tablet, in terms of both rate and extent of absorption.

Declaration of interest

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