

Original Article

Estimation of Amlodipine in human plasma by liquid chromatography tandem mass spectrometry

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ABSTRACT

A simple, accurate, precise and cost effective bioanalytical method was developed and validated in human plasma by high performance liquid chromatography/positive ion electro spray tandem mass spectrometry. Liquid liquid extraction with ethyl acetate was used for extraction of the drug from plasma and the analytes were separated using isocratic mode on a reversed- phase column Hypurity C18 50X 2.1 mm, 5 μ and analyzed by mass spectrometry in the multiple reaction monitoring mode using the respective [M+H]⁺ ions, m/z was 409.2/238.0 for Amlodipine along with m/z 406.3.2/151.1 for Nebivolol as internal standard. The method exhibited a linear dynamic range between 0.10 to 20.12 ng/mL concentrations for Amlodipine in human plasma. Method development and full method validation was performed along with all stability parameter. The validated method can be used to analyze human plasma samples for its pharmacokinetics application, bioavailability and bioequivalence studies.

1. INTRODUCTION

Amlodipine, (R,S)-2-[(2-aminoethoxy) methyl]-4-(2-chlorophenyl)-3-ethoxycarbonyl-5-methoxycarbonyl-6-methyl-1,4-dihydropyridine, is a potent calcium antagonist, that has been widely prescribed for the treatment of angina and hypertension [1]. Amlodipine is a peripheral arterial vasodilator which acts directly on the vascular smooth muscle leading to reduction in peripheral vascular resistance and blood pressure [2]. It has high bioavailability, large volume of distribution and long elimination half- life (t_{1/2}) of up to 35 to 45 h [3]. As very low plasma concentrations of Amlodipine are achieved after oral administration [4], it requires a sensitive and specific analytical method for determination of amlodipine in human plasma.

As per the literature, several analytical methods including GC

with electron capture [5], HPLC with fluorimetric detection [6] or with UV detection [7], and LC–MS/MS have been reported for the determination of amlodipine [11-14] individually or with other drugs in biological samples. However the sensitivity of the UV detection methods is inadequate for the pharmacokinetic studies and therapeutic monitoring of the drug in human plasma [15]. All the previous work was based on methodology of solid phase extraction using and sophisticated sample pre-treatment that makes them very difficult for routine quantitative analysis. This prompted us to develop a new method with a simpler sample pre-treatment and extraction technique as well as cost effectiveness over solid phase extraction. Extraction of drug from plasma samples which would be easier and high throughput for day-to-day analysis.

2. EXPERIMENTAL

2.1 Materials

All solvents were used of HPLC grade or higher. All other chemicals were of analytical grade or higher. Amlodipine besylate and nebivolol were supplied by Splendid Lab, India. HPLC grade methanol and acetonitrile were purchased from J.T. Baker; formic acid AR (85%) was procured from Finar fine chemicals. Ethyl acetate was purchased from Rankem. Blank human plasma was procured from the blood bank. Milli Q-water was used throughout the development and validation.

2.2 Methods

2.2.1 LC-MS/MS instrumentation and conditions

The HPLC System (Shimadzu) is equipped with Autosampler SIL-HTC, solvent delivery mode LC-10ADvp and column oven CTO-10ASvp. The chromatography was performed on a Hypurity C18 (50 x 2.1 mm, 5 μ) at 40 °C. The isocratic mobile phase was acetonitrile: 0.1% Formic acid (90:10 v/v) which was pumped at 0.4 mL/min. Injection volume was 10 μ L and total run time was 1.8 minute. Mass spectrometric detection was performed on AB SCIEX API 3200 with positive ionization mode. Mass transition [M+H]⁺ ions, m/z was 409.2/238.0 for Amlodipine along with m/z 406.3/151.1 for Nebivolol as internal standard.

2.2.2 Preparation of standard solutions

An approximately weighed quantity of 13.89 mg Amlodipine besylate and 10.85 mg of Nebivolol and transferred in 10 mL volumetric flask and volume was made up to 10 mL each with methanol separately. All stock solutions of 1 mg/mL were prepared. Further dilutions were prepared for desirable concentration range. These stock solutions and dilution were stored between 2-8°C until the time of use.

The internal standard solution of concentration 1000 μ g/mL was prepared by dissolving an accurately weighed quantity of 10.85 mg of Nebivolol in 10 mL of methanol. Further dilutions were prepared up to 500 ng concentration with diluents used as internal standard.

2.2.3 Spiking in pooled plasma

The K₃EDTA plasma lots were thawed and pooled at room temperature and a volume of 9.5 mL of the screened pooled plasma was transferred to 10 mL of volumetric flask & spiked with 0.5 mL of spiking solution to obtained all eight non zero standards & quality control samples (low, medium & high) and vortexed to ensure proper mixing of analytes. Non-zero standards were prepared 0.10 ng/mL to 20.12 ng/mL for Amlodipine.

2.2.4 Preparation & extraction of drug from plasma samples

Aliquot (0.475 mL) of spiked plasma of non-zero standard & quality control samples were taken in RIA vials and followed

by addition of 25 μ L of internal standard stock solution (500 ng/mL) and vortexed for 15 seconds except blank samples. Approximately 3.0 mL of extraction solvent was added to the spiked plasma samples and was vortexed for 2 minute. The mixture was centrifuged at 4000 rpm for 5 minutes at 4 °C and flash freeze the plasma layer and transfer organic layer in to pre-labeled tube. The sample was evaporated to dryness at 40°C under the stream of Nitrogen then dried extract was reconstituted with 300 μ L of mobile phase and vortexed for 15 seconds and transferred into pre labeled vials. 10 μ L was injected into chromatographic system. For preparation of blank sample, 5% diluents were in pooled plasma.

2.2.5 Bioanalytical method validation

A standard stock solution of Amlodipine (1 mg/mL) was prepared separately up to stock dilution. Stock solution of internal standard (Nebivolol) was prepared in methanol. Spiking solution for calibration and quality control were prepared by appropriate dilution in methanol: water (90:10 v/v). Spiking solution (0.5 mL) was added to drug free human plasma (9.5 mL) as a bulk to obtained concentration levels of 0.10, 0.20, 0.50, 2.01, 5.03, 10.06, 15.10 and 20.12 ng/mL for Amlodipine. Quality control samples (low, medium, high) were also prepared at concentration 0.29, 9.56 and 15.20 ng/mL for Amlodipine. The spiked samples were stored in freezer at below -20 °C until analysis.

Calibration curve was constructed from a blank sample, blank zero, eight non-zero standards covering the stated range including LLOQ. The calibration curve was generated using the analyte to IS Peak area ratio by best fit weighted (1/X²) least squares linear regression on consecutive days. The acceptance criteria was a correlation coefficient (r) of 0.99 or better, and each back calculated standard concentration must be within 15% deviation except at the LLOQ which the maximum acceptable deviation was set at 20 %.

The assay of Amlodipine in K₃EDTA human plasma was validated for assay specificity, sensitivity, matrix effect, recovery, linearity, precision, accuracy, dilution integrity and all stabilities in accordance with the USFDA guidelines.

2.2.6 Specificity

The chromatogram obtained from six different lots along with lipemic & hemolytic blank human plasma was identified and retrieved in order to analyze the potential interference of endogenous substances at the peak region. The specificity of the method was evaluated by comparing chromatograms of blank plasma, blank plasma spiked with Amlodipine and IS.

2.2.7 Matrix Effect

Blank plasma samples were extracted and spiked with Amlodipine at three low concentration levels and high concentration in six different blank matrices to evaluate the matrix effects of plasma. The corresponding peak areas were compared with those of

the standards solutions, and peak area ratio was defined as the matrix effect.

2.2.8 Recovery

The extraction recovery of Amlodipine and IS were evaluated by comparing the bioanalytical results for the extracted QC samples with solutions equivalent to 100% recovery of low, medium and high QCs. Six replicates for each QC level were performed with the established extraction procedure.

2.2.9 Calibration curve

The calibration curve was prepared by analyzing spiked calibration samples at eight different concentrations. Every calibration standard was injected in five replicates. The linearity of calibration curve was assessed by linear regression. The low limit of quantification (LLOQ) was determined by analyzing five replicates of spiked samples.

2.2.10 Accuracy and precision

The within run accuracy and precision batches were assessed by analyzing six replicates of LLOQ, low QC, medium QC, high QC samples and all batches were meeting acceptance criteria.

2.2.11 Dilution integrity

Dilution quality control (DQC) is diluted fifth and tenth in human plasma. Prior to extraction, six samples each of fifth and tenth diluted samples was processed and analyzed with freshly processed calibration samples.

2.2.12 Hemolysis effect

For evaluation of potential interference of hemolysed plasma sample with Amlodipine and internal standard, the hemolysed plasma was collected and stored in deep freezer at $-20 \pm 5^\circ\text{C}$, six samples each of blank, low QC and high QC were processed and analyzed with freshly processed calibration set. Area response was used to determine any significant interference and concentrations were calculated to determine % nominal.

2.2.13 Stability studies

The stability of Amlodipine and Nebivolol (IS) in human plasma was evaluated under different temperature and condition as short term stock solution stability at room temperature and refrigerator ($2-8^\circ\text{C}$), long term stock working solution stability, bench top stability, freeze thaw stability, autosampler stability and dry extract stability ($20^\circ\text{C} \pm 5$).

3. RESULTS AND DISCUSSION

3.1 Results

The representative chromatogram blank plasma and upper limit of quantification for Amlodipine and internal standard are illustrated in Fig. 1 and 2. The retention times of the Amlodipine and Nebivolol internal standard were approximately 0.62 and 0.63 minutes. The overall chromatography run time was 1.8 minutes.

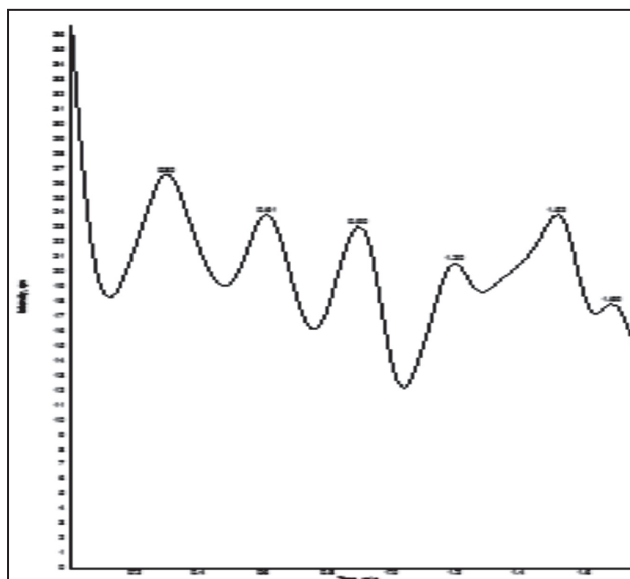
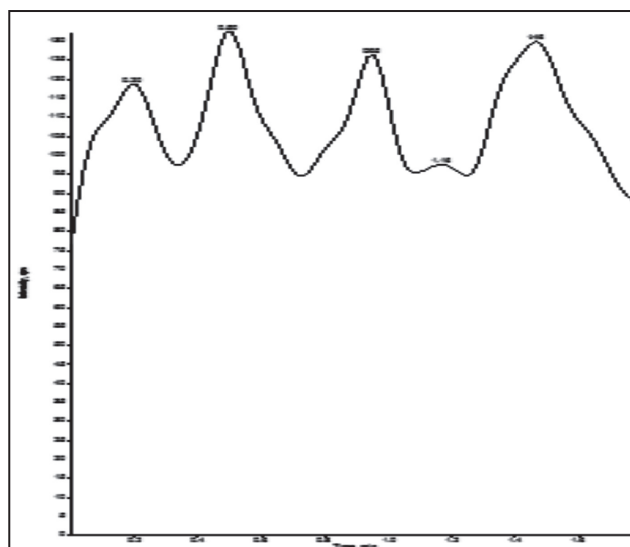
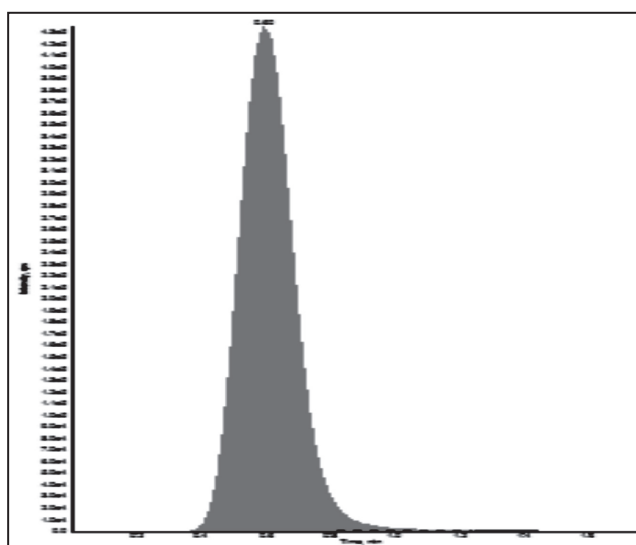


Fig. 1. Representative chromatogram of blank human plasma sample of Amlodipine and Nebivolol



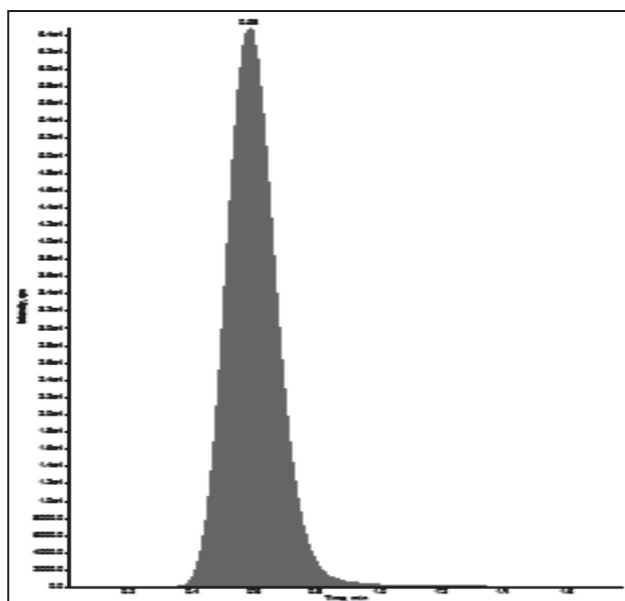


Fig. 2. Representative chromatogram of upper limit of quantification sample of Amlodipine and Nebivolol (IS)

3.1.1 Specificity

Selected blank human plasma sources were carried through the extraction procedure and chromatographed to determine the extent to which endogenous human plasma components may contribute to chromatographic interference with the Amlodipine and Nebivolol. No significant interference was observed in six different lots of human plasma samples.

3.1.2 Matrix effect

One calibration curve along with 18 low QC samples and 18 high QC (three each from six different lots of human plasma) was processed and analysed with freshly processed calibration samples in a single run. The % nominal value for Amlodipine was found to be 96.67% and 95.54 % for LQC and HQC levels. The results are presented in Table 1.

Table 1. Matrix Effect

Amlodipine		
QC Level	Concentration (ng/mL)	Mean ± SD (%)
LQC	0.29	96.67 ± 0.023
HQC	15.20	95.54 ± 0.994

3.1.3 Recovery

Recovery of analyte and metabolites was evaluated by comparing mean analyte response of six extracted samples of low, medium and high quality control samples to mean analyte response of six replicates injection of un-extracted quality control samples. The mean recovery for LQC, MQC and HQC of Amlodipine are

61.32 %, 58.65 % and 72.92 % at LQC, MQC and HQC level respectively (Table 2). The mean recovery for internal standard is 76.90 %.

Table 2. Extraction recovery

QC Level	Amlodipine (% Recovery)
LQC	61.32
MQC	58.65
HQC	72.92
Mean	64.30
SD	7.586
% CV	11.80

3.1.4 Calibration curve

Calibration curve of Amlodipine was found to be consistently accurate and precise over the range 0.10 to 20.12 ng/mL. The regression coefficient (r) was greater than or equal to 0.99. Back-calculations were made from the calibration curves to determine Amlodipine concentrations of each calibration standard and a typical calibration curve of Amlodipine is presented in Fig 3.

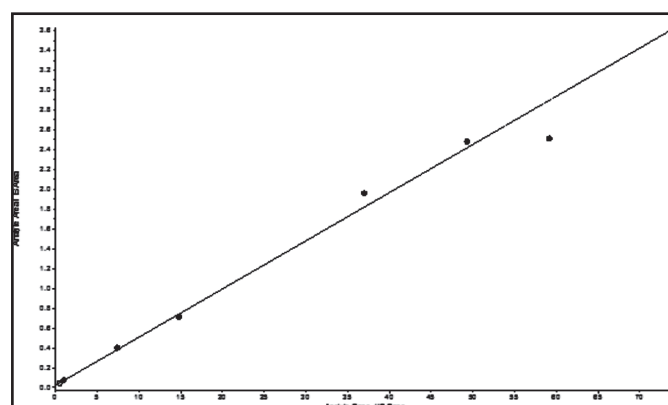


Fig. 3. Calibration curve of Amlodipine

3.1.5 Accuracy and precision

The between-run accuracy and precision evaluation were assessed by the repeated analysis of human plasma samples containing different concentrations of Amlodipine on separate occasions. A single run consisted of a calibration curve, 6 replicates of lower limit of quantification, low, medium and high quality control samples. The between-run % coefficient of variation and between-run percentage of nominal value of Amlodipine are presented in Table 3.

Table 3. Between run accuracy and precision of plasma samples

Amlodipine				
QC Level	Concentration (ng/mL)	Mean ± SD	% CV	Accuracy %
LLOQC	0.10	0.10 ± 0.009	8.40	101.25
LQC	0.29	0.30 ± 0.020	6.68	102.01

MQC	9.56	9.70 ± 0.428	4.41	101.46
HQC	15.2	15.01 ± 0.571	3.80	98.77

Replicate concentrations of Amlodipine in human plasma were analysed for within-run accuracy and precision evaluations. The run consisted of a calibration curve plus a total of 30 spiked samples, 6 replicate each of the lower limit of quality control (LLOQ), low, medium and high quality control samples. The within-run % coefficient of variation of Amlodipine ranged from 2.87 to 9.99 while the within-run percentage of nominal value of Amlodipine ranged from 98.28 to 104.04 (Table 4).

Table 4. Within-run % CV and % nominal for Amlodipine

Amlodipine				
QC Level	Concentration (ng/mL)	Mean ± SD	% CV	Accuracy %
LLOQC	0.10	0.10±0.010	9.99	103.33
LQC	0.29	0.30±0.015	4.88	104.04
MQC	9.56	9.61±0.405	4.21	100.51
HQC	15.2	14.94±0.429	2.87	98.28

3.1.6 Stability

3.1.6.1 Short term stock solution stability at room temperature

One solution of Amlodipine at working calibration standard at ULOQ level and internal standard solution at working internal standard level were prepared in diluent from stock solution. Solutions were kept on bench as such at room temperature. After approximately 26 h, fresh solution each of amlodipine at ULOQ level and internal standard at working standard level, were prepared in diluent. Two vials were prepared (each from bench top and freshly prepared) by spiking 50µL of ULOQ, 50 µL of IS in 900µL of diluent. Six replicates injection from each vial was given and the area response was used to determine % change over time. Amlodipine and Nebivolol internal standard are found to be stable in diluent on bench at room temperature for approximately 26 h. The % change was 2.34 whereas for internal standard it was found to be 4.55.

3.1.6.2 Short term stock solution stability at refrigerator (2-8°C)

Stability samples were prepared as for room temperature stability and the solutions were kept into refrigerator as such. After approximately 72 h, fresh solution of Amlodipine at working calibration level ULOQ level and internal standard at working internal standard level were prepared in diluent. Two vials were prepared (each from refrigerator and freshly prepared) by spiking 50µL of ULOQ, 50µL of internal standard in 900µL of diluent. Six replicates from each vial were injected and the area response

was used to determine % change over time. Amlodipine and Nebivolol internal standard were found to be stable in diluent respectively at refrigerator at 2-8°C for approximately 72 h. The % change for Amlodipine was found to be 0.14 whereas for internal standard the % change was 5.26.

3.1.6.3 Bench top stability

Six samples each of low and high QC (stability samples) were kept on bench at room temperature for approximately 12 h. Stability samples were processed and analysed (six samples each of low and high QC). Concentrations were calculated to determine % change over time (Table 5).

Table 5. Bench top stability

Analyte	LQC (% Change)	HQC (% Change)
Amlodipine	-5.09	-4.06

3.1.6.4 Freeze thaw stability at -20 ± 5°C

Eighteen samples each of low and high QC were retrieved from -20 ± 5°C after 24 h of storage of samples. After thawing, the stability samples were restored for at least 12 h and again the same samples were retrieved and kept on bench at room temperature for thaw. The samples were restored and after at least 12 h again retrieve and thawed. Six stability samples (after three cycles) and six comparison samples at each level (Low QC and High QC) were processed and analyzed along with freshly processed quality control samples. Concentrations were calculated to determine % change over time (Table 6).

Table 6. Freeze Thaw Stability at -20 ± 5°C

Analyte	LQC (% Change)	HQC (% Change)
Amlodipine	-1.67	-1.86

3.1.6.5 Autosampler stability

Six samples (stability samples) each of low and high QC were processed and kept in auto sampler (at 5±2°C) for approximately 72 h. The stability samples were analyzed along with freshly processed calibration and comparison samples (six samples each of Low and High QC). Concentrations were calculated to determine % change over time (Table 7).

Table 7. Autosampler stability

Analyte	LQC (% Change)	HQC (% Change)
Amlodipine	-1.05	-2.5

3.1.6.6 Dry extract stability (-20±5°C)

Six samples (stability samples) each of low and high QC were processed and kept in deep freezer (at -20±5°C) for approximately 80 h. The stability samples were analysed along

with freshly processed calibration and comparison samples (six samples each of Low and High QC). Concentrations were calculated to determine % change over time (Table 8).

Table 8. Dry extract stability (-20±5°C)

Analyte	LQC (% Change)	HQC (% Change)
Amlodipine	-1.32	0.65

3.1.6.7 Dilution integrity

Dilution quality control (DQC) is diluted fifth and tenth in human plasma. Prior to extraction, six samples each of fifth and tenth diluted samples was processed and analyzed with freshly processed calibration samples. The calculated concentrations, including the dilution factor for 1/5th and 1/10th yielded coefficient of variation of 1.23 and 9.92 respectively. Percentage of nominal values for dilution factor 1/5th and 1/10th are 97.05 and 100.50 respectively. Results are presented in Table 9.

Table 9. Dilution integrity

Dilution factor	Mean ± SD	% CV	% Accuracy
1/5	58.56 ± 0.722	1.23	97.05
1/10	60.66 ± 6.017	9.92	100.50

3.2 Discussion

3.2.1 Selection of IS

The IS used should be able to negate the sample matrix effects. The chosen IS must be able to match with the physiochemical parameter like solubility, pH, pK_a, structure similarity, category and compatibility with desired analyte. Nebivolol, an easily available compound was selected as the IS in the positive mode. The extraction recovery and chromatographic properties were similar to that of Amlodipine. It was stable in plasma and the results were reproducible in the LC-MS/MS system. Additionally, it caused no interferences to the analytes under study.

3.2.2 Sample pretreatment

An ideal sample pre-treatment method should be able to remove the interferences from the biological matrix and it must also be reproducible with high recovery in minimum number of steps. LLE was found to be the best suited method as it was able to produce clean chromatograms with sufficient efficiency and specificity. LLE was also able to minimize the ion suppression and matrix effects in LC-MS/MS as well as cost effective hence ethyl acetate was adopted as the extraction solvent.

3.2.3 Mass spectrometry

Pharmacokinetic application requires highly selective assays with high sample throughput capacity. Quantification of drugs in biological matrices by LC-MS/MS is becoming more common due to the improved sensitivity and selectivity of this technique [12]. Hence to achieve the desired LLQC Level, LC-MS/MS detection was chosen.

4. CONCLUSION

Amlodipine is highly effective drug being used in treatment of angina and hypertension. Many attempts have being made to determine Amlodipine in human plasma by simple and cost effective process. In the present full validation we tried to explore a simpler method for extraction of Amlopdipine from human plasma and quantify its concentration in the same. Ethyl acetate was used for extraction of Amlodipine from plasma and its concentration was determined using a newly developed and validated bioanalytical method based on LC-MS. The stability studies were also performed to assess the routine viability of the method. The method was found to be simple, accurate, precise and convenient for routine analysis of Amlodipine in human plasma. The validated method can be used to analyze human plasma samples for its pharmacokinetics application, bioavailability and bioequivalence studies.

REFERENCES

- [1] Murdoch D, Heel RC. Amlodipine. A review of its pharmacodynamic and pharmacokinetic properties and therapeutic use in cardiovascular disease. *Drugs* 1991; 41(3): 478-505.
- [2] Bhatt J, Singh S, Subbaiah G, Shah B, Kambli S, Ameta S. A rapid and sensitive liquid chromatography - tandem mass spectrometry (LC-MS/MS) method for the estimation of Amlodipine in human plasma. *Biomed Chromatogr* 2007; 21(2): 169-75.
- [3] Tatar S, Atmaca S. Determination of Amlodipine in human plasma by high-performance liquid chromatography with fluorescence detection. *J Chromatogr B* 2001; 758(2): 305-10.
- [4] Yuanyuan M, Feng Q, Xiaohong S, Xiumei L, Famei L. Determination and pharmacokinetic study of amlodipine in human plasma by ultra performance liquid chromatography–electrospray ionization mass spectrometry. *J Pharmaceut Biomed* 2007; 43:1540–5
- [5] Scharpf F, Riedel KD, Laufen H, Leitold M. Enantioselective gas chromatographic assay with electron-capture detection for Amlodipine in biological samples. *J Chromatogr B* 1994; 655(2): 225-33.
- [6] Bahrami G, Mirzaeei S. Simple and rapid HPLC method for determination of amlodipine in human serum with fluorescence detection and its use in pharmacokinetic studies. *J Chromatogr B* 2004; 36(1): 163-8.

- [7] Zarghi A, Foroutan SM, Shafaati A, Khoddam A. Validated HPLC method for determination of Amlodipine in human plasma and its application to pharmacokinetic studies. *Farmaco* 2005; 60(9): 789-92.
- [8] Strel A, Laine C, Zimmer C, Sibenaler R, Ceccato A. Enantiomeric determination of Amlodipine in human plasma by liquid chromatography coupled to tandem mass spectrometry. *J Biochem Bioph Meth* 2002; 54: 357-68.
- [9] Shentu J, Fu L, Zhou H, Hu XJ, Liu J, Chen J, Wu G. Determination of Amlodipine in human plasma using automated online solid-phase extraction hplc-tandem mass spectrometry: Application to a bioequivalence study of chinese volunteers. *J Pharmaceut Biomed* 2012; 70: 614-8.
- [10] Carvalho M, Oliveira CH, Mendes GD, Sucupira M, Moraes ME, De Nucci G. Amlodipine bioequivalence study: Quantification by liquid chromatography coupled to tandem mass spectrometry. *Biofarm Drug Disposition* 2001; 22(9): 383- 90
- [11] Sarkar AK, Ghosh D, Das A, Selvan PS, Gowda KV, Mandal U, Bose A, Agarwal S, Bhaumik U, Pal TK. Simultaneous determination of metoprolol succinate and amlodipine besylate in human plasma by liquid chromatography-tandem mass spectrometry method and its application in bioequivalence study. *J Chromatogr B Analyt Technol Biomed Life Sci* 2008; 873(1): 77-85
- [12] Addepalli RV, Sengupta P, Mullangi R. Development and validation of a highly sensitive and robust LC-ESI-MS/MS method for simultaneous quantitation of simvastatin acid, amlodipine and valsartan in human plasma: application to a clinical pharmacokinetic study. *Biomed Chromatogr* 2009; 23(6): 615-22
- [13] Nirogi RVS, Kandikere VN, Mudigonda K, Shukla M, Maurya S. Sensitive and rapid liquid chromatography/tandem mass spectrometry assay for the quantification of amlodipine in human plasma. *Biomed Chromatogr* 2006; 20(9): 833–42.
- [14] Massaroti P, Moraes LAB, Marchioretto MAM, Cassiano NM, Bernasconi G, Calafatti SA, Barros FAP, Meurer EC, Pedrazzoli J. Development and validation of a selective and robust LC-MS/MS method for quantifying amlodipine in human plasma. *Anal Bioanal Chem* 2005; 382(4): 1049-54
- [15] Klinkenberg R, Strel B, Ceccato A. Development and validation of a liquid chromatographic method for the determination of amlodipine residues on manufacturing equipment surfaces. *J Pharmaceut Biomed* 2003; 32:345–52.