CHAPTER VII

LC-MS METHOD FOR THE ESTIMATION OF AMLODIPINE

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INTRODUCTION

Amlodipine (ALP)\textsuperscript{14}, 5-Methyl 3-ethyl 2-(2-aminoethoxymethyl)-4-(2-chlorophenyl)-6-methyl-1,4-dihydropyridine-3,5-dicarboxylate (Table 7.01, Page no. 291), is a long-acting calcium channel blocker with cardiovascular activity. It is used as antihypertensive, antiarrhythmic agent and in the treatment of angina\textsuperscript{5-8}. Amlodipine belongs to the dihydropyridine type third generation calcium channel blocker. It acts by relaxing (dilating) blood vessels, lowering blood pressure, and decreasing heart rate, which lowers the workload on the heart. It also dilates coronary arteries increasing blood flow to the heart. In angina it increases blood flow to the heart muscle. The therapeutic importance and characteristic features of Amlodipine are represented in Table 7.02 (Page no. 292)

A limited number of methods have been reported for the estimation of amlodipine in biological fluids. The literature suggested and reported only a few HPLC\textsuperscript{9-12}, HPTLC\textsuperscript{13}, LC/MS\textsuperscript{14-17}, ELISA\textsuperscript{18}, Gas chromatography\textsuperscript{19}, UPLC-MS\textsuperscript{20} and Voltametric methods\textsuperscript{21} for the determination of amlodipine in human plasma.

In the present chapter author had developed a sensitive and specific liquid chromatographic method coupled with mass spectrometry (MS\textsubscript{26}) for the quantification of amlodipine in human plasma. The chromatographic separation was carried out on a C18 analytical column with an isocratic mobile phase consisting of acetonitrile: 0.1% formic acid (80:20 v/v) at a flow rate of 0.5 ml/min (Table 7.06, Page no. 310). An electron spray ionization interface (ESI) in positive ionization mode was used as the ion source and the analysis was performed in the multiple reaction monitoring (MRM) mode (Table 7.05, Page no. 309). Citalopram was used as the internal standard. Serum sample preparation was based on liquid–liquid extraction using a mixture of diethylether and dichloromethane in the ratio of 70:30 (v/v).
### Analytically important functional groups
Imino group, Primary amino group, Keto group and Aromatic benzene ring.

### Chemical name
5-Methyl 3-ethyl 2-(2-aminoethoxymethyl)-4-(2-chlorophenyl)-6-methyl-1,4-dihydropyridine-3,5-dicarboxylate

### Official name
Amlodipine

### Table 7.01
Structural features and active functional groups of Amlodipine

<table>
<thead>
<tr>
<th>S. No</th>
<th>Official name</th>
<th>Chemical name</th>
<th>Structure</th>
<th>Analytically important functional groups</th>
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<tr>
<td>1.</td>
<td>Amlodipine</td>
<td>5-Methyl 3-ethyl 2-(2-aminoethoxymethyl)-4-(2-chlorophenyl)-6-methyl-1,4-dihydropyridine-3,5-dicarboxylate</td>
<td><img src="image" alt="Structure" /></td>
<td>Imino group, Primary amino group, Keto group and Aromatic benzene ring.</td>
</tr>
</tbody>
</table>
# Table - 7.02
Therapeutic importance and characteristic features of Amlodipine

<table>
<thead>
<tr>
<th>Pharmacodynamic/Therapeutic category</th>
<th>Characteristics</th>
<th>Therapeutic importance</th>
</tr>
</thead>
</table>
| Calcium channel blocker/Dihydropyridine | **Molecular formula:** $\text{C}_{26}\text{H}_{22}\text{ClN}_{2}\text{O}_{5}$  
**Molecular weight:** 408.879 g/mol  
**Solubility:** Slightly soluble in water and propanol, freely soluble in methanol and sparingly soluble in ethanol.  
**Physical state:** White crystalline powder.  
**Melting point:** 178 - 179°C | Amlodipine selectively inhibits the entry of excess calcium (without changing the serum calcium levels) into cells and/or prevents the mobilization of calcium from intracellular stores, resulting in relaxation of blood vessel walls and cardiac muscle for blood to flow more freely, lowering blood pressure thereby reducing oxygen demand in the heart and relieving anginal pain. |
I. EXPERIMENTAL:

1. Instrumentation:

- The separation was performed on a Shimadzu HPLC system (Shimadzu), which consisted of a LC-10ADvp pump, a SCL-10Avp system controller (accompanied by an auto sampler), a CTO-10Avp column oven, an FCV-10ALvp low pressure gradient unit, and DGU-14A degasser.

- API 4000 triple quadrupole tandem mass spectrometer, (Applied Biosystems MDS SCIEX) equipped with an electrospray ionization (ESI) source in positive ionization mode was used for detection.

- Data processing was performed on Analyst software version 1.4.1 (Applied Biosystems MDS SCIEX).

- The water was purified using a Milli-Q system (Milford, MA).

- Vortex shaker (Spinix).

- Ultra sonicator (Bandolin sonorex).

2. Drugs, Chemicals and Reagents:

- Amlodipine
- Citalopram
- Methanol (HPLC grade)
- Water (HPLC grade)
- Acetonitrile (HPLC grade)
- Diethylether (Merck)
- Dichloromethane (Merck)
- Ethylene diamine tetra acetic acid (Merck)
- Citrate phosphate dextrose adenine (Merck)

3. Diluent solution:

- Prepared by mixing methanol and water in the ratio of 50:50 (v/v)
4. Extraction solvent:

- Prepared by mixing diethyl ether and dichloromethane in the ratio of 70:30 (v/v).

5. Mobile phase:

- Mobile phase ‘A’ consisted of acetonitrile. Mobile phase ‘B’ was 0.1% formic acid. The mobile phase used for analysis was prepared by mixing mobile phase ‘A’ and mobile phase ‘B’ in the ratio, 80:20 (v/v).

- 0.1% Formic acid: Measure accurately 1 ml of formic acid and mix it with 250 ml of HPLC grade water in 1 liter volumetric flask. Stopper the flask. Mix well and made up to the mark with HPLC grade water.

6. Preparation of standard solutions:

6.1. Amlodipine standard solution:

The standard stock solution (1 mg/ml) of amlodipine was prepared by dissolving 100 mg of the drug in 20 ml of diluent and made up to 100 ml with diluent to get a clear solution. A portion of this stock solution was diluted step wise to get the working standard solutions 100–10000 pg/ml. All the solutions were stored at 4°C and were brought to room temperature before use.

6.2. Internal standard solution (Citalopram):

The standard stock solution (1 mg/ml) of citalopram was prepared by dissolving 100 mg of the drug in 20 ml of diluent and made up to 100 ml with diluent to get a clear solution. A portion of this stock solution was diluted step wise to get the working standard solution of 1 ng/ml, stored at 4°C and brought to room temperature before use.

6.3. Spiked plasma calibration curve standards:

Calibration plot standards were prepared by spiking blank plasma with aqueous working amlodipine (100 µg/ml) at concentration of 100, 250, 500, 1000, 2500, 5000,
7500, 10000 pg/ml. All the standards were stored at -20\(^\circ\)C and brought to room temperature before use. The preparations of spiked plasma calibration curve standards are summarized in Table-7.03 (Page no. 307).

6.4. Spiked plasma quality control samples:

Quality control samples were prepared using drug-free human plasma to obtain four different concentrations by spiking blank plasma with aqueous working amlodipine (100 \(\mu\)g/ml) at concentrations of LLOQ (110 pg/ml), LQC (290 pg/ml), MQC (4500 pg/ml) and HQC (8000 pg/ml). 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 stored at -20\(^\circ\)C. The preparations of spiked plasma quality control samples are summarized in Table-7.04 (Page no. 308).

7. Operating Conditions:

7.1. Liquid chromatography:

The chromatographic separation was carried out on a 50 mm \(\times\) 4.6 mm i.d., 5 \(\mu\)m particle, Zorax XDB C18 analytical column with an isocratic mobile phase consisting of acetonitrile: 0.1% formic acid (80:20 v/v) at a flow rate of 0.5 ml/min. Column oven temperature was set at 35\(^\circ\)C. The autosampler temperature was kept at 5\(^\circ\)C and 20 \(\mu\)l of sample solution was injected with partial loop mode (Table-7.06, Page no. 310).

7.2. Mass spectrometer:

Mass spectrometric detection was performed on an API 4000 triple quadrupole instrument using multiple reaction monitoring. A electron spray ionization interface in positive ionization mode was used (Table-7.05, Page no. 309).
8. Method development:

The development of the method involves the tuning of the molecule, optimizing the chromatographic conditions, selection of internal standard and selection of extraction procedure.

8.1. Tuning of molecule:

Tuning of the molecule is made by optimizing mass spectrophotometer parameters like collision gas, curtain gas, gas1 (nebulizer gas), gas2 (heater gas), ion spray voltage, ion source temperature, declustering potential, collision energy, collision cell exit potential and dwell time per transition by using a 10 µl continuous infusion of 50 ng/ml of ALP and internal (CTP) standards.

8.2. Chromatographic conditions:

The chromatographic conditions, especially selection of analytical column, composition & flow rate of mobile phase and injection volume to achieve good resolution and symmetric peak shapes for the analyte (ALP) and internal standard (CTP), as well as a short run time were studied using a 10 µl continuous infusion of 50 ng/ml of ALP and internal (CTP) standards.

8.3. Plasma sample preparation

A liquid phase extraction technique was used to extract ALP and CTP (ISTD) from plasma. All frozen subject samples, calibration standards and quality control samples were thawed at room temperature and homogenized with a vortex mixer. To each glass test tube (10 ml) 500 µl of aliquot of plasma, 20 µl of CTP working standard (1 ng/ml) was added. The samples were vortex-mixed for about 30 s and 3.5 ml of extraction solvent (Diethylether:Dichloromethane (70:30) v/v) was added. The mixture was 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 and injected into the LC-MS system.

9. Method validation:

Method validation includes all of the procedures required to demonstrate that a method to quantify the concentration of ALP in plasma is reliable for the intended application.

9.1. Selectivity:

The selectivity of the method was 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 are compared with those obtained with an aqueous solution of the ALP and CTP (ISTD) at a lower limit of quantification (LLOQ) concentration.

Acceptance criteria: Area of the peak at the retention time of ALP in standard blank samples was ≤20% of the area of the ALP in the extracted LLOQ sample, area of the peak at the retention time of CTP (ISTD) in standard blank samples was <5% of the area of the CTP (ISTD) in the extracted LLOQ sample.

9.2. Linearity and standard calibration curve:

Calibration curve (three replicate analyses) of ALP was prepared using a set of 8 calibrations points, prepared in human blank plasma in the 100-10000 pg/ml concentration range (Table 7.03, Page no. 307), and stored frozen. The aliquots were not thawed until needed for use. The linearity of calibration curve was determined by plotting the peak area ratios (y) of amlodipine to internal standard versus the amlodipine concentrations (x) in spiked plasma samples.
**Acceptance criteria:** 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. At least 75% non-zero standards should meet the following criteria, including the LLOQ (lower limit of quantitation) & the LQC (low quality control).

9.3. Accuracy:

The accuracy of the assay was defined as the absolute value of the ratio of back calculated mean values of the quality control samples to their respective nominal values, expressed as percentage of theoretical concentration. Within-batch and between-batch accuracy were evaluated at four quality control samples concentrations (LLOQ, LQC, MQC and HQC).

\[
\text{Accuracy} \, (\%) = \left( \frac{\text{found concentration}}{\text{theoretical concentration}} \right) \times 100
\]

Within-batch assays involved five replicates per run and between-batch assays were performed on five separate runs.

**Acceptance criteria:** For within- and between-run accuracy, the mean value of samples at each concentration level should be within 85-115% of the actual value. 67% of all quality control samples should be within acceptance criteria and minimum 50% of each concentration level should be within acceptance criteria.

9.4. Precision:

It was measured by percent coefficient of variation (CA) over the concentration range of quality control sample of ALP during course of validation. Within-batch and between-batch precision was evaluated at four quality control samples concentrations (LLOQ, LQC, MQC and HQC). Within-batch assays involved five replicates per run and between-batch assays were performed on five separate runs.
Acceptance criteria: The within and between batch mean precision for LLOQ, LQC, MQC, and HQC samples should be <15%. 67% of all quality control samples should be within acceptance criteria and minimum 50% of each concentration level should be within acceptance criteria.

10. Recovery:

10.1. Analyte drug (ALP):

The percentage recovery of ALP was determined by measuring peak area response of six replicates after extraction of quality control samples containing low, middle and high drug concentration against the peak area response of six replicates of aqueous quality control samples containing equivalent concentrations.

10.2. ISTD (CTP):

The percentage recovery of CTP was determined by measuring peak area response of six replicates after extraction of ISTD samples against the peak area response of six replicates of samples containing equivalent concentrations.

The extraction yield was calculated by dividing the mean peak area of the extracted samples by the corresponding mean peak area of the aqueous samples. The recovery of the analyte and ISTD need not be 100%, but the extent of recovery of an analyte and ISTD should be consistent, precise and reproducible.

11. Stability of ALP and CTP (ISTD) in stock solutions:

11.1. Short term stability of drugs in stock solutions:

Stock solutions of 0.100 mg/ml for ALP and CTP (ISTD) were prepared freshly and aliquots of freshly prepared stock solutions were left at room temperature of 25°C (stability samples) for 7 hours. The freshly prepared stock solution stored in refrigerator below 10°C, were used as comparison samples. The short-term stock solution stability of ALP and CTP (ISTD) in methanol:water (50:50 w/v) was successfully assessed by
comparing mean responses of six replicates of stability samples versus six replicates of comparison samples. The samples qualified the test if the deviation was within ±15%.

11.2. Long term stability of drugs in stock solutions:

Stock solutions of 0.100 mg/ml for ALP and CTP (ISTD) were prepared freshly and aliquots of freshly prepared stock solutions were stored in refrigerator below 10°C (stability samples) for 8 days. On the day of analysis, freshly prepared stock solution stored in refrigerator below 10°C, were used as comparison samples. The long-term stock solution stability of ALP and CTP (ISTD) in methanol:water (50:50 v/v) was successfully assessed by comparing mean responses of six replicates of stability samples versus six replicates of comparison samples. The samples qualified the test if the deviation was within ±15%.

12. Stability of the drug in biological matrix:

12.1. Freeze-thaw stability:

The freeze-thaw stability of ALP in human sample was assessed by analyzing six replicates of quality control samples at low and high levels (LQC and HQC), previously frozen and thawed over 3 cycles along with freshly prepared calibration curve and by calculating the percent nominal of mean concentrations of stability samples at low and high quality control levels. The samples qualified the test if the deviation was within ±15%.

12.2. Bench top stability:

To evaluate the bench top stability, six replicates human plasma samples at two quality control concentration levels (low and high) of ALP were frozen, thawed and left to stand at room temperature for 7 hours 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 ±15%.
12.3. In-injector stability:

In-injector stability of the ALP was assessed by analyzing six replicates of processed quality control samples at low and high concentrations kept in auto-injector at 5°C after 38 hours. These samples were run along with the freshly spiked calibration curve and quality control samples. Concentration was calculated against the freshly spiked calibration curve. The percentage stability was determined by calculating the percentage nominal of mean concentrations of quality control samples stored at 5°C for 38 hours in auto sampler. The samples qualified the test if the deviation was within ±15%.

12.4. Long term stability of drug in biological matrix:

The long term stability of ALP in human plasma was assessed by analyzing quality control samples at high and low concentrations after storage for 30 days at -20°C and at -70°C. The concentrations of stability quality control samples were determined with freshly prepared calibration curve. The percentage stability was determined by calculating the percent nominal of mean concentrations of stability quality control samples at high and low quality control levels.

12.5. Effect of anticoagulant:

Effect of anticoagulant on the stability of ALP was assessed by analyzing six replicates of quality control samples at LQC and HQC concentrations spiked in human plasma containing EDTA (Ethylene diamine tetra acetic acid) and CPDA (Citrate phosphate dextrose adenine). These samples were run along with the freshly processed calibration curve prepared in CPDA plasma. Concentration was calculated against the freshly processed calibration curve. The percentage stability was determined by calculating the percentage nominal of mean concentrations of quality control samples. The samples qualified the test if the deviation was within ±15%.
II. RESULTS AND DISCUSSION:

1. Method development:

Tuning of the molecule is made by optimizing the mass spectrometric parameters for analyte (ALP) and ISTD (CTP). The results are summarized in Table-7.05 (Page no. 309). After ALP and CTP (ISTD) 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 $[\text{M+H}]^+$ \textit{m/z} 409.4 and 325.5 for ALP and CTP (ISTD) and the major fragment ions observed in each product spectrum were at \textit{m/z} 238.1 and 109.3, respectively.

The chromatographic conditions to achieve good resolution and symmetric peak shapes for ALP and CTP (ISTD) were optimized and the results are summarized in Table-7.06 (Page no. 310).

Liquid-liquid extraction was used for the sample preparation. Liquid-liquid extraction can be helpful in producing a spectroscopically clean sample and avoiding the introduction of non volatile materials onto the HPLC column and mass spectrophotometer system. Clean samples are essential for minimizing ion suppression and matrix effect in LC-MS analyses. A mixture of diethyl ether and dichloromethane in the ratio of 70:30 (v/v) was found to be optimal, which can produce a clean chromatogram for blank plasma blank.

Choosing the appropriate ISTD is an important aspect to achieving acceptable method performance, especially with LC-MS, where matrix effects can lead to poor analytical results. Ideally, isotopically labeled internal standards for all analytes should be used, but these are not commercially available. Therefore, we have opted CTP, which is relevant to ALP. In addition, its retention behavior is similar to that of the target analyte. Clean chromatograms were obtained and no significant direct interferences in multiple reaction monitoring channels at the relevant retention times were observed.
2. Selectivity:

The selectivity of the method was investigated by comparing the chromatograms of six different batches of blank human plasma with the peak response of aqueous solution of ALP and CTP at LLOQ (Table-7.07, Page no. 311). The product ion chromatograms are depicted in Fig: 7.01 (Page no. 317). As shown in the chromatogram, retention times for ALP and CTP (ISTD) were about 1.0 and 0.95 min, respectively, and there was no interference from endogenous substances observed at the retention time of the analytes. The total LC-MS analysis time was 1.8 min per sample. The chromatograms of the blank plasma of ALP and CTP are represented in Figs: 7.02 & 7.03 (Page no. 318).

3. Linearity:

The linearity of the method was determined by a weighted (1/x²) least square regression analysis of standard plots associated with eight point standard curve for ALP. The linearity regression of the peak ratios versus concentrations were fitted over the concentration range of 100-10000 pg/ml (Fig: 7.04, Page no. 319). The calibration curve exhibited good linearity (r = 0.9965) and the curve showed good back-calculated precision and accuracy. The linear regression equation of the calibration curve was y = 7.66e-005 x + 0.0014. The back-calculated concentration of a calibration samples were within the limits of acceptance; ±20 % at the LLOQ and ±15 % for the other concentration levels. The results of back calculated concentrations of calibration standards for ALP are given in Table-7.08 (Page no. 312).

4. Accuracy:

The within-batch accuracy for the low, middle and high quality control samples ranged from 90.74% to 98.74% and between-batch ranged from 97.04% to 101.65%, within the acceptance criteria ±15% of nominal concentration. The within-batch accuracy for the lower limit of quantification quality control samples ranged from 94.09% to 106.45% and between-batch was 99.86% to 112.74%, within the acceptance criteria ±20% of nominal concentration for ALP. The results (Table-7.09, Page no. 313) obtained were reproducible and satisfied the criteria for acceptance of accuracy.
5. Precision:

The within-batch precision for low, middle and high quality control samples ranged from 2.79 to 4.25%, within the acceptance criteria 15% of %CV. The within-batch precision for the lower limit of quantification quality control sample was 4.47% and between-batch precision was in the range of 4.88% to 6.79%, within the acceptance criteria 20% of %CV for amlodipine. The between batch precision for low, middle and high quality control samples were ranged from 3.27% to 6.71%, which is within the acceptance limits of ±15%. The results (Table-7.09, Page no. 313) obtained were reproducible and satisfied the criteria for acceptance of precision.

6. Recovery:

The areas of extracted quality control samples were compared against the areas of respective aqueous quality control samples. The percentage mean recovery of ALP in HQC, MQC and LQC was 62.20, 66.03 and 67.39% respectively. The CTP (ISTD) peak areas of plasma samples were compared to the CTP (ISTD) peak areas of the aqueous samples. The percentage mean recovery of CTP (ISTD) was 57.03%. The results showed that the recoveries of ALP and CTP (ISTD) were consistent, precise and reproducible, which has proved to be satisfied in bioanalysis. The chromatograms of LQC, MQC and HQC are represented in Figs: 7.06, 7.07 and 7.08 (Page no. 321, 322 & 323). The calculated mean peak area, standard deviation, coefficient of variation and percent recovery was represented in Table-7.10 (Page no. 314).

7. Stability of ALP and CTP (ISTD) in stock solution:

7.1. Short Term stock Solution Stability:

The percentage stability after about 7.0 hours was found to be 97.49% for ALP and for CTP (ISTD) the percentage stability after about 7.0 hours was found to be 102.16%, which were within the acceptance limits of 85 to 115% for drug and ISTD.
7.2. Long Term Stock Solution Stability:

The percentage mean stability after 8 days for ALP was found to be 97.34%, which is within the acceptance range of 85 to 115%. The % mean stability after 8 days was found as 102.90 for CTP (ISTD), which is within the acceptance range of 85 to 115%.

The calculated mean peak area, standard deviation, coefficient of variation and percent stability for short and long term stability of stock solution was summarized in Table-7.11 (Page no. 315).

8. Stability of drug in biological matrix:

8.1. Freeze Thaw Stability:

The stability of high and low quality control samples of ALP was determined after three freeze thaw cycles. The percentage mean for low and high quality control samples was 99.42% and 99.13% respectively. This is within the acceptance range of 85.0 to 115.0%.

8.2. Bench top stability:

The short term stability of replicate concentrations of low and high quality control samples at room temperature after 7 hours was 100.30% and 95.84% respectively, which is within the acceptance range of 85.0 to 115.0% for ALP.

8.3. In-injector stability:

The stability of high and low quality control samples in auto sampler at 10°C after 38 hours was 100.72% and 98.52% respectively for ALP. This is within the acceptance range of 85.0 to 115.0%.
8.4. Long term stability of drug in biological matrix:

The stability of high and low quality control samples of ALP in human plasma at -20°C after 30 days was 95.25% and 100.45% respectively. At -70°C after 30 days the stability of LQC and HQC 102.31% and 99.04% respectively for ALP. This is within the acceptance range of 85.0 to 115.0%.

8.5. Effect of anticoagulant:

The stability of high and low quality control samples of ALP in EDTA plasma was 97.22% and 97.92% respectively, whereas in CDPA plasma it was 97.05% and 94.84% respectively. The values obtained are within the acceptance range of 85.0 to 115.0%.

The mean concentration, standard deviation, coefficient of variation and percent stability for all the types of stability of ALP in biological matrix was calculated and summarized in the Table-7.12 (Page no. 316). 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 ALP plasma samples. The findings from these stability tests indicated that storage of ALP in plasma samples is adequate and no-stability-related problems would be expected during the samples routine analysis for the bioequivalence or pharmacokinetic studies.
## Table – 7.03
### Preparation of spiked plasma calibration curve standards

<table>
<thead>
<tr>
<th>S.No</th>
<th>Calibration standard</th>
<th>Volume of Working Amlodipine (μL)</th>
<th>Volume of Plasma (μL)</th>
<th>Concentration (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>S1</td>
<td>10</td>
<td>9990</td>
<td>100</td>
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<td>2</td>
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<td>8</td>
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<td>1000</td>
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</table>

* Working Amlodipine concentration – 100 μg/ml*
Table – 7.04
Preparation of spiked plasma quality control samples

<table>
<thead>
<tr>
<th>S.No</th>
<th>Quality control sample</th>
<th>Volume of Working Amlodipine (µl)</th>
<th>Volume of Plasma (µl)</th>
<th>Concentration (pg/ml)</th>
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<tr>
<td>1</td>
<td>LLOQ</td>
<td>11</td>
<td>9989</td>
<td>110</td>
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<tr>
<td>2</td>
<td>LQC</td>
<td>29</td>
<td>9971</td>
<td>290</td>
</tr>
<tr>
<td>3</td>
<td>MQC</td>
<td>450</td>
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<tr>
<td>4</td>
<td>HQC</td>
<td>800</td>
<td>9200</td>
<td>8000</td>
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* Working Amlodipine concentration – 100 µg/ml
### Table – 7.05

**Working Mass spectrophotometric parameters**

<table>
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<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
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<td>Collision gas (psi)</td>
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</tr>
<tr>
<td>Curtain gas (nitrogen) (psi)</td>
<td>20</td>
</tr>
<tr>
<td>Gas1 (nebulizer gas) (psi)</td>
<td>60</td>
</tr>
<tr>
<td>Gas2 (heater gas) (psi)</td>
<td>40</td>
</tr>
<tr>
<td>Ion spray voltage (V)</td>
<td>5500</td>
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<tr>
<td>Ion source temperature (°C)</td>
<td>500</td>
</tr>
<tr>
<td>Declustering potential (V)</td>
<td>19 (Amlodipine), 50 (Citalopram)</td>
</tr>
<tr>
<td>Collision energy (V)</td>
<td>16 (Amlodipine), 46 (Citalopram)</td>
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<tr>
<td>Collision cell Exit Potential (V)</td>
<td>15 (Amlodipine), 5 (Citalopram)</td>
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<tr>
<td>Dwell time per transition (ms)</td>
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Table – 7.06  
Working Chromatographic conditions

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<th>Parameter</th>
<th>Mobile Phase</th>
<th>Flow rate (ml/min)</th>
<th>Run time (min)</th>
<th>Volume of injection (µl)</th>
<th>Retention time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acetonitrile + 0.1% Formic acid (80:20)</td>
<td>0.5</td>
<td>1.8</td>
<td>20</td>
<td>1.00</td>
</tr>
</tbody>
</table>
**Table - 7.07**

Selectivity of the method

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Plasma sample No.</th>
<th>ALP</th>
<th>CTP (ISTD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Area response</td>
<td>Interference response</td>
</tr>
<tr>
<td>1</td>
<td>I</td>
<td>7091</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>II</td>
<td>7596</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>III</td>
<td>7172</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>IV</td>
<td>8002</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>V</td>
<td>148</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>VI</td>
<td>7169</td>
<td>0</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>7363</td>
<td>0</td>
</tr>
</tbody>
</table>
Table – 7.08
Back calculated concentrations for calibration curve standards of ALP

<table>
<thead>
<tr>
<th>Concentration added (pg/ml)</th>
<th>Mean concentration found (pg/ml) (n=3)</th>
<th>± S.D</th>
<th>% CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>98.07</td>
<td>1.43</td>
<td>1.46</td>
</tr>
<tr>
<td>250</td>
<td>251.48</td>
<td>9.63</td>
<td>3.83</td>
</tr>
<tr>
<td>500</td>
<td>553.95</td>
<td>18.32</td>
<td>3.31</td>
</tr>
<tr>
<td>1000</td>
<td>1076.29</td>
<td>57.28</td>
<td>5.32</td>
</tr>
<tr>
<td>2500</td>
<td>2388.71</td>
<td>113.22</td>
<td>4.74</td>
</tr>
<tr>
<td>5000</td>
<td>4736.55</td>
<td>143.07</td>
<td>3.02</td>
</tr>
<tr>
<td>7500</td>
<td>7385.96</td>
<td>328.74</td>
<td>4.45</td>
</tr>
<tr>
<td>10000</td>
<td>9723.91</td>
<td>201.97</td>
<td>2.08</td>
</tr>
</tbody>
</table>
Table – 7.09
Within and Between batch precision and accuracy for quality control samples of ALP

<table>
<thead>
<tr>
<th>Level</th>
<th>Concentration added (pg/ml)</th>
<th>Within Batch</th>
<th>Between Batch</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean concentration found* (pg/ml) ±S.D</td>
<td>% Accuracy</td>
</tr>
<tr>
<td>LLOQ</td>
<td>110</td>
<td>101.52 ± 4.58</td>
<td>94.09</td>
</tr>
<tr>
<td>LQC</td>
<td>290</td>
<td>283.22 ± 7.89</td>
<td>94.40</td>
</tr>
<tr>
<td>MQC</td>
<td>4500</td>
<td>4443.6 ± 150.312</td>
<td>98.74</td>
</tr>
<tr>
<td>HQC</td>
<td>8000</td>
<td>7259.21 ± 308.750</td>
<td>90.74</td>
</tr>
</tbody>
</table>

* Average of five determinants
Table 7.10
Recovery of ALP and CTP

<table>
<thead>
<tr>
<th>Parameters</th>
<th>ALP</th>
<th>CTP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LQC (290 pg/ml)</td>
<td>MQC (4500 pg/ml)</td>
</tr>
<tr>
<td>Unextracted</td>
<td>Extracted</td>
<td>Unextracted</td>
</tr>
<tr>
<td>Mean area</td>
<td>43430</td>
<td>29268</td>
</tr>
<tr>
<td>±S.D</td>
<td>518.8</td>
<td>589</td>
</tr>
<tr>
<td>%CV</td>
<td>1.19</td>
<td>2.01</td>
</tr>
<tr>
<td>% Recovery</td>
<td>67.39</td>
<td>66.03</td>
</tr>
</tbody>
</table>

* Average of six determinants
Table 7.11
Short term and long term stability of ALP and CTP in stock solution

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Short term stability</th>
<th>Long term stability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ALP</td>
<td>CTP</td>
</tr>
<tr>
<td>Mean area*</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2037463</td>
<td>1986416</td>
</tr>
<tr>
<td>±S.D</td>
<td>40330.7</td>
<td>68898.2</td>
</tr>
<tr>
<td>%CV</td>
<td>1.98</td>
<td>3.47</td>
</tr>
<tr>
<td>% Stability</td>
<td>97.49</td>
<td>102.16</td>
</tr>
</tbody>
</table>

* Average of six determinants
### Table – 7.12
Stability of ALP in biological matrix

<table>
<thead>
<tr>
<th>Stability</th>
<th>LQC (290 pg/ml)</th>
<th></th>
<th></th>
<th>HQC (8000 pg/ml)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean concentration (pg/ml)</td>
<td>±S.D</td>
<td>%CV</td>
<td>% Stability</td>
<td>Mean concentration (pg/ml)</td>
<td>±S.D</td>
</tr>
<tr>
<td>Freeze thaw</td>
<td>299.31</td>
<td>8.218</td>
<td>2.75</td>
<td>99.42</td>
<td>7881.73</td>
<td>266.658</td>
</tr>
<tr>
<td>Bench top</td>
<td>294.67</td>
<td>9.388</td>
<td>3.19</td>
<td>100.30</td>
<td>7735.59</td>
<td>268.236</td>
</tr>
<tr>
<td>In-injector</td>
<td>289.46</td>
<td>7.977</td>
<td>2.76</td>
<td>98.52</td>
<td>8129.73</td>
<td>191.460</td>
</tr>
<tr>
<td>Long term at -20°C</td>
<td>295.13</td>
<td>19.524</td>
<td>6.62</td>
<td>100.45</td>
<td>7687.67</td>
<td>410.012</td>
</tr>
<tr>
<td>Long term at -70°C</td>
<td>300.58</td>
<td>12.033</td>
<td>4.00</td>
<td>102.31</td>
<td>7993.50</td>
<td>290.806</td>
</tr>
<tr>
<td>EDTA plasma</td>
<td>287.69</td>
<td>11.900</td>
<td>4.14</td>
<td>97.92</td>
<td>7846.53</td>
<td>301.396</td>
</tr>
<tr>
<td>CPDA plasma</td>
<td>278.65</td>
<td>14.863</td>
<td>5.33</td>
<td>94.84</td>
<td>7833.00</td>
<td>248.685</td>
</tr>
</tbody>
</table>

- Average of six determinants
Fig: 7.01 Chromatograms of aqueous standards of ALP and CTP
Fig: 7.02 Chromatogram of blank plasma (ALP)

Fig: 7.03 Chromatogram of blank plasma (CTP)
Fig: 7.04 Calibration curve of ALP

Untitled 34 (Amlodipine): "Linear" Regression ("1 / (x * x)" weighting): $y = 7.66e-005 \times + 0.0014 \ (r = 0.9965)$
Fig: 7.05 Chromatogram of LLOQ sample (ALP)

**Sample Index:** 1
**Sample Type:** QC
**Concentration:** 110.17 ppb
**Calculated Conc.:** 106.56 ppb
**Start Time:** 01:32:04 PM
**End Time:** 01:32:04 PM

**Modified:** No
**Proc. Algorithm:** Analyst Classic
**Bunching Factor:** 1
**Noise Threshold:** 2.00 cps
**Area Threshold:** 100.00 cps

**Sep. Width:** 0.20
**Sep. Height:** 0.01
**Exp. Peak Ratio:** 0.01
**Exp. Adj. Ratio:** 0.01
**Exp. Val. Ratio:** 0.00

**Observed RT:** 1.90 min
**Use Relative RT:** No

**Int. Type:** Base To Base
**Retention Time:** 1.902 min
**Area:** 5944 counts
**Height:** 1731.016 cps
**Start Time:** 0.942 min
**End Time:** 1.90 min

**Time (min):** 0.00 0.20 0.40 0.60 0.80 1.00 1.20 1.40 1.60 1.80

**Intensity (a.u.):** 90 180 270 360 450 540 630 720 810 900

**Calculated Conc.:** 106.56 ppb
**Date:** 09/16/2009
**Time:** 01:32:04 PM

**Expected Rt:** 1.95 min
**Obs. Relative Rt:** 1.90 min

**Expected A T:**

**Expected Adj. A T:**

**Expected Val. A T:**

**Expected Rat. A T:**

**Calculated Rat. A T:**

**Sample Index:** 1
**Sample Type:** QC
**Concentration:** 1.00 ng/ml
**Calculated Conc.:** 0.998 ng/ml
**Start Time:** 01:32:04 PM
**End Time:** 01:32:04 PM

**Modified:** No
**Proc. Algorithm:** Analyst Classic
**Bunching Factor:** 1
**Noise Threshold:** 5.00 cps
**Area Threshold:** 100.00 cps

**Sep. Width:** 0.20
**Sep. Height:** 0.01
**Exp. Peak Ratio:** 0.01
**Exp. Adj. Ratio:** 0.01
**Exp. Val. Ratio:** 0.00

**Observed RT:** 1.50 min
**Use Relative RT:** No

**Int. Type:** Base To Base
**Retention Time:** 1.502 min
**Area:** 935599 counts
**Height:** 113931.239 cps
**Start Time:** 0.627 min
**End Time:** 1.50 min

**Time (min):** 0.00 0.20 0.40 0.60 0.80 1.00 1.20 1.40 1.60 1.80

**Intensity (a.u.):** 10 20 30 40 50 60 70 80 90 100

**Calculated Conc.:** 0.998 ng/ml
**Date:** 09/16/2009
**Time:** 01:32:04 PM

**Expected Rt:** 1.50 min
**Obs. Relative Rt:** 1.50 min

**Expected A T:**

**Expected Adj. A T:**

**Expected Val. A T:**

**Expected Rat. A T:**

**Calculated Rat. A T:**
Fig: 7.06 Chromatogram of LQC sample (ALP)

- **Sample Index:** 1
- **Sample Type:** QC
- **Concentration:** 2.00 ng/µl
- **Calculated Concentration:** 2.00 ng/µl
- **Acq. Date:** 09/16/2009
- **Acq. Time:** 01:34:48 PM
- **Modified:** No
- **Proc. Algorithm:** Analyst Classic
- **Bunching Factor:** 1
- **Baseline Threshold:** 2.00 cps
- **Area Threshold:** 100.00 cps
- **Num. Smooths:** 10
- **Sep. Width:** 0.10
- **Sep. Height:** 0.01
- **Exp. Peak Ratio:** 5.00
- **Exp. Adj. Ratio:** 4.00
- **Exp. Val. Ratio:** 3.00
- **Exp. Val. Window:** 3.0 sec
- **Expected RT:** 0.96 min
- **Use Relative RT:** No
- **Int. Type:** Base To Base
- **Retention Time:** 0.94 min
- **Areas:** 107512 counts
- **Height:** 379.752 cps
- **Start Time:** 9.682 min
- **End Time:** 1.96 min

- **Sample Index:** 1
- **Sample Type:** QC
- **Concentration:** 1,00 ng/µl
- **Calculated Concentration:** 1,00 ng/µl
- **Acq. Date:** 09/16/2009
- **Acq. Time:** 01:34:48 PM
- **Modified:** No
- **Proc. Algorithm:** Analyst Classic
- **Bunching Factor:** 1
- **Baseline Threshold:** 2.00 cps
- **Area Threshold:** 100.00 cps
- **Num. Smooths:** 10
- **Sep. Width:** 0.10
- **Sep. Height:** 0.01
- **Exp. Peak Ratio:** 5.00
- **Exp. Adj. Ratio:** 4.00
- **Exp. Val. Ratio:** 3.00
- **Exp. Val. Window:** 3.0 sec
- **Expected RT:** 0.96 min
- **Use Relative RT:** No
- **Int. Type:** Base To Base
- **Retention Time:** 0.94 min
- **Areas:** 107512 counts
- **Height:** 379.752 cps
- **Start Time:** 9.682 min
- **End Time:** 1.96 min
Fig: 7.07 Chromatogram of MQC sample (ALP)

**Peak Name:** Chlorpropam<br>**Method:** 325.SVM03.4.mna<br>**Comment:** Filename: 325.SVM03.4.mna<br><br>**Sample Index:** 1<br>**Sample Type:** QC<br>**Concentration:** 300.00 pg/mL<br>**Calculated Conc:** 301.91 pg/mL<br>**Run Date:** 09/16/2009<br>**Run Time:** 01:31:33 PM<br>**Modified:** No<br>**Proc Algorithm:** Analyst Classic<br>**Bunching Factor:** 1<br>**Noise Threshold:** 100.00 cps<br>**Area Threshold:** 10.00 cps<br>**Area Smoothing:** 10<br>**Sep. Height:** 0.01<br>**Exp. Peak Ratio:** 5.00<br>**Exp. Adj. Ratio:** 4.00<br>**Exp. Val. Ratio:** 3.00 RP Window: 20.0 m sec<br>**Expected RT:** 1.01 min<br>**Use Relative RT:** No<br><br>**Int. Type:** Base To Base<br>**Retention Time:** 1.995 min<br>**Area:** 201394 counts<br>**Height:** 0.001 000 cps<br>**Start Time:** 0.002 min<br>**End Time:** 1.01 min

**Expected RT:** 0.350 min
**Use Relative RT:** No
**Int. Type:** Base To Base
**Retention Time:** 0.948 min
**Area:** 129646 000 counts
**Height:** 171075.422 cps
**Start Time:** 0.834 min
**End Time:** 1.54 min

---

**Peak Name:** Nalidixic acid<br>**Method:** 325.SVM03.1.mna<br>**Comment:** Filename: 325.SVM03.1.mna<br><br>**Sample Index:** 1<br>**Sample Type:** QC<br>**Concentration:** 1.00 ng/mL<br>**Calculated Conc:** N/A<br>**Run Date:** 09/16/2009<br>**Run Time:** 01:37:33 PM<br>**Modified:** No<br>**Proc Algorithm:** Analyst Classic<br>**Bunching Factor:** 1<br>**Noise Threshold:** 5.00 cps<br>**Area Threshold:** 100.00 cps<br>**Area Smoothing:** 10<br>**Sep. Height:** 0.20<br>**Exp. Peak Ratio:** 3.90<br>**Exp. Adj. Ratio:** 4.40<br>**Exp. Val. Ratio:** 3.00 RP Window: 30.0 m sec<br>**Expected RT:** 0.350 min
**Use Relative RT:** No
**Int. Type:** Base To Base
**Retention Time:** 0.948 min
**Area:** 129646 000 counts
**Height:** 171075.422 cps
**Start Time:** 0.834 min
**End Time:** 1.54 min

---

322
Fig: 7.08 Chromatogram of HQC sample (ALP)

Sample Index: 1
Sample Type: QC
Concentration: 8600.00 pg/mL
Calculated Conc: 9975.20 pg/mL
Acq. Date: 09/16/2009
Acq. Time: 01:40:16 AM

Modified: No
Proc. Algorithm: Analyst Classic
Bunching Factor: 1
Noise Threshold: 1.00 cps
Area Threshold: 100.00 cps
Num. Smooths: 10
Sep. Width: 0.20
Sep. Height: 0.01
Exp. Peak Ratio: 5.00
Exp. Adj. Ratio: 0.01
Exp. Val. Ratio: 3.00 RT Window: 10.0 sec
Expected RT: 1.00 min
Use Relative RT: No

Int. Type: Base To Base
Retention Time: 1.066 min
Area: 765144 counts
Height: 59610.136 cps
Start Time: 0.954 min
End Time: 1.149 min

Sample Index: 1
Sample Type: QC
Concentration: 1.40 pg/mL
Calculated Conc: 0/A
Acq. Date: 09/16/2009
Acq. Time: 01:40:16 AM

Modified: No
Proc. Algorithm: Analyst Classic
Bunching Factor: 1
Noise Threshold: 5.00 cps
Area Threshold: 100.00 cps
Num. Smooths: 10
Sep. Width: 0.20
Sep. Height: 0.01
Exp. Peak Ratio: 5.00
Exp. Adj. Ratio: 0.01
Exp. Val. Ratio: 5.00 RT Window: 30.8 sec
Expected RT: 0.356 min
Use Relative RT: No

Int. Type: Base To Base
Retention Time: 0.469 min
Area: 151647 counts
Height: 15423.993 cps
Start Time: 0.420 min
End Time: 1.471 min
CONCLUSION:

The method for determination of ALP in human plasma with HPLC with mass spectrometric detection has met the acceptance criteria with respect to selectivity, precision, accuracy, linearity, recovery and anticoagulant effect. The assay described in this work is highly specific, sensitive and reproducible for the quantitative analysis of ALP in non-biological solvents as well as human plasma samples. The calibration curves were highly linear over a wide range of concentrations.

The specificity of the LC-MS method makes possible the determination of these compounds in the presence of other endogenous and exogenous plasma components. The chromatograms of ALP and CTP (ISTD) in human plasma showed no significant interfering peaks.

This analytical method demonstrates the ruggedness and reproducibility of the developed method as well as its high sensitivity. The detection limit of the method is as low as 100 to 10000 pg/ml in human plasma allowing the quantification of ALP at low and high concentrations in body fluids. Stability evaluations performed in human plasma and stock solutions have met the acceptance criteria, demonstrating insignificant degradation of ALP over the specified storage durations and conditions.

The present work demonstrated a novel LC-MS method for the quantitative analysis of ALP in human plasma. The reproducibility, specificity and high sensitive of this method allows for the selective and reliable determination of ALP in human plasma.

REFERENCES:

4. Michio Hashimoto, Satomi Kagota, Yoko Kubota, Masanori Katakura, Budbazar Enkhjargal, Shuji Gamoh, Haque Md Abdul, Osamu Shido, Masaru Kunitomo


