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APPLICATIONS

# Development of a Highly Selective Bioanalytical Quantitation Method for the LC-MS/MS Analysis of (R)-Amlodipine and (S)-Amlodipine Enantiomers in Human Plasma Using Lux® Cellulose-4 and Strata®-X

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In this technical note we report the development of an improved selective and rugged validated LC-MS/MS method for the quantitation of Amlodipine enantiomers in human plasma samples. Sample extraction utilized Strata-X polymeric solid phase extraction cartridges and chromatographic separation of (R)–Amlodipine and (S)–Amlodipine enantiomers was achieved on a polysaccharide-based chiral stationary phase, Lux Cellulose-4, under polar organic conditions. This method can be used in pre-clinical and bioequivalence studies for rapid analysis of both pharmacologically active and inactive enantiomers of Amlodipine in human plasma.

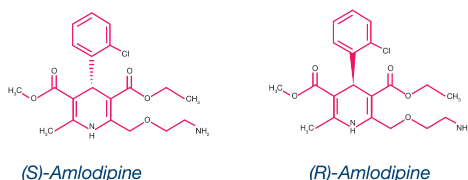
## Introduction

Amlodipine is a calcium channel blocker and although (S)-Amlodipine is the pharmacologically active enantiomer, a racemic mixture of Amlodipine is used for therapeutic purposes. (S)-Amlodipine and (R)-Amlodipine exhibit different pharmacokinetics (PK) and pharmacodynamics (PD); and according to regulatory guidelines[1,2] individual enantiomers should be measured in cases where enantiomers exhibit different pharmacokinetic or pharmacodynamic properties, or the exposure ratio of enantiomers is modified by a difference in the rate of absorption. If one enantiomer is pharmacologically active and the other is inactive or has low contribution to activity, it is sufficient to demonstrate bioequivalence (BE) for the active enantiomer; in this case (S)-Amlodipine. This validated LC-MS/MS method was developed to monitor concentrations of (S)-Amlodipine and (R)- Amlodipine in human plasma samples to evaluate the pharmaceutical equivalence for both racemic and (S)-Amlodipine formulations of Amlodipine.

## Method Development

Several methods have been published for the separation of (S)-Amlodipine and (R)-Amlodipine in various biological matrices[3-13]. However, the major challenge in developing an adequate LC-MS/MS method was in achieving good peak shape for the Amlodipine enantiomers. Published methods using various chiral columns and mobile phase with various basic mobile phase additives such as ammonium hydroxide, trimethylamine and diethylamine, typically gave poor peak shapes and required long analysis times. During method development we tried various additives with 0.05 % (v/v) ethanolamine providing the best peak shapes and signal intensity for amlodipine isomers. It is important to set up a rapid, selective and sensitive LC-MS/MS method for bioanalysis approach to ensure wide concentration range (50 pg/mL to 50 ng/mL) to cover PK and BE studies for all dosage forms ranging from 2.5 mg to 10 mg per day.

**Structures of (S)-Amlodipine and (R)-Amlodipine**



*(S)-Amlodipine* *(R)-Amlodipine*

To minimize potential matrix interferences compromising quantitation due to ion suppression, Strata-X SPE tubes were used for sample preparation of the human plasma samples prior to LC-MS/MS analysis.

## Reagents and Solutions

Analytical Standards of (S)-Amlodipine, (R)-Amlodipine, (S)-Amlodipine-d4 and (R)-Amlodipine-d4 were purchased from Toronto Research Chemicals, Canada with the purity of 99 %. Acetonitrile, Water, Methanol, Ethanolamine, Formic acid and Isopropyl alcohol were purchased from Sigma Aldrich.

A stock solution of (S)-Amlodipine and (R)-Amlodipine enantiomers was prepared in methanol at 1 mg/mL. Working standard solutions at various concentrations for spiking in plasma were prepared with 50:50 (Methanol/Water). Working stock solution of internal standard was prepared at 100 ng/mL.

## Sample Preparation

The extraction of Amlodipine enantiomers from human plasma was carried out using Strata-X cartridges per the SPE method described below:

Spiked Plasma Samples: 5 µL of internal standard working solution was added to 100 µL spiked human plasma (spiked at 0.050, 0.150, 20.0, 40.0, and 50.0 ng/mL) and mixed, 500 µL of 0.2 % (v/v) Ethanolamine in water was added to sample and vortexed.

**SPE Protocol:**

| | |
|---|---|
| **Cartridge:** | Strata-X, 30 mg/1 mL tubes |
| **Part Number:** | 8B-S100-TAK |
| **Condition:** | 1 mL Methanol |
| **Equilibrate:** | 1 mL Water |
| **Load:** | Spiked plasma sample was loaded and allowed to pass through the cartridge at moderate speed |
| **Wash 1:** | 1 mL Water (to remove polar interferences) |
| **Wash 2:** | 1 mL of 20 % Methanol in Water (to remove non-polar interferences) |
| **Elute:** | 1 mL of 0.1 % Formic Acid in Methanol |
| **Dry:** | Take to dryness under a gentle stream of Nitrogen at 50 °C |
| **Reconstitute:** | 100 µL of Mobile Phase |

**LC-MS/MS Conditions:**

| | |
|---|---|
| **Column:** | Lux 3 µm Cellulose-4 |
| **Dimensions:** | 150 x 2.0 mm |
| **Part No.:** | 00F-4490-B0 |
| **Mobile Phase:** | 0.05 % Ethanolamine in Acetonitrile and Isopropyl Alcohol (96:4 v/v) |
| **LC condition:** | Isocratic |
| **Flow Rate:** | 0.3 mL/min |
| **Run time:** | 4 min |
| **Temperature:** | 25 °C |
| **Injection Volume:** | 10 µL |
| **HPLC System:** | SCIEX® ExionLC™ AD HPLC (AB SCIEX PTE, Ltd.) |
| **Mass Spectrometer:** | SCIEX QTRAP® 4500, ESI positive (AB SCIEX PTE, Ltd.) |
| **Scan Type:** | MRM |
| **IS Voltage:** | 5500V |
| **Source Temperature:** | 300 °C |
| **Collision Energy:** | 15V |

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**QTRAP 4500 Compound Dependent Parameters**

| *Compound* | *Precursor m/z* | *Fragment m/z* |
|---|---|---|
| (R)-Amlodipine | 409.3 | 237.9 |
| (S)-Amlodipine | 409.3 | 237.9 |
| (R)-Amlodipine-d4 | 413.1 | 237.9 |
| (S)-Amlodipine-d4 | 413.1 | 237.9 |

## Results and Discussion

(S)-Amlodipine and (R)-Amlodipine enantiomers were analyzed on a Lux Cellulose-4 chiral column with the simple isocratic mobile phase conditions shown and detected using electrospray ionization (ESI) positive acquisition mode with multiple reaction monitoring (MRM) scan type using the SCIEX QTRAP® 4500. The optimized LC-MS/MS conditions and MS/MS compound dependent parameters are shown above. The chromatogram for the separation on the Lux Cellulose-4 column is shown in **Figure 1**; full baseline separation with excellent peak shape was achieved in less than 4 minutes.

The developed LC-MS/MS method was fully validated, and these results will be discussed below.

System suitability was assessed every day at the start of the experiment by injecting six replicates of analyte and internal standard mixture with concentration 1 ng/mL to ensure instrument performance at the start of each day. The criteria for system suitability was established at ≤5 % for the analyte-to-internal standard peak area, and retention time deviation for both the analytes and ISTD was less than 2 % for system suitability. Chromatograms of (R)-Amlodipine and (S)-Amlodipine and the respective deuterated internal standards is shown in **Figure 2**.

Calibration curves were generated by spiking analytes into blank human plasma at 8 different concentration levels ranging from 0.050 ng/mL to 50 ng/mL (**Figure 3**). The calibration curve was found to be linear over the specified range for both (R)-Amlodipine and (S)-Amlodipine enantiomers. Excellent linearity across the calibration range was exhibited, with the regression coefficient (r) greater than 0.99 for all calibration curves generated from the accuracy and precision sample batches.

For specificity and carryover studies eight different lots of blank plasma were extracted and analysed using the LC-MS/MS method and injected along with the corresponding lots of plasma spiked at the lower limit of quantitation (LLOQ) and working internal standard concentration. Carryover was evaluated by injecting the following sample sets: extracted plasma blank, LLOQ, ULOQ and extracted plasma blank. The percentage interference at the retention time of the analyte was monitored and calculated against the LLOQ area response and internal standard area response. Analytes and internal standard showed high degree of selectivity and specificity in the proposed method. Blank samples injected after ULOQ samples did not show quantifiable response (<20 % of the LLOQ peak area response), which demonstrates that the proposed method is free of carryover. Chromatograms of extracted plasma blank and plasma spiked LLOQ QC sample are shown in **Figure 4**, and a summary of carryover data is presented in **Table 1**.

**Table 1. Evaluation of Carry Over**

| Sample | *Precursor m/z* (R)-Amlodipine Peak Area | *Fragment m/z* (S)-Amlodipine Peak Area |
|---|---|---|
| Plasma Blank | 22 | 66 |
| LLOQ | 3761 | 3706 |
| ULOQ | 6536117 | 6468086 |
| Plasma Blank | 82 | 119 |
| Below 20% of LLOQ area response | | |

**Figure 1.**
Chromatogram for Amlodipine Enantiomers on Lux Cellulose-4

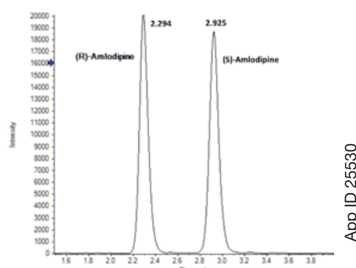


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**Figure 2.**
Chromatogram for Amlodipine Enantiomers and Internal Standards

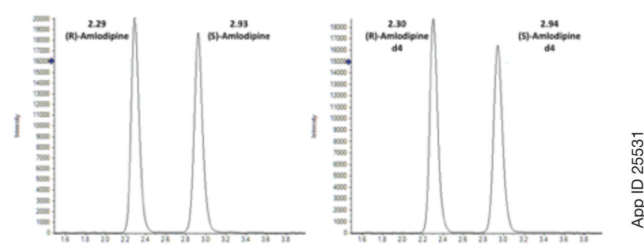


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**Figure 3.**
Calibration Curves for (R)-Amlodipine (blue) and (S)-Amlodipine (purple)

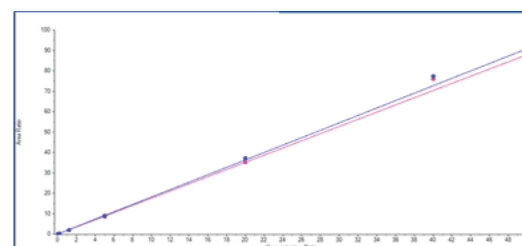

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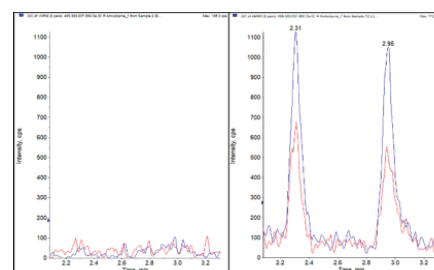
**Figure 4.**
Chromatograms for Plasma Blank (left) and Plasma Spiked at LLOQ QC Sample (right); (m/z 409.3⇨237.9)

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Intraday and interday precision and accuracy was evaluated using 6 replicates of extracted quality control (QC) samples in human plasma at LLOQ, low QC (LQC), mid QC (MQC), and high QC (HQC) as follows: LLOQ QC (0.050 ng/mL), LQC (0.150 ng/mL), MQC (20.000 ng/mL) and HQC (40.000 ng/mL). According to the US FDA Regulatory guidelines, mean % nominal concentration at each QC level, other than LLOQ QC, must be within 85 % to 115 % and precision (% CV) should be ≤15 %. Mean % of nominal concentration at LLOQ QC level must be within 80 % to 120 % and the precision (% CV) should be ≤20 %. Intraday and interday precision and accuracy was studied and met both the precision and accuracy requirements (**Table 2**).

**Table 2.** Evaluation of Interday and Intraday Accuracy and Precision

| | Interday Precision and Accuracy, n = 6 | | | Intraday Precision and Accuracy, n = 6 | | |
|---|---|---|---|---|---|---|
| **(R)-Amlodipine** | | | | | | |
| Nominal Conc., ng/mL | Measured Conc., ng/mL | Precision (% CV) | Accuracy (%) | Measured Conc., ng/mL | Precision (% CV) | Accuracy (%) |
| 0.050 | 0.048 | 6.00 | 96 | 0.047 | 8.66 | 94 |
| 0.150 | 0.151 | 4.05 | 101 | 0.149 | 3.47 | 99 |
| 20 | 20.399 | 3.11 | 102 | 20.576 | 2.46 | 103 |
| 40 | 41.922 | 2.79 | 105 | 42.410 | 2.74 | 106 |
| **(S)-Amlodipine** | | | | | | |
| 0.050 | 0.048 | 9.67 | 96 | 0.047 | 8.51 | 94 |
| 0.150 | 0.152 | 3.10 | 101 | 0.151 | 4.64 | 101 |
| 20 | 20.812 | 2.89 | 104 | 20.693 | 2.38 | 103 |
| 40 | 42.599 | 3.19 | 107 | 42.279 | 2.98 | 106 |

A significant benefit associated with SPE for sample preparation is the removal of endogenous compounds present in plasma, such as proteins and phospholipids, prior to LC-MS/MS analysis. The presence of these matrix compounds in injected samples can impact analyte peak shapes, HPLC column lifetimes, and negatively affect accurate quantitation with MS detection due to potential ion suppression effects. The polymeric Strata®-X sorbent used in this method allows for both a simple water wash to remove polar endogenous compounds (sugars, salts, etc), as well as a stronger wash (20 % methanol in this case) to help remove some of the non-polar matrix compounds while retaining the analytes of interest (improved recoveries). To confirm that matrix effect was minimized for this method, the matrix effect was assessed by measuring the corresponding analyte peak area response in reconstituted matrix samples from 6 different plasma lots against the analyte mean peak area response in reference solution at 2 different concentration levels (low and high QC concentrations). Matrix effect was also studied for all 6 replicates and the developed method showed no matrix effect.

Extraction recoveries for both (R)-Amlodipine and (S)-Amlodipine at three concentration levels were determined by measuring the mean peak area response of 6 replicates of extracted QC samples and comparing to the mean peak area response of extracted blank matrix that was spiked after preparation with the nominal amount of both analytes at the low, medium and high QC levels. The extraction recoveries of analytes were uniform and reproducible, with 94.14 % extraction recovery for (R)-Amlodipine and 92.23 % for (S)-Amlodipine. This confirms that the stronger second solvent wash (20 % methanol) did not negatively impact recovery of (R)-Amlodipine and (S)-Amlodipine.

For this method, the upper level of quantitation (ULOQ) for (R)-Amlodipine and (S)-Amlodipine was found to be 50 ng/mL. A dilution integrity test was performed by spiking 1.6x concentrations of ULOQ in plasma samples and diluted 4-fold and 2-fold with blank plasma. The average percentage recoveries for 4-fold and 2-fold dilutions for 6 replicate injections each were 94.90 % for (R)-Amlodipine and 96.40 % for (S)-Amlodipine.

Stability studies in human plasma were evaluated to cover a wide range of expected experimental and storage conditions. Analytes and internal standard were found to be stable on the benchtop for 6 hours, in the autosampler for 48 hours and for 3 freeze thaw cycles of stability studies. Sample stock solutions were also found to be stable when refrigerated at 2 °C to 8 °C for 4 days.

## Conclusions

This technical note highlights the selective and sensitive LC-MS/MS method which was developed for the separation and quantitation of (R)-Amlodipine and (S)-Amlodipine enantiomers in human plasma using a Lux® Cellulose-4 chiral column and SCIEX QTRAP® 4500 mass spec system. A sample preparation method for extraction of Amlodipine from human plasma, with high analyte recoveries, was developed using polymeric Strata-X SPE tubes, reducing potential matrix interference from proteins and phospholipids that could impact accuracy of the quantitative method. The Lux Cellulose-4 column provided baseline chromatographic separation of the (R) and (S) enantiomers of Amlodipine with excellent peak shape, and the SCIEX QTRAP 4500 provides the sensitivity required to detect the Amlodipine enantiomers at therapeutic levels in human plasma. This method is unique in that it can quantify both pharmacologically active and inactive enantiomers of Amlodipine in human plasma samples with LLOQ of 0.050 ng/mL. We believe that this simple, sensitive and reproducible method for the quantitation of (R)-Amlodipine and (S)-Amlodipine in human plasma will have application in bioequivalence, bioavailability and preclinical studies. We anticipate that this method can be easily extended to other preclinical species with little or no modification.

Acknowledgement: the original SCIEX Technical Note can be found at https://sciex.com

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## Ordering Information

| 3 µm Minibore, MidBore, and Analytical Columns (mm) | | | | | | | | | SecurityGuard™ Cartridges (mm) | |
|---|---|---|---|---|---|---|---|---|---|---|
| Phases | 50 x 2.0 | 150 x 2.0 | 100 x 3.0 | 150 x 3.0 | 50 x 4.6 | 100 x 4.6 | 150 x 4.6 | 250 x 4.6 | 4 x 2.0* | 4 x 3.0* |
| | | | | | | | | | /10pk | /10pk |
| i-Cellulose-5 | 00B-4755-B0 | 00F-4755-B0 | 00D-4755-Y0 | 00F-4755-Y0 | 00B-4755-E0 | 00D-4755-E0 | 00F-4755-E0 | 00G-4755-E0 | AJ0-8631 | AJ0-8632 |
| Cellulose-1 | 00B-4458-B0 | 00F-4458-B0 | 00D-4458-Y0 | 00F-4458-Y0 | 00B-4458-E0 | 00D-4458-E0 | 00F-4458-E0 | 00G-4458-E0 | AJ0-8402 | AJ0-8403 |
| Cellulose-2 | 00B-4456-B0 | 00F-4456-B0 | 00D-4456-Y0 | 00F-4456-Y0 | 00B-4456-E0 | 00D-4456-E0 | 00F-4456-E0 | 00G-4456-E0 | AJ0-8398 | AJ0-8366 |
| Cellulose-3 | 00B-4492-B0 | 00F-4492-B0 | 00D-4492-Y0 | 00F-4492-Y0 | 00B-4492-E0 | 00D-4492-E0 | 00F-4492-E0 | 00G-4492-E0 | AJ0-8621 | AJ0-8622 |
| Cellulose-4 | 00B-4490-B0 | 00F-4490-B0 | 00D-4490-Y0 | 00F-4490-Y0 | 00B-4490-E0 | 00D-4490-E0 | 00F-4490-E0 | 00G-4490-E0 | AJ0-8626 | AJ0-8627 |
| Amylose-1 | 00B-4729-B0 | 00F-4729-B0 | 00D-4729-Y0 | 00F-4729-Y0 | 00B-4729-E0 | 00D-4729-E0 | 00F-4729-E0 | 00G-4729-E0 | AJ0-9337 | AJ0-9336 |
| Amylose-2 | 00B-4471-B0 | 00F-4471-B0 | 00D-4471-Y0 | 00F-4471-Y0 | 00B-4471-E0 | 00D-4471-E0 | 00F-4471-E0 | 00G-4471-E0 | AJ0-8471 | AJ0-8470 |
| | | | | | | | | for ID: | 2.0–3.0 mm | 3.2–8.0 mm |

## Strata-X
## Ordering Information

| Format | Sorbent Mass | Part Number | Unit |
|---|---|---|---|
| Tube | | | |
| | 30 mg | 8B-S100-TAK** | 1 mL (100/box) |

*SecurityGuard Analytical Cartridges require holder, Part No.: KJ0-4282

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