

# Determination of S-amlodipine in Human Plasma Using Tizanidine as Internal Standard by Lc/Ms/Ms Method

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## Abstract

High performance liquid chromatographic tandem mass spectrometric method for the estimation of S-amlodipine in human plasma has been developed and validated using Tizanidine as internal standard. Sample process was accomplished by Liquid-Liquid Extraction technique. The processed sample was chromatographed and analysed on Phenomenex, Lux 3u Cellulose-2 (150 × 4.6) mm column using mobile phase [0.1% formic acid in water and Acetonitrile (50:50% v/v)]. S-amlodipine was chromatographed and analysed by MS detector. Amlodipine has two basic moiety (one primary and one secondary amino) so it shows pKa=9.45 with two ester linkages and one ether linkage, so it is highly lipid soluble (logP=2.22) whereas Tizanidine has two secondary amino and three tertiary amino groups, so it shows pKa=7.49. Primary amine -NH<sub>2</sub> is aliphatic in nature in amlodipine and secondary amine -NH is in ring so it has less action of basicity. Tizanidine has secondary amine -NH is in chain which predominates the basicity (pKa=7.49) and other secondary and tertiary amines are in ring so they show less action with formic acid/ACN mobile phase. Formic acid ionizes (primary amino) -NH<sub>2</sub> part, then (secondary amino) -NH part and then (tertiary amino) =N part. So amlodipine elutes 1st then tizanidine comes out. LogP of amlodipine is 2.22 and for tizanidine it is 1.4, so due to Formic acid-water/CAN mobile phase Amlodipine has higher retention time than tizanidine and comparison Rt. The analytical method described is valid the determination of S-amlodipine (over a range of 0.77 ng/ml to 50.98 ng/ml) using Tizanidine as internal standard in human plasma. Signals from the detector were captured in a computer and processed using Analyst software. This validation report provides results of various validation parameters.

**Keywords:** S-amlodipine; Tizanidine; Internal standard; LC/MS/MS; Validation

## Introduction

Levamlodipine (INN), also known as Levoamlodipine or S-amlodipine is a pharmacologically active enantiomer of Amlodipine. Amlodipine belongs to the dihydropyridine group of calcium channel blocker used as an antihypertensive and antianginal agent. Amlodipine blocks the transmembrane influx of calcium into the vascular and cardiac smooth muscles resulting in vasodilation and hence a fall in blood pressure. Levamlodipine is an allosteric modulator and acts on the L- type of calcium channels. Receptor binding studies have shown that out of the two forms only the (S) enantiomer of Amlodipine binds to and blocks L-type calcium channels whereas the (R) enantiomer has no activity on these channels. Oral administration of Levamlodipine (2.5 mg) as a single dose gives maximum plasma concentration (C<sub>max</sub>) of 8.3 to 9.3ng/ml in 2 to 3 hrs (T<sub>max</sub>). It is extensively (about 90%) converted to inactive metabolites via hepatic metabolism with 10% of the parent compound and 60% of the metabolites excreted in the urine. Levamlodipine shows approximately 93% plasma protein binding in hypertensive patients. The mean AUC<sub>0t</sub> value (t=48 hrs) of Levamlodipine tablets (2.5 mg) is 95 ± 14 ng hr/ml. The plasma elimination half-life of Levamlodipine has been found to be 31 ± 13 hrs. Different methods for the determination of Amlodipine in biological fluids, such as gas chromatography (GC) [1] or liquid chromatography (LC) [2-4] or mass spectrometric analysis [5-7], previously reported methods [8-12] either had long retention time (10–15min) or suffered from low sensitivity and in some cases required large sample injection volumes. Hence the main objective of this work was to develop a simple, sensitive, rapid and reliable mass spectrometry method for the quantification of S-amlodipine in human plasma. Only limited methods have been reported in the HPLC and GC. The objective of the work was to develop and validate LC-MS/MS method for quantification

in Human Plasma. The method shows more sensitive limit of detection and Limit of Quantification is very less to the previous reported methods.

## Materials and Methods

### Chemicals and reagents

S-amlodipine [13] (Figure 1) and Tizanidine (IS) [14] (Figure 2) working standards were used. HPLC grade Acetonitrile and

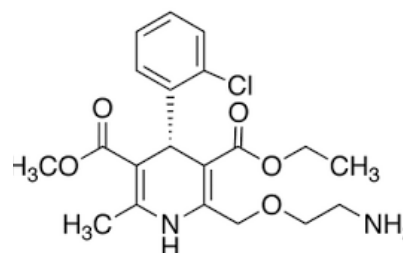


Figure 1: Structure of S-amlodipine.

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Methanol were manufactured by Qualigens Fine Chemicals, Formic acid AR grade manufactured by S.D. Fine chemicals, Water HPLC grade from Milli-Q RO system was used throughout the analysis. Diluent (50% methanol in water) and extraction solvent diethyl ether were used.

### Chromatographic conditions

The chromatography was performed on an ACQUITY™ UPLC system (Waters Corp, USA) [15,16] with cooling auto sampler and column oven enabling temperature control of the analytical column. Phenomenex, Lux 3u Cellulose-2 (150 × 4.6) mm column was employed. The column temperature was maintained at 45°C and chromatographic separations were achieved with isocratic elution using a mobile phase composed of [0.1% formic acid in water, Acetonitrile (50:50% v/v)]. The flow rate was set at 0.4 ml/min, run time was 5.5 minutes and retention time for S-amlodipine and Tizanidine were 3.9 min. and 3.4 min respectively. The auto sampler was conditioned at 4°C and the injection volume was 10 µl using Auto sampler mode for sample injection.

### Compound Setting

Ion source ESI Positive mode, Ion spray voltage at 4.5 kV, temperature at 500°C, curtain gas at 6 L/min, nebulizer gas at 12 (psi) and collision gas at 6L/min were set. The Parent mass For S-amlodipine (409.3) m/z and Tizanidine (254.2) m/z, Product mass S-amlodipine (238.1) m/z and Tizanidine (44.1) m/z, and collision energy was 15 eV and 46 eV respectively.

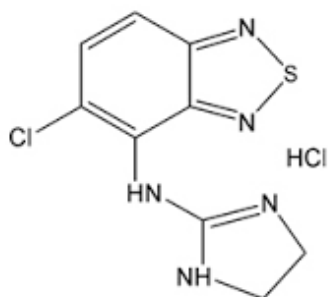


Figure 2: Structure of Tizanidine.

### Plasma sample extraction procedure

50 µl (1000 ng/ml) of internal standard solution was admixed with 0.500 ml of sample in a RIA-vials and vortexed for 1 minute, 2.5 ml of extraction solvent was added. The content was vortexed for 10 minutes at 14000 rpm; 1.8 ml organic layer transferred into RIA vials and evaporated upto dryness at 35°C under a stream of nitrogen gas. The dried residue was reconstituted with 0.150 ml of diluent and vortex for 60 minutes. Samples were injected into the LC-MS/MS system for analysis.

### Validation parameters

Blank screening, selectivity, linearity, accuracy, precision, stability (freeze thaw, bench top, long term, auto sampler, stock solution at RT and RF) Each batch of spiked plasma samples includes one complete calibration curve (consisting of one blank plasma, one blank plasma with internal standard and eight different non-zero concentration samples, six replicates quality control samples includes six replicate quality control samples LLOQ, LQC, MQC and HQC), except bench top, freeze thaw stability, long term stability, auto sampler stability and dilution integrity in which four replicate quality control samples were used. Quality control samples for S-amlodipine prepared by using CPD as an anticoagulant and stored at a temperature below -20°C (deep freezer), protected from light with concentrations taken as LLOQ (0.79 ng/ml) (Figure 3), LQC (2.13 ng/ml) (Figure 4), MQC (22.62 ng/ml) (Figure 5) and HQC (45.25 ng/ml) (Figure 6)

### Standardization and Calculation

The chromatographic data were acquired and processed using computer based Analyst software. The best-fit lines using weighting factor (1/concentration) linear least square regression analysis were obtained by peak area ratio of S-amlodipine (Figure 7) to its internal standard (Tizanidine) (Figure 8). The concentration of S-amlodipine in plasma samples were calculated using linear regression parameters by corresponding calibration curve.

## Results and Discussion

### Chromatography

Representative chromatograms containing solvent reference mix solution, blank plasma (Figure 9), blank plasma with internal standard

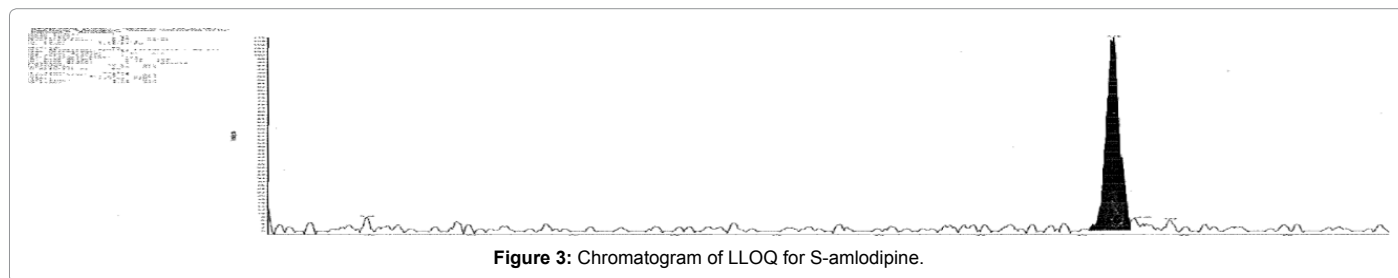


Figure 3: Chromatogram of LLOQ for S-amlodipine.

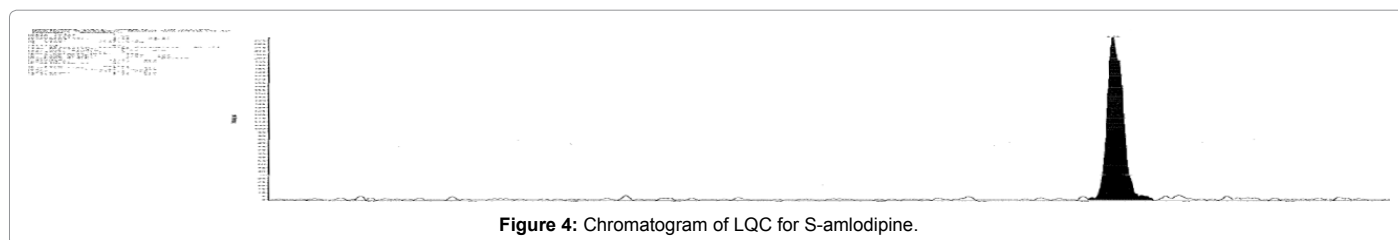
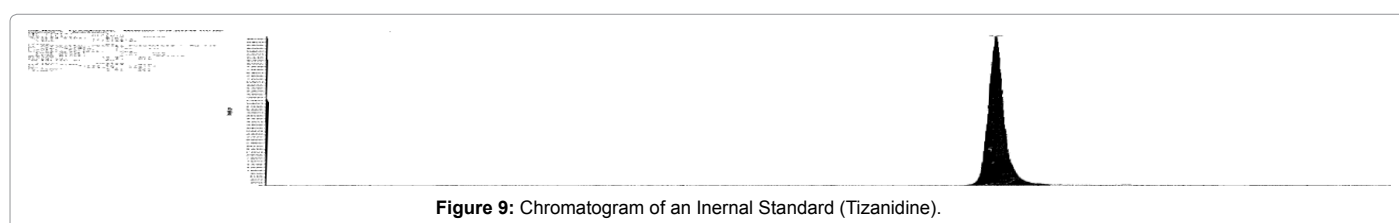
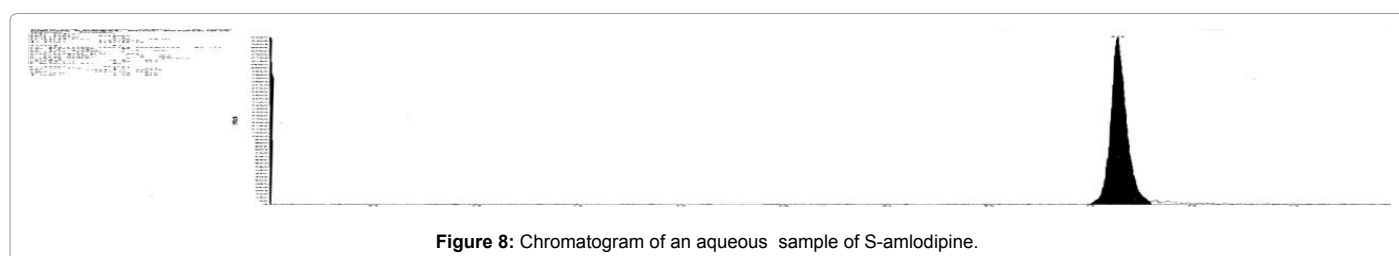
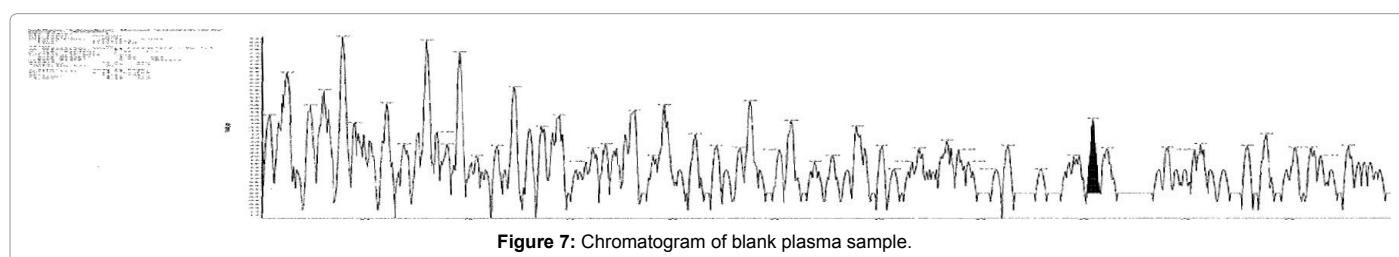
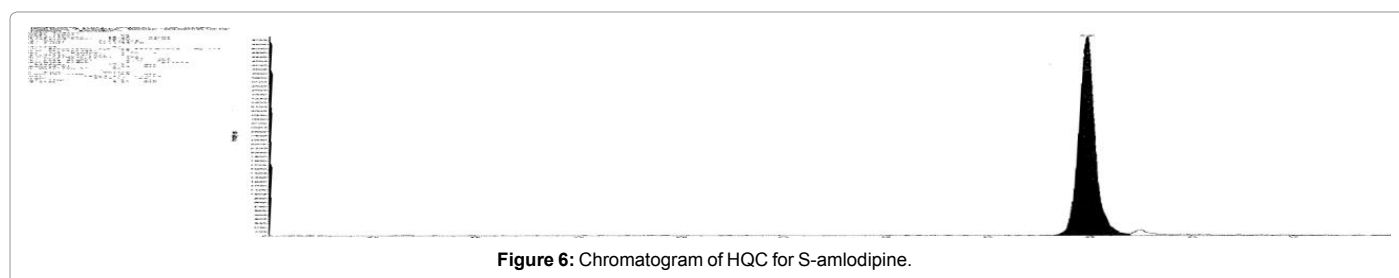
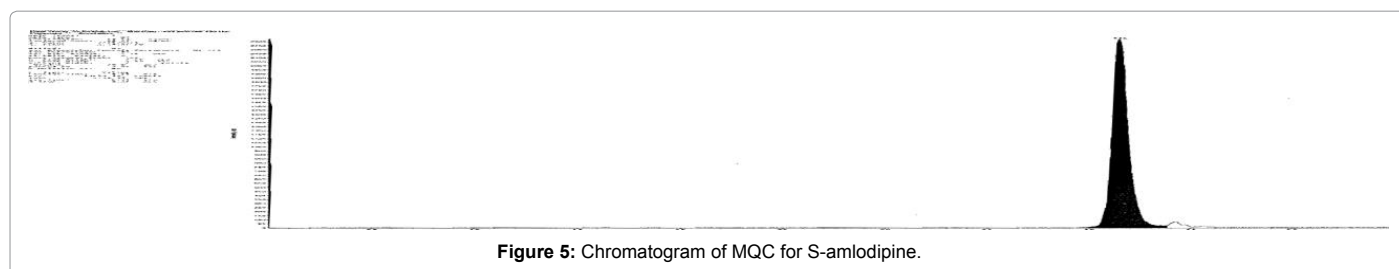


Figure 4: Chromatogram of LQC for S-amlodipine.



and quality control samples (LLOQ, LQC, MQC and HQC) and a calibration curve of representative regression analysis for S-amlodipine.

### Selectivity

Six lots of plasma with CPD anticoagulant were evaluated and none showed significant interfering peaks at the retention time of S-amlodipine and internal standard (Tizanidine).

### Linearity

The linearity of S-amlodipine was determined by weighted least square regression analysis of standard plot associated with eight point standard curve. The calibration was shown to be linear from 0.77 ng/ml

to 50.98 ng/ml for S-amlodipine (Figure 10). Best-fit calibration lines of chromatographic response versus concentration were determined by weighted least square regression analysis with weighting factor of 1/concentration. The coefficient of correlation ( $r^2$ ) was consistently greater than or equal to 0.99 during the course of validation, which are within limits.

### Sensitivity

The Limit of Quantitation was 0.79 ng/ml for S-amlodipine. The between batch precision and accuracy at LLOQ concentration for S-amlodipine using internal standard ratio method was 6.0% and 106.9% respectively.

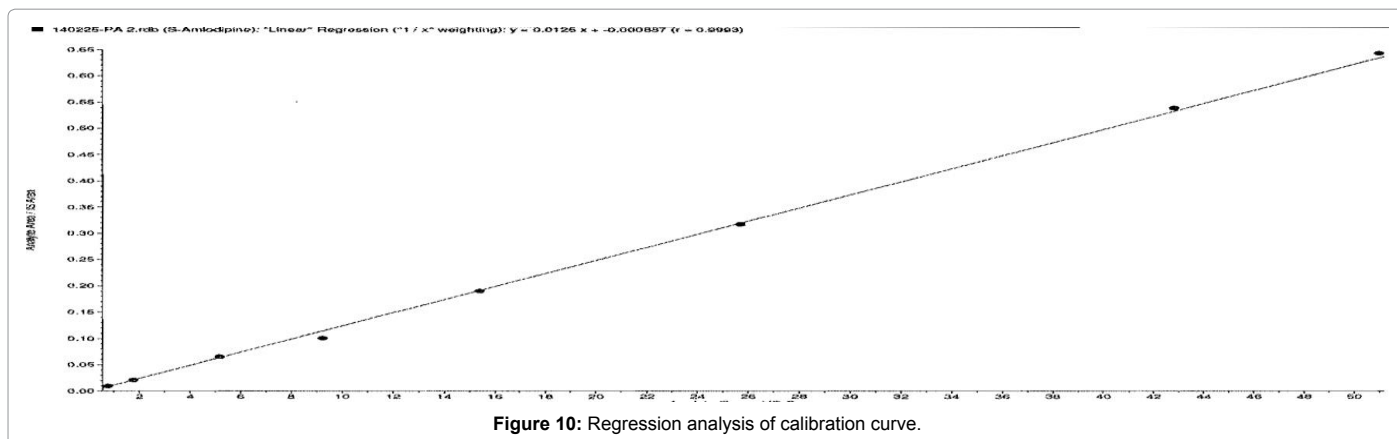


Figure 10: Regression analysis of calibration curve.

### Accuracy

The accuracy of the assay was defined as the absolute value of calculated mean values of the quality control samples to their respective nominal values, expressed as percentage.

### Within batch accuracy

The within batch accuracy using internal standard area ratio method ranged from 103.0% to 111.7% for S-amlodipine, which are within limits (Table 1).

### Between batch accuracy

The between batch accuracy using internal standard area ratio method ranged from 106.6% to 110.5% for S-amlodipine, which are within limits (Table 1).

### Precision

The precision of the assay was measured by the percent coefficient of variation over the concentration range of LLOQ, LQC, MQC and HQC quality control samples of S-amlodipine.

### Within Batch Precision

The within batch precision using internal standard area ratio method ranged from 1.5% to 6.2% for S-amlodipine, which are within limits (Table 1).

### Between batch precision

Between batch precision using internal standard area ratio method ranged from 2.1% to 6.0% for S-amlodipine which is within limits (Table 1).

### Recovery of analyte

The percentage recovery of S-amlodipine was determined by

S. No	Parameters	Acceptance Criteria	Results (%)
1	Within Batch Accuracy	Nominal concentration: 85-115%	103-111.7
2	Between Batch Accuracy	Nominal concentration: 85-115%	106.6-110.5
3	Within Batch Precision	% CV: 15%	1.5-6.2
4	Between Batch Precision	% CV: 15%	2.1-6.0
5	Recovery of Analyte	% CV: 20%	0.7
6	Recovery of internal standard	% CV: 20%	3.3

Table 1: Results of accuracy, precision and recovery.

measuring the peak area response of spiked (extracted) quality control samples (LQC, MQC and HQC) against the peak area response of aqueous (Unextracted) quality control samples (LQC, MQC and HQC) of equivalent concentrations. The percent recovery of S-amlodipine at LQC, MQC and HQC quality control samples were 98.41%, 97.22%, 98.42% respectively. The % CV for recovery of inter quality control samples for S-amlodipine was 0.7%, which is within limits. The % mean of recovery was 96.1%, which are within limits (Table 1).

### Recovery of internal standard

The percentage recovery of Tizanidine (IS) was determined by measuring the peak area response of spiked (extracted) quality control samples (MQC) against the peak area response of aqueous (unextracted) IS dilution of same concentration. The % mean recovery and % CV for recovery of Tizanidine (IS) were 99.65% and 3.3%, which are within limits (Table 1).

### Stability

**Freeze thaw stability:** The stability of the spiked plasma samples was determined during three freeze-thaw cycles. Four replicates numbers of LQC and MQC samples (Stability samples) kept at 80°C and were analysed after third freeze thaw cycle against freshly spiked calibration curve standards and freshly spiked QC samples (comparison samples). The comparative stability ranged from 98.6% to 99.9% for S-amlodipine, which are within limits (Table 2).

**Bench top stability:** The bench top stability (short term stability in matrix) determined by analyzing four replicates of low and high QC stability samples, which had been kept at room temperature for a designed time against the freshly spiked QC samples (comparison samples). The comparative stability ranged from 100.8% to 102.3% for S-amlodipine, which are within limits (Table 2).

**Auto sampler stability:** The auto sampler stability (post-processing stability) determined by analyzing four replicates of low and high QC stability samples, which had been processed and kept in auto sampler and were analyzed after against freshly spiked calibration curve standards and freshly spiked QC samples (comparison samples). The comparative stability ranged from 102.10% to 102.3% for S-amlodipine, which are within limits (Table 2).

**Long term stability:** For long term stability (long term stability in matrix) four replicates of low and high QC stability samples were analyzed against freshly spiked calibration curve. The low and high QC samples were stored for 04 days in deep freezer (at below -20°C) with

S. No	Parameters	Acceptance Criteria	Results (%)
1	Freeze and thaw cycle at -80°C	Mean% change after 3 cycles ± 15%	98.6-99.9
2	Bench top stability	Mean% change after 10.14 h ± 15%	100.8-102.3
3	Auto sampler stability	Mean% change after 50.04 h: 85-115%	102.10-102.3
4	Long term Stability	Mean% change after 7 Days: ± 15%	99.1-101.3
5	Stock solution stability at room temp.	% Nominal concentration: ± 15%	97.5 (Analyte) 103.3 (IS)
6	Dilution integrity	Accuracy (% Nominal ): ± 15%	101.2
		Precision(%CV): ≤ 15%	4.0
7	Matrix effect	% CV ≤ 15%	LQC 9.35 HQC 6.84
8	Ruggedness	% Nominal: ± 15%	107.1-111.4
		%CV: ≤ 15%	1.8-4.7

**Table 2:** Result of stability, dilution integrity, matrix effect and ruggedness.

CPD as an anticoagulant in polypropylene tubes. The stability ranged from 99.1% to 101.3%, which are within limits (Table 2).

### Stock solution stability at room temperature

Stock solution stability at room temperature was performed by storing S-amlodipine and internal standard (Tizanidine) stock solutions at room temperature. The evaluation of stability was done by assaying six replicate injections of appropriately prepared dilutions of stored stock aliquot solutions of S-amlodipine and internal standard (Tizanidine) against six replicate injection of appropriately prepared dilution from fresh stock solutions of S-amlodipine and internal standard (Tizanidine). The stock solution of S-amlodipine and internal standard (Tizanidine) was found to be stable, when stored at room temperature. The percent stability of the stock solution stability was 97.5% and 103.3% respectively, which are within limits (Table 2).

### Dilution integrity

Dilution Integrity was determined by assaying four replicates QCs spiked approximately two times the concentration of ULOQ. Samples were diluted by an appropriate factor (two) prior to extraction, against freshly spiked calibration standard samples. The accuracy for two times diluted concentration was 101.9% and four times diluted concentration were 101.2% for S-amlodipine. The precision for two times diluted concentration were 4.0% and four times diluted concentration were 2.8% for S-amlodipine, which are within limits (Table 2).

### Matrix effect

Matrix effect was calculated by spiking analyte and IS at LQC and HQC levels into each of blank plasma extracts from six different batches of matrix respectively and analyzed in duplicate against six replicate injections of aqueous samples at low and high QCs samples. At low and QCs samples, the % coefficient of variation for matrix factor was found to be 9.35% and 6.84%, respectively for S-amlodipine which is within limits (Table 2).

### Ruggedness

The ruggedness of the extraction procedure and chromatographic method was evaluated by analysis of a batch of six sets of quality control samples and a set of calibration standards using different column (same type) by another analyst. The within batch accuracy ranged from 107.1% to 111.4% and within batch precision ranged from 1.8% to 4.7% for S-amlodipine. The results indicated that the batch met the acceptance criteria of linearity, precision and accuracy data of the quality control

samples, which are within limits (Table 2).

### Stock solution stability at refrigerated temperature (4.0 days)

Stock solution stability at refrigerated temperature was performed by S-amlodipine and internal standard (Tizanidine) stock solutions at refrigerated temperature. The evaluation of stability was done by assaying six replicates injections of appropriately prepared dilution from stored aliquot solutions of S-amlodipine and internal standard (Tizanidine) against six replicate injection of appropriately prepared dilution from fresh stock solutions of S-amlodipine and internal standard (Tizanidine). The percent stability of the stock solution for S-amlodipine and internal standard (Tizanidine) were 101.3.0% and 101.5% respectively which are within limits.

### Conclusion

The above analytical method described is valid the determination of S-amlodipine (over a range of 0.77 ng/ml to 50.98 ng/ml) using Tizanidine as internal standard in human plasma using a Phenomenex, Lux 3u Cellulose, (150 × 4.6) mm column.

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