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ORIGINAL RESEARCH

# A rapid and sensitive liquid chromatography–tandem mass spectrometry (LC-MS/MS) method for the estimation of amlodipine in human plasma

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ABSTRACT: A rapid and sensitive liquid chromatography–tandem mass spectrometry (LC-MS/MS) method has been developed and validated for the estimation of amlodipine in human plasma. Amlodipine was extracted from human plasma by using a solid-phase extraction technique. Imipramine was used as the internal standard. A Hypersil BDS $C_{18}$ column provided chromatographic separation of analytes followed by detection with mass spectrometry. The method involves a rapid solid-phase extraction from plasma, simple isocratic chromatography conditions and mass spectrometric detection that enables detection at sub-nanogram levels. The proposed method has been validated for a linear range of 0.1–10.0 ng/mL with correlation coefficient ≥0.9990. The intrarun and interrun precision and accuracy were within 10.0%. The overall recovery for amlodipine was 63.67%. Total run time was 3.2 min only. Copyright © 2007 John Wiley & Sons, Ltd.

KEYWORDS: amlodipine; LC-MS/MS; human plasma

## INTRODUCTION

Amlodipine, (*R*,*S*)-2-[(2-aminoethoxy) methyl]-4-(2-chlorophenyl)-3-ethoxycarbonyl-5-methoxycarbonyl-6-methyl-1,4-dihydropyridine (Fig. 1), is a potent calcium channel blocker. It is clinically used in the treatment of hypertension and angina, and has the highest oral bioavailability and the longest half-life of elimination among several dihydropyridine derivatives with calcium antagonist activity (Murdoch and Heel, 1991; Abernethy and Schwartz, 1998).

It has low plasma concentration because amlodipine has a long elimination half-life in humans ranging from 35 to 45 h due to the large volume of distribution and moreover it is highly bound to plasma proteins (>95.0%; Stopher *et al.*, 1988). Therefore, a sensitive and specific analytical method for the assay of amlodipine plasma levels is necessary.

Several analytical methods have been reported in the literature for monitoring plasma levels of amlodipine. The techniques used in these methods include HPLC with fluorimetric detection (Tatar and Atmaca, 2001), HPLC with ultraviolet detection (Luska *et al.*, 1997), HPLC with electrochemical detection (Shimooka *et al.*, 1989; Josefsson *et al.*, 1995), high-performance thin-layer chromatography–densitometry (Pandya *et al.*, 1995) and gas chromatography (GC) with electron capture detection (Scharpf *et al.*, 1994; Monkman *et al.*, 1996). The reported HPLC methods require laborious extraction procedures like liquid–liquid extraction or solid-phase extraction involving drying and reconstitution, long run-time, high quantification limit, low recovery and large plasma volume. The major disadvantage of GC methods is thermal decomposition of amlodipine at high temperatures to the pyridine analogue, which is already present in plasma as a metabolite. Liquid chromatography tandem mass spectrometric (LC-MS/MS) methods (Carvalho *et al.*, 2001; Massaroti *et al.*, 2005) have also been reported for the determination

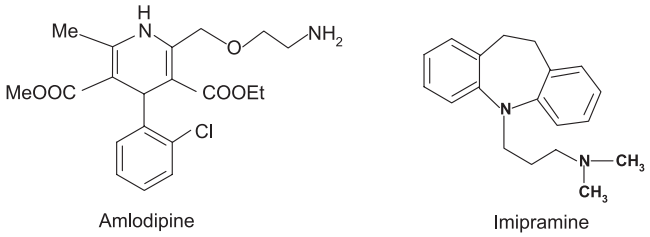


**Figure 1.** Chemical structure of amlodipine and imipramine.

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**Abbreviations used:** QC, quality control; SRM, selected reaction monitoring.

of amlodipine in human plasma. Although these assays are sufficiently sensitive, the method requires laborious liquid–liquid extraction involving drying and reconstitution with a long chromatographic run time of 5.0 min. Therefore, it was necessary to develop a simple, specific, rapid and sensitive analytical method for the quantification of amlodipine in human plasma.

This paper focuses on the development and validation of a sensitive LC-MS/MS method for the quantification of amlodipine in human plasma having reduced sample preparation and analysis time relative to other commonly employed techniques. Imipramine (Fig. 1) was used as an internal standard.

## EXPERIMENTAL

**Chemicals and reagents.** The working standards of amlodipine besylate and imipramine hydrochloride were obtained from Torrent Research Centre (Ahmedabad, India). High-purity water was prepared in-house using a Milli-Q water purification system obtained from Millipore (Bangalore, India). HPLC methanol was purchased from E. Merck (Mumbai, India). Suprapure ammonium formate and formic acid were purchased from Merck (Germany). Drug-free (blank) buffered human plasma was obtained from Green Cross laboratory (Ahmedabad, India) and was stored at –20°C prior to use.

**Calibration curves.** The stock solutions of amlodipine and internal standard imipramine were prepared in methanol at a free base concentration of 1 mg/mL. Secondary standard solutions were prepared from stock solutions by dilution with methanol. Working standard solutions were prepared from secondary standard solutions by dilution with water:methanol (70:30). These diluted working standard solutions were used to prepare the calibration curve and quality control samples.

A nine-point standard calibration curve for amlodipine was prepared by spiking the blank plasma with an appropriate amount of amlodipine. The calibration curve ranged from 0.1 to 10.0 ng/mL. Quality control samples were prepared at three concentration levels of 0.3 ng/mL (low QC, LQC), 3.0 ng/mL (medium QC, MQC) and 7.5 ng/mL (high QC, HQC) for amlodipine in a manner similar to the standard from the stock solution.

**Sample preparation.** A 1.0 mL aliquot of human plasma sample was mixed with 25 µL of internal standard working solution (25.0 ng/mL of imipramine). The sample mixture was loaded into an Oasis HLB extraction cartridge that was pre-conditioned with 1.0 mL methanol followed by 2.0 mL water. The extraction cartridge was washed with 2.0 mL water followed by 1.0 mL 5% methanol. Amlodipine and imipramine were eluted with 0.5 mL of 0.2% acetic acid in methanol; 20.0 µL of the extract were injected into the LC-MS/MS system.

**Instrumentation.** Chromatographic separation was carried out on Surveyor HPLC with Hypersil BDS $C_{18}$ column (5.0 µm, 50 × 4.6 mm) purchased from Thermo Electron Corporation, UK. A mobile phase consisting of methanol and ammonium formate (pH 4.5, 10.0 mM; 80:20) was delivered with a flow rate of 0.5 mL/min. The total run time for each sample analysis was 3.2 min. The sample injection volume was 20.0 µL. Mass spectra were obtained using a TSQ Quantum mass spectrometer (Thermo Finnigan Ltd, UK) equipped with electrospray ionization source. The mass spectrometer was operated in the selected reaction monitoring (SRM) mode. The spray voltage and capillary temperature were 3500 V and 375°C, respectively. The data acquisition was ascertained by Xcalibur 1.3 software. For quantification the peak area ratios of the target ions of the drug to those of the internal standard were compared with weighted ($1/c$) least squares calibration curves in which the peak area ratios of the calibration standards were plotted vs their concentrations.

**Validation.** The method has been validated for selectivity, sensitivity, linearity, precision, accuracy, recovery, stability and dilution integrity. Selectivity was performed by analyzing the blank plasma samples from different sources (or donors) to test for interference at the retention time of amlodipine and internal standard imipramine. Sensitivity was determined by analyzing five replicates of blank human plasma and plasma spiked with the analyte at the lowest level of the calibration curve. The intrarun and interrun accuracy was determined by replicate ($n = 5$) analysis of quality control samples and at limit of quantification (LOQ) that were extracted from the sample batch. Interrun precision and accuracy of the calibration standards was assessed using the five calibration curves used for assay validation.

Accuracy is defined as the percent relative error (%RE) and was calculated using the formula $\%RE = (E - T) \times (100/T)$ where $E$ is the experimentally determined concentration and $T$ is theoretical concentration. Assay precision was calculated using the formula $\%RSD = (SD/M) \times (100)$ where $M$ is the mean of the experimentally determined concentrations and SD is the standard deviation of $M$.

The extraction efficiencies of amlodipine and imipramine were determined by comparing the peak area of extracted analytes with the peak area of non-extracted standards. Dilution integrity was performed to extend the upper concentration limits with acceptable precision and accuracy. Five replicates each at a concentration of double the uppermost calibration standard were diluted 2-fold and 4-fold with blank plasma. The diluted samples were processed and analyzed.

The processed sample stability was evaluated by comparing the extracted plasma samples that were injected immediately (time 0), with the samples that were re-injected after keeping in the auto sampler at 5°C for 24.0 h. The stability of spiked human plasma stored at room temperature (bench-top stability) was evaluated for 6 h and compared with freshly prepared extracted samples. The freeze–thaw stability was conducted by comparing the stability samples that had been frozen and thawed three times, with freshly spiked quality control samples. The stability of spiked human plasma stored at –70°C (long-term stability) was evaluated by analyzing low, medium and high quality control samples that were stored at –70°C for 122 days together with freshly spiked calibration standard and quality control samples. All stability evaluations were based on back-calculated concentrations. Analytes were



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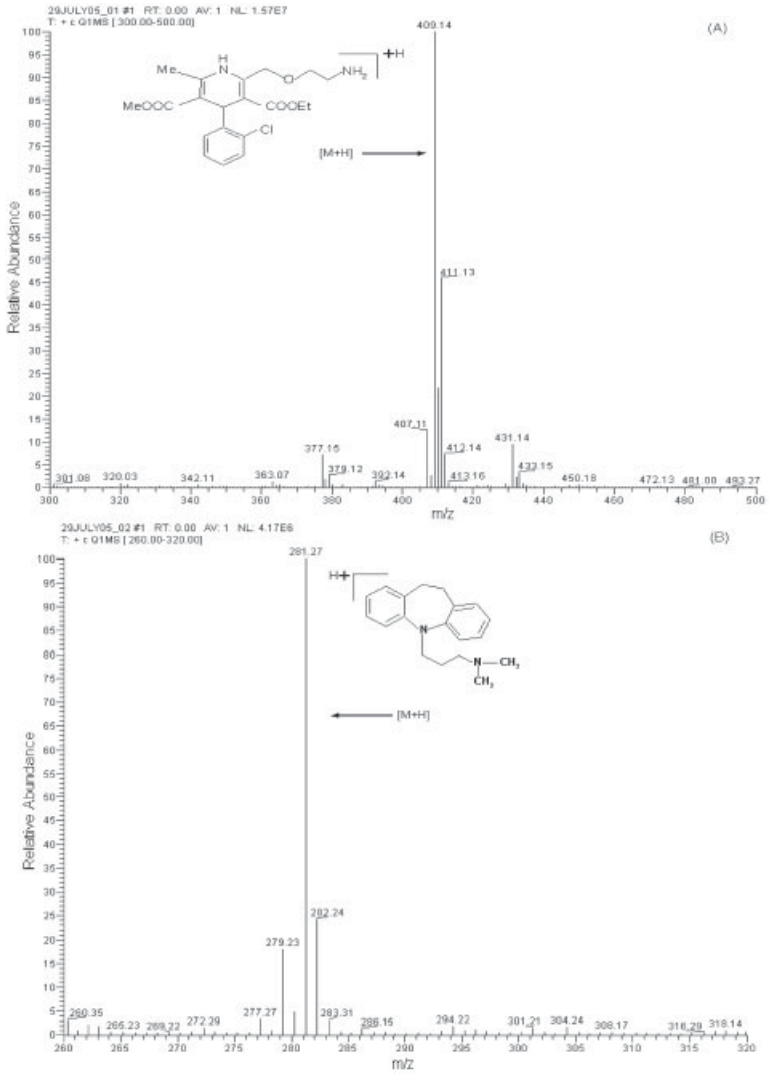


**Figure 2.** Electrospray positive ion mass spectra of the precursor ion of (A) amlodipine and (B) imipramine.

considered stable if the deviation of the mean test responses were within 15% of freshly prepared or comparison samples.

## RESULTS AND DISCUSSION

### Method development

To develop a rapid, sensitive and simple assay method for the extraction and quantification of amlodipine during method development different options were evaluated to optimize detection and chromatography parameters. Amlodipine accepts the proton in an acidic mobile phase to produce a protonated precursor ion ($[M+H]^+$) at *m/z* 409.2. The mass spectra of precursor ions of amlodipine and imipramine are presented in Fig. 2. Electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) were evaluated to get a better response of analytes. It was found that the best signal was achieved with ESI in positive ion mode. Two product ions, *m/z* 294.1 and *m/z* 238.0, of amlodipine were monitored together to increase sensitivity and selectivity. The selected fragments of each compound, as product ions to be monitored, are indicated in Fig. 3.

Further optimization of the chromatography conditions increased the signal of analytes. A mobile phase containing 10 mM ammonium formate (pH 4.5) buffer in combination with methanol resulted in improved signal. Use of short Hypersil BDS $C_{18}$ (50 × 4.6 mm i.d., 5 μm) column resulted in reduced flow rate and reduce run time as low as 3.2 min. The resulting signal with optimized chromatography and detection parameters enabled the elimination of the laborious extraction steps of evaporation of eluent and reconstitution involved in generic solid-phase extraction (SPE) methods without



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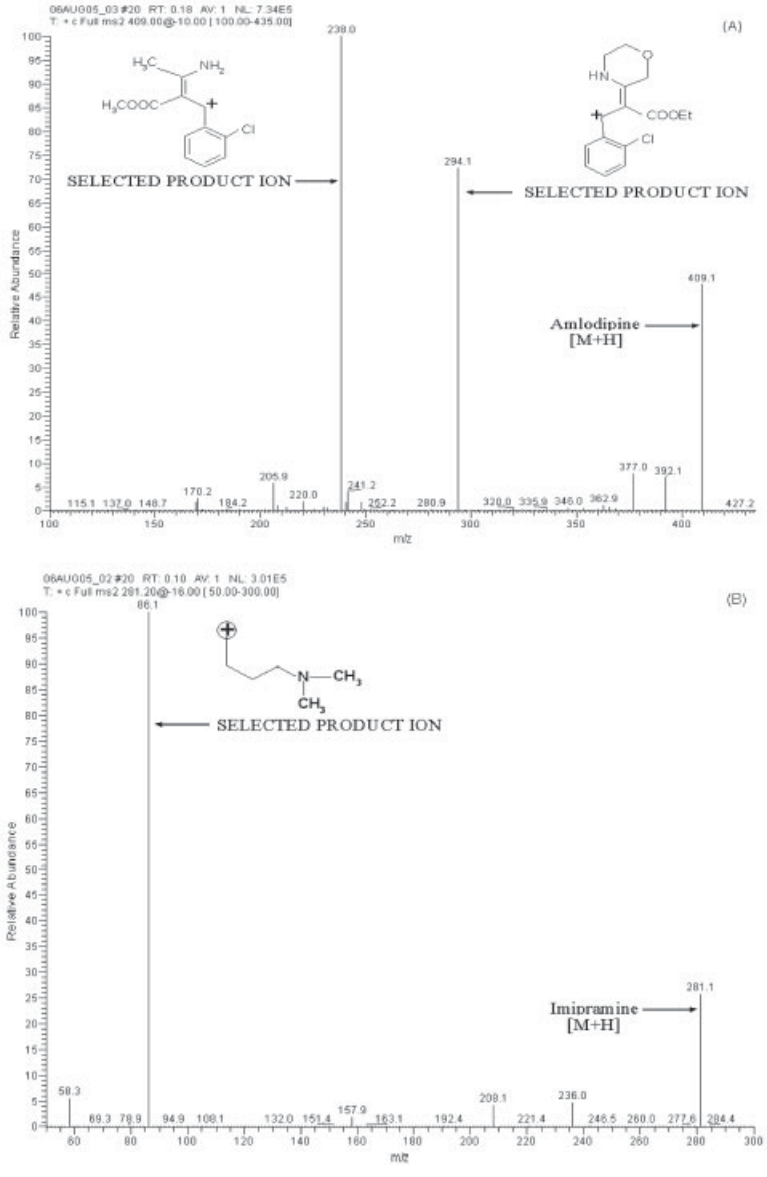


**Figure 3.** Electrospray product ion mass spectra for (A) amlodipine and (B) imipramine.

compromising the sensitivity, which further resulted in reduced processing and analysis time.

## Selectivity

Utilization of predominant product ions for each compound enhanced mass spectrometric selectivity. The mass transition ion-pair was selected as, 409.20 → 238.00, 409.20 → 294.06 for amlodipine and 281.20 → 86.10 for imipramine. The predominant product ions of *m*/*z* 238.00 and 294.06 were specific for amlodipine and *m*/*z* 86.10 was specific for imipramine.

Chromatographic selectivity of the method was demonstrated by the absence of endogenous interfering peaks at the retention times for amlodipine and imipramine in six different lots of extracted blank plasma. Representative chromatograms of extracted blank plasma and extracted plasma samples containing 0.10 ng/mL amlodipine (low standard) are presented in Fig. 4.

## Linearity

The peak area ratios of calibration standards were proportional to the concentration of analytes in each assay over the nominal concentration range of 0.1–10.0 ng/mL for amlodipine. The calibration curves appeared linear and were well described by least squares lines. The slopes intercepts and correlation coefficients are presented in Table 1. The results of the calibration standard are presented in Table 2. The correlation coefficients were ≥0.9990.



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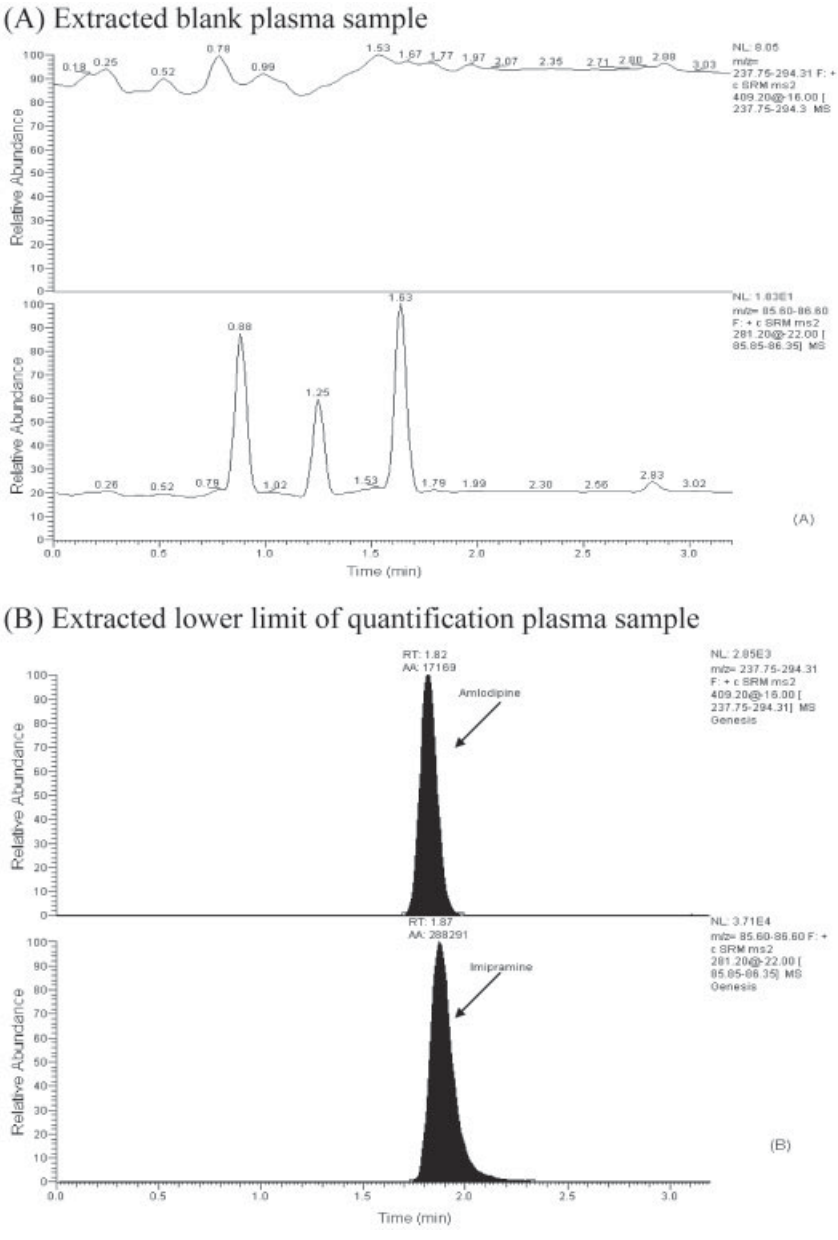


**Figure 4.** Representative chromatograms for amlodipine: (A) extracted blank plasma sample; (B) extracted lower limit of quantification plasma sample.

**Table 1. Summary of calibration curve parameters for amlodipine**

| Calibration curve | Slope | Intercept | Correlation coefficient |
|---|---|---|---|
| 1 | 0.601086 | –0.0214601 | 0.9997 |
| 2 | 0.600925 | –0.0253201 | 0.9991 |
| 3 | 0.621311 | –0.0201560 | 0.9990 |
| 4 | 0.509452 | –0.0149036 | 0.9995 |
| 5 | 0.546440 | –0.0201212 | 0.9995 |

**Table 2. Interrun accuracy and precision of plasma calibration standard ($n$ = 5) for amlodipine**

| Spiked concentration (ng/mL) | Mean calculated concentration (ng/mL) | %RSD | %RE |
|---|---|---|---|
| 0.100 | 0.100 | 8.58 | 0.00 |
| 0.200 | 0.210 | 6.93 | 5.00 |
| 0.400 | 0.417 | 2.43 | 4.25 |
| 1.000 | 0.895 | 2.73 | –10.50 |
| 2.000 | 1.977 | 5.02 | –1.15 |
| 4.000 | 4.068 | 3.66 | 1.70 |
| 6.000 | 6.201 | 2.81 | 3.35 |
| 8.000 | 7.798 | 0.77 | –2.53 |
| 10.000 | 10.035 | 1.34 | 0.35 |



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**Table 3. Intrarun accuracy and precision ($n$ = 5) of amlodipine in human plasma**

| Analyte | Concentration (ng/mL) | Mean (ng/mL) | %RSD | %RE |
|---|---|---|---|---|
| Amlodipine | 0.100 | 0.102 | 4.54 | 2.00 |
| | 0.300 | 0.310 | 8.94 | 3.33 |
| | 3.000 | 2.806 | 1.60 | –6.47 |
| | 7.500 | 7.344 | 2.77 | –2.08 |

## Sensitivity (lower limit of quantification)

The LOQ is defined as the lowest concentration of the calibration standard yielding accuracy ±20% and precision of ≤20%. The LOQ for amlodipine was 0.1 ng/mL. These data are tabulated in Table 3 for amlodipine. The intrarun precision of the LOQ plasma samples containing amlodipine was 4.54%. The mean intrarun accuracy of the LOQ plasma samples containing amlodipine was 2.0%.

## Precision and accuracy

The results of interrun precision and accuracy for amlodipine plasma calibration standards are summarized in Table 2. The interrun precision and accuracy for calibration standards was ≤8.58 and ≤10.50% respectively. The results for intrarun precision and accuracy for amlodipine in plasma quality control samples are summarized in Table 3. The intrarun precision and accuracy was ≤8.94 and ≤6.47%, respectively. The results of interrun ($n$ = 5) precision and accuracy for amlodipine plasma quality control samples are summarized in Table 4. The interrun precision was ≤7.39% and interrun accuracy was 5.65% for amlodipine.

## Recovery

Five replicates at low, medium and high quality control concentration for amlodipine were prepared for recovery determination. The mean recovery for amlodipine was 63.67% with precision of 6.94%. The mean recovery for imipramine was 91.96%.

## Dilution integrity

The upper concentration limits can be extended to 20 ng/mL for amlodipine by a 2-fold or 4-fold dilution with human plasma with a precision of ≤3.87% and an accuracy of ≤7.96% for amlodipine.

## Stability

The results of the stability studies are enumerated in Table 5. The bench top stability, process stability and freeze and thaw stability of amlodipine in plasma were

**Table 4. Interrun accuracy and precision of amlodipine in human plasma**

| Analyte | $n$ | Spiked concentration (ng/mL) | Mean calculated concentration (ng/mL) | %RSD | %RE |
|---|---|---|---|---|---|
| Amlodipine | 5 | 0.100 | 0.101 | 7.08 | 1.47 |
| | 5 | 0.300 | 0.297 | 7.39 | –1.02 |
| | 5 | 3.000 | 2.831 | 4.44 | –5.65 |
| | 5 | 7.500 | 7.753 | 6.13 | 3.37 |

**Table 5. Stability sample results for amlodipine**

| Stability | $n$ | Spiked concentration (ng/mL) | Mean calculated comparison sample concentration (ng/mL) | Mean calculated stability sample concentration (ng/mL) | Mean percentage change |
|---|---|---|---|---|---|
| Process[a] | 5 | 0.300 | 0.276 | 0.292 | 5.80 |
| | 5 | 7.500 | 7.835 | 7.555 | –3.58 |
| Bench top[b] | 5 | 0.300 | 0.310 | 0.309 | –0.22 |
| | 5 | 7.500 | 7.344 | 7.252 | –1.26 |
| Freeze and thaw[c] | 5 | 0.300 | 0.295 | 0.332 | 12.59 |
| | 5 | 7.500 | 7.391 | 8.023 | 8.55 |
| Long-term[d] | 5 | 0.300 | 0.291 | 0.277 | –4.94 |
| | 5 | 3.000 | 3.108 | 2.750 | –11.51 |
| | 5 | 7.500 | 7.726 | 7.422 | –3.93 |

[a] After 24 h in autosampler at 5°C; [b] after 6 h at room temperature; [c] after three freeze and thaw cycles at –70°C; [d] at –70°C for 122 days.



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investigated by analyzing quality control samples in replicates ($n = 5$) at LQC and HQC levels. For process stability, the results indicated that the difference in the back-calculated concentration from time 0 to 24 h is ≤5.80%, which allowed us to conclude that processed samples are stable at least for 24 h at 5°C in the auto-sampler. For bench top stability, the results allowed us to conclude that amlodipine is stable for at least 6 h at room temperature in plasma samples. Freeze and thaw stability results indicated that the repeated freeze and thawing (three cycles) did not affect the stability of amlodipine. Long-term stability of amlodipine in plasma at –70°C was performed at LQC, MQC and HQC levels and it was found to be stable for at least 122 days at –70°C.

## CONCLUSION

A simple, specific, rapid and sensitive LC-MS/MS method has been developed for the determination of amlodipine in human plasma. The proposed method provided excellent specificity and reproducibility with a limit of quantification of 0.1 ng/mL for amlodipine.

It is concluded that this sensitive and specific method is applicable for the quantitative determination of amlodipine in human plasma in pharmacokinetic and bioavailability studies of amlodipine.

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