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Short communication

Determination and pharmacokinetic study of amlodipine in human plasma by ultra performance liquid chromatography–electrospray ionization mass spectrometry

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Abstract

A novel, specific and sensitive ultra performance liquid chromatography tandem mass spectrometry (UPLC–MS/MS) method was developed for the determination and pharmacokinetic study of amlodipine in human plasma. The analysis was carried out on an ACQUITY UPLC^{TM} BEH C_{18} column (50 mm × 2.1 mm, i.d., 1.7 μm) with gradient elution at a flow-rate of 0.35 ml/min. The mobile phase was water and acetonitrile under gradient conditions (both containing 0.3% formic acid) and nimodipine was used as the internal standard. Detection was performed on a triple-quadrupole tandem mass spectrometer by multiple reaction monitoring (MRM) mode via Turbo ion spray ionization (ESI). Linear calibration curves were obtained over the concentration range 0.15–16.0 ng/ml, with a lower limit of quantification of 0.15 ng/ml. The intra- and inter-day precision (R.S.D.) values were below 15% and the accuracy (R.E.) was −2.3% to 6.9% at all three QC levels. The method was used to support clinical pharmacokinetic studies of amlodipine in healthy volunteers following oral administration.

Keywords: Amlodipine; UPLC–ESI-MS/MS; Human plasma; Pharmacokinetic

1. Introduction

Amlodipine is a calcium channel blocker, which is used as an anti-hypertensive and anti-anginal agent. It has a long elimination half-life and large volume of distribution. It has been reported that low plasma concentrations are achieved after oral administration of amlodipine [1], thus, a sensitive and specific analytical method is needed for determination of amlodipine in human plasma. Several analytical methods for amlodipine in biological samples have been reported. The sensitivity of published HPLC–UV methods [2] is inadequate for pharmacokinetic studies and therapeutic drug monitoring due to low absorbance of the drug. In the case of published gas chromatography (GC) methods [3,4] involving capillary column and electron capture detection, although the sensitivity was improved, thermal decomposition of the drug under GC conditions was the major problem. Determination of amlodipine at the nanogram level was achieved by HPLC-coupled to electrochemical detection [5–7], however, the method was limited by a low recovery [5], and the long retention time of the drug and internal standard (I.S.) [6,7]. Several HPLC procedures have been also reported for the analysis of amlodipine based on MS–MS detection using tandem mass spectrometry [8,9]. These reported methods are sensitive, having low quantification limits.

This paper describes a novel, selective and highly sensitive approach, which enables the determination of amlodipine with good accuracy at low drug concentrations in plasma using ultra performance liquid chromatography coupled to tandem mass spectrometry (UPLC–MS/MS). UPLC, with a stationary phase below 2 μm, is able to operate at a very high mobile phase linear velocity and allow rapid analysis, good chromatographic performance, and high sensitivity. The total run time of the method per sample was just 3.0 min which is shorter than the reported ones [8,9]. The LLOQ corresponding to an on-column sensitivity was lower than that reported in the literature [9]. This method was fully validated and applied to a pharmacokinetic study in healthy volunteers after oral administration of 10 mg tablets of amlodipine.

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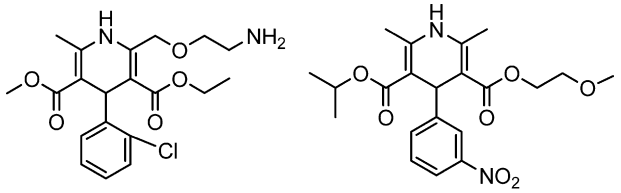

Fig. 1. Structures of amlodipine (I) and nimodipine (II).

2. Experimental

2.1. Chemicals and reagents

Amlodipine tablets (batch no. 55805028, 5 mg per tablet) were supplied by Sy Biopharmaceutical Corporation (Liaoning, PR China). The formulation contained 5 mg amlodipine. Reference standards of amlodipine (99.2% of purity), and nimodipine (internal standard, I.S., 99.4% purity) (Fig. 1) were purchased from the National Institute for Control of Pharmaceutical and Biological Products (Beijing, PR China). Acetonitrile, methanol and formic acid (HPLC grade) were purchased from Dikma (Richmond Hill, NY, USA). All Other chemicals were of analytical grade. Sodium hydroxide and diethyl ether were purchased from Yuwang (Chemical Reagent Plant, Shandong, China). Water was purified by redistillation and passed through a 0.22 μm membrane filter before use.

2.2. Apparatus and operating conditions

2.2.1. Liquid chromatography

The chromatography was performed on an ACQUITY^{TM} UPLC system (Waters Corp, Milford, MA, USA) with cooling autosampler and column oven enabling temperature control of the analytical column. An ACQUITY UPLC^{TM} BEH C_{18} column (50 mm × 2.1 mm, 1.7 μm; Waters Corp, Milford, MA, USA) was employed. The column temperature was maintained at 45 °C and chromatographic separations were achieved with gradient elution using a mobile phase composed of water and acetonitrile each containing 0.3% formic acid. The gradient elution started at 60% water, changed linearly to 10% water (0–1.5 min), was maintained at 10% for 0.5 min and then returned to the initial condition. The flow rate was set at 0.35 ml/min. The auto-sampler was conditioned at 4 °C and the injection volume was 5 μl using partial loop mode for sample injection.

2.2.2. Mass spectrometry

Triple-quadrupole tandem mass spectrometric detection was carried out on a Micromass® Quattro micro^{TM} API mass spectrometer (Waters Corp, Milford, MA, USA) with an electrospray ionization (ESI) interface. The ESI source was operated in positive ionization mode. Quantification was performed using multiple reaction monitoring (MRM) of the transitions of m/z 409 → 238 for amlodipine and m/z 419 → 343 for nimodipine (I.S.), respectively, with a scan time of 0.05 s per transition. The optimal MS parameters obtained were as follows: capillary 3.0 kV, cone 14 kV, source temperature 105 °C and desolvation temperature 300 °C. Nitrogen was used as the desolvation and cone gas with a flow rate of 400 and 70 L/h, respectively. Argon was used as the collision gas at a pressure of approximately 2.73×10^{-3} mbar. All data collected in centroid mode were processed using MassLynx^{TM} NT 4.0 software with a QuanLynx^{TM} program (Waters Corp., Milford, MA, USA).

2.3. Preparation of standards and quality control samples

Standard stock solution of amlodipine was prepared in methanol at a concentration of 200 μg/ml. A standard solution was prepared by adding 0.5 ml standard stock solution to a 100 ml volumetric flask and made up to the mark with a mixture of methanol–water (50:50, v/v) to give a solution with a final concentration of 1 μg/ml amlodipine. The solution was then serially diluted with methanol–water (50:50, v/v) to provide working standard solutions of desired concentrations. The I.S. working solution (50 ng/ml) was also prepared by diluting the 50 μg/ml stock solution of nimodipine with a mixture of methanol–water (50:50, v/v).

Calibration standards were prepared by spiking 0.5 ml of blank human plasma with 50 μl of the appropriate standard solutions. The effective concentrations in the plasma samples were 0.15, 0.50, 1.00, 2.00, 4.00, 8.00 and 16.0 ng/ml. One calibration curve was constructed on each analysis day using freshly prepared standards. The quality control samples (QCs) were prepared in blank plasma at concentrations of 0.50, 2.0 and 12.0 ng/ml and stored at −20 °C after preparation at the beginning of the experiment. The standards and quality controls were extracted on each analysis day along with the unknown samples.

2.4. Plasma sample preparation

To a 0.5 ml aliquot of plasma in 10 ml clean glass tubes, 50 μl internal standard (50 ng/ml) and 200 μl 1 mol/l sodium hydroxide solution were added. The samples were vortexed for 60 s and 3 ml diethyl ether was added. The mixture was vortex-mixed for another 60 s, then shaken on a mechanical shaker for 10 min. After centrifugation at 3500 × g for 10 min, the upper organic layer was then transferred to another set of clean glass tubes and evaporated to dryness at 40 °C under a gentle stream of nitrogen. The residue was reconstituted in 100 μl acetonitrile: water (70:30, v/v), and transferred to 700 μl glass vials, and an aliquot of 5 μl was injected into the UPLC–MS/MS system for analysis.

2.5. Method validation

The method was validated for linearity, selectivity, lower limit of quantification (LLOQ), accuracy and precision. To evaluate linearity, plasma calibration curves were prepared and assayed in duplicate on 3 separate days. Precision and accuracy were also assessed by determination of a minimum of one set of calibration standards and six sets of QC plasma samples at three concentrations. Calibration curves for standards were obtained using weighted ($1/x^2$) least squares linear regression. Peak area ratios of amlodipine to I.S. were then interpolated from the cal-

ibration curve to give the concentrations of unknown samples. During routine analysis, each analytical run included a set of calibration standards, a set of QC plasma samples in duplicate at intervals per batch and plasma samples to be determined.

2.5.1. Selectivity

Selectivity was assessed by comparing chromatograms of six different batches of blank plasma obtained from six subjects with those of corresponding standard plasma samples spiked with amlodipine and nimodipine (50 ng/ml) and a plasma sample after two oral doses of amlodipine tablets.

2.5.2. Linearity and lower limit of quantification (LLOQ)

Calibration curves were prepared using seven standard plasma samples in the 0.15–16.0 ng/ml range. The linearity of each calibration curve was determined by plotting the peak area ratio (y) of amlodipine to I.S. versus the nominal concentration (x) of amlodipine. The calibration curves were constructed by weighted ($1/x^2$) least squares linear regression.

The lower limit of quantification (LLOQ) is defined as the lowest concentration on the calibration curve at which an acceptable accuracy within ±20% and a precision below 20% can be obtained.

2.5.3. Precision and accuracy

For determining the intra-day accuracy and precision, a replicate analysis of plasma samples of amlodipine in human plasma was performed on the same day. The run consisted of a calibration curve and six replicates of each LLOQ, low, mid, and high concentration quality control samples. The inter-day accuracy and precision were assessed by analysis of three batches on different days. The precision was expressed as the relative standard deviation (R.S.D.%) and the accuracy as the relative error (R.E.%).

2.5.4. Extraction recovery

The extraction recovery of amlodipine was determined by calculating the mean of the response of each concentration and dividing the extracted sample mean by the unextracted (spiked blank plasma extract) sample mean of the corresponding concentration. Comparison with the unextracted samples, and spiked plasma residues, was carried out in order to eliminate matrix effects, giving the true recovery. This procedure was repeated for the three concentrations of 0.50, 2.0 and 12.0 ng/ml.

2.5.5. Stability

2.5.5.1. Freeze and thaw stability. The effect of three freeze and thaw cycles on the stability of plasma samples containing amlodipine was determined by subjecting six aliquots each of low, mid and high-concentration unextracted quality control samples to three freeze–thaw cycles. After completion of the three cycles, the samples were analyzed and the experimental concentrations were compared with the nominal values.

2.5.5.2. Long-term stability. Six aliquots each of low, mid and high concentration QC samples were stored at −20 °C for 30 days. Then, the samples were processed and analyzed and the concentrations obtained were compared with the nominal values.

2.5.5.3. Short-term stability. Six aliquots each of low, mid and high concentration unextracted QC samples were kept at ambient temperature (25 °C) for 4 h in order to establish the short-term stability of amlodipine in human plasma. Then, the samples were processed and analyzed. The concentrations obtained were compared with the nominal values of QC samples.

2.5.5.4. Stability of post-preparation samples. In order to estimate the stability of amlodipine stored in the prepared sample, aliquots of low, mid and high concentration QC samples were kept in an autosampler maintained at 4 °C for about 4 h. Then, the samples were analyzed and the concentrations obtained were compared with the nominal values.

2.6. Pharmacokinetic study

The method was applied to determine the plasma concentrations of amlodipine from a clinical trial in which 20 healthy male volunteers received two tablets (containing 10 mg amlodipine). The pharmacokinetic study was approved by the local Ethics Committee and all volunteers gave their signed informed consent to participate in the study according to the principles of the Declaration of Helsinki. Blood samples were collected before and 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 8.0, 10.0, 14.0, 24.0, 48.0, 72.0, 96.0 and 120 h post-dosing. Samples were centrifuged and plasma was separated and stored at −20 °C until analyzed.

3. Results and discussion

3.1. Mass spectrometry

When amlodipine and nimodipine were injected directly into the mass spectrometer along with the mobile phase with a positive ion interface, the full scan spectrum was dominated by protonated molecules $[M+H]^+$ m/z 409 and 419 for amlodipine and nimodipine, and the major fragment ions observed in each product spectrum were at m/z 238 and 343, respectively. The analysis temperature, nebulizer gas, and ESI temperature were investigated to optimize the specificity and sensitivity of m/z 238 and m/z 343 ions detection. Fig. 2 shows the product the ion mass spectra of $[M+H]^+$of amlodipine and nimodipine.

3.2. Chromatography

Chromatographic separation was performed by gradient elution using a mobile phase composed of acetonitrile–water (both containing 0.3% formic acid). The presence of a small amount of formic acid in the mobile phase improved the detection of the analytes in positive ion mode of the LC–MS/MS and, consequently, improved the sensitivity. Gradient elution is usually used in order to extend the column life and elute the analyte rapidly. The use of small particles of stationary phase allowed UPLC to push the limits of both peak capacity (due to higher

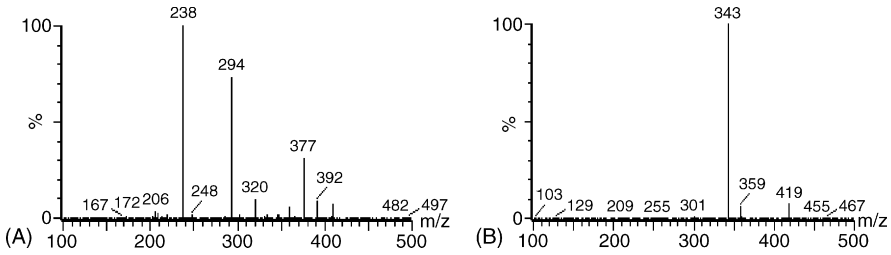

Fig. 2. Full scan product ion mass spectra of $[M+H]^+$ of amlodipine (A) and nimodipine (B).

efficiency) and speed of analysis (due to higher linear velocities) without compromising resolution. However, the column with sub-2 μm particles was more easily blocked. Therefore, gradient elution was used to provide a better peak shape and increase the column life.

Two channels were used for recording, channel 1 for amlodipine with a retention time of 0.75 min, and channel 2 for the I.S. with a retention time of 1.38 min. As shown in Fig. 3, both amlodipine and I.S. were well separated with excellent peak shapes, and no interfering peaks were observed in the blank plasma and in all samples tested. The very narrow chromatographic peaks with a peak width about 6 s, produced by UPLC™ resulted in an increase in the chromatographic efficiency and sensitivity. Both amlodipine and I.S. were rapidly eluted with retention times less than 2 min, and the total run time was just 3.0 min per sample, which was less than the values (4.5 and 5 min) in the literature [8,9] and this met the requirement for a high sample throughput.

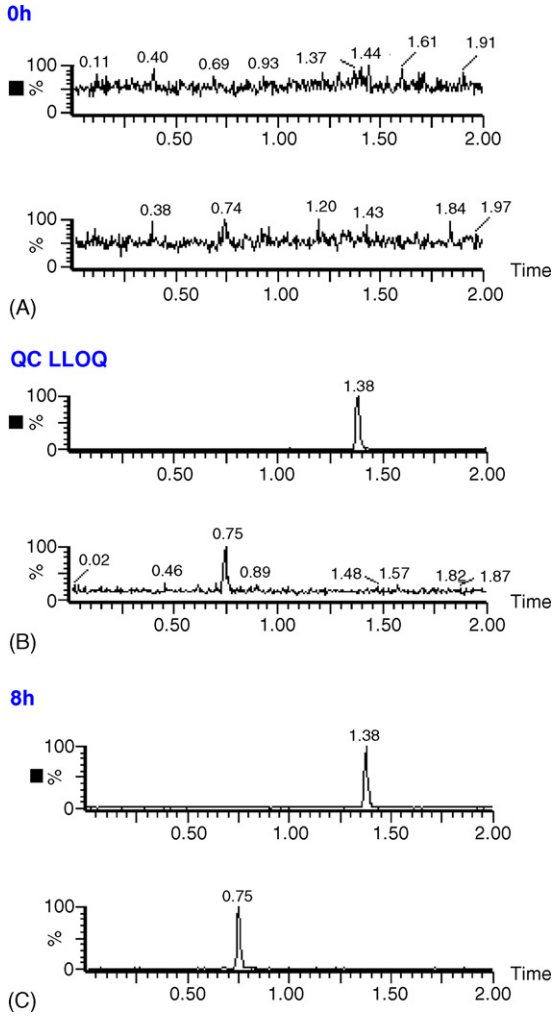

Fig. 3. Representive MRM chromatograms of amlodipine (peak 1, channel 1) and nimodipine (peak 2, channel 2) in human plasma samples. (A) A blank plasma sample; (B) a blank plasma sample spiked with amlodipine at the LLOQ of 0.15 ng/ml and I.S. (50 ng/ml); (C) plasma sample from a volunteer 8.0 h after oral administration of amlodipine. The retention times for amlodipine and I.S. were 0.75 and 1.38 min, respectively.

3.3. Method validation

3.3.1. Selectivity

Selectivity was assessed by comparing the chromatograms of six different batches of blank human plasma with the corresponding spiked plasma. As shown in Fig. 3A–C, there was no interference from endogenous substances observed at the retention time of the analytes. At the same time, due to the high selectivity of tandem mass spectrometry, only required ion reactions were monitored and, so, there was no interference from its metabolites.

3.3.2. Matrix effect

The possibility of a matrix effect caused by ionization competition between the analytes and co-eluents exists when using MS/MS for analysis. To evaluate the matrix effect, the peak areas of blank plasma extracts spiked with analyte post-extraction (A) were compared with those of the standard solutions dried directly and reconstituted with mobile phase (B). All the ratios $(A/B \times 100)\%$ were between 85% and 115%, which meant no significant matrix effect for amlodipine and nimodipine in this method. Thus, ion suppression or enhancement from the plasma matrix was negligible for this method.

3.3.3. Linearity and LLOQ

The linear regression of the peak area ratios versus concentrations were fitted over the concentration range 0.15–16.0 ng/ml for amlodipine in human plasma. A typical equation for the calibration curve was: $y = 2.159 \times 10^{-1}x + 5.730 \times 10^{-4}$, $r = 0.9984$.

The lower limit of quantification for amlodipine was 0.15 ng/ml with 5 μl injected onto the UPLC column. The LLOQ corresponded to an on-column sensitivity of 0.75 pg (0.15 ng/ml × 5 μl) amlodipine, which was lower than that (0.10 ng/ml × 40 μl = 4 pg) reported in the literature [9]. The

Table 1
Precision and accuracy for the determination of amlodipine in human plasma (intra-day: $n = 6$; inter-day: $n = 6$ series per day, 3 days)

Concentrations (ng/ml)		R.S.D. (%)		Relative error (%)
Added	Found (mean)	Intra-day	Inter-day	
0.15	0.16	9.0	5.3	3.3
0.50	0.50	5.6	7.8	−0.7
2.00	1.95	5.0	8.4	−2.3
12.00	12.83	4.4	5.2	6.9

precision and accuracy are presented in Table 1 and these are sufficient for clinical pharmacokinetic studies following oral administration. The high sensitivity could be attributed to the extra resolution and peak sharpness produced by the UPLC chromatographic system and the improved ionization efficiency.

3.3.4. Precision and accuracy

The intra-day precision (R.S.D.) for QC samples (0.5, 2.0, 2.0 ng/ml) was 5.6%, 5.0% and 4.4%, respectively and that of inter-day analysis was 7.8%, 8.4% and 5.2% with an accuracy (R.E.) within −2.3 to 6.9%. All these data indicated the adequate precision and accuracy of the present method for the determination of amlodipine in human plasma. The accuracy and precision data are shown in Table 1.

3.3.5. Extraction recovery and stability

The extraction recoveries of amlodipine from human plasma were 90.2 ± 3.6%, 91.3 ± 2.8%, and 92.3 ± 3.7% at concentration levels of 0.50, 2.0 and 12.0 ng/ml, respectively, and the mean extraction recovery of nimodipine was 85.4 ± 2.7%. In the published methods [8,9] mixed solvents such as ether/hexane (80:20 v/v) and ethyl acetate/hexane (80:20 v/v) were used as extraction solvents. Higher extraction efficiency was obtained by using ether as the extraction solvent compared with the reported mixed solvents (mean recovery of about 80%). Moreover, the avoidance of using mixed solvents made the extraction process much simpler.

The results of stability experiments showed that no significant degradation occurred during the chromatography, extraction and sample storage of amlodipine plasma samples. Stability data are shown in Table 2.

A stock solution of the I.S. (0.01 mg/ml) in methanol was stored at −20 °C for 30 days. From the stock solution, dilutions of 50 ng/ml in the mobile phase were made on days 1, 5, 8, 12, 19, 25 and 30. The resulting solutions were injected directly. The R.S.D.% calculated for the I.S. peak areas was 8.7%.

3.4. Application of the method to a pharmacokinetic study in healthy volunteers

This validated analytical method was used to support clinical pharmacokinetic studies of amlodipine in healthy volunteers following oral administration. The profile of the mean plasma concentration of amlodipine versus time is shown in Fig. 4. The pharmacokinetic parameters were computed using 3p97 software. The maximum plasma concentration (C_{max}) was

Table 2
Stability of amlodipine in plasma samples ($n = 6$)

Nominal concentration (ng/ml; $n = 6$)	Concentration found (ng/ml)	Precision (%)	Accuracy (%)
Three freeze-thaw cycles			
0.50	0.49	9.2	−2.9
2.00	2.05	6.8	2.5
12.00	13.03	4.3	8.6
Long term (−20 °C for 30 days)			
0.50	0.49	9.6	−1.9
2.00	1.88	4.0	−0.6
12.00	12.46	3.2	3.8
Short term (room temperature for 4 h)			
0.50	0.53	4.8	6.2
2.00	2.06	5.3	3.2
12.00	12.75	4.5	6.3
Post-preparative (4 °C for 4 h)			
0.50	0.53	3.8	5.8
2.00	2.14	5.4	6.4
12.00	12.76	4.9	6.4

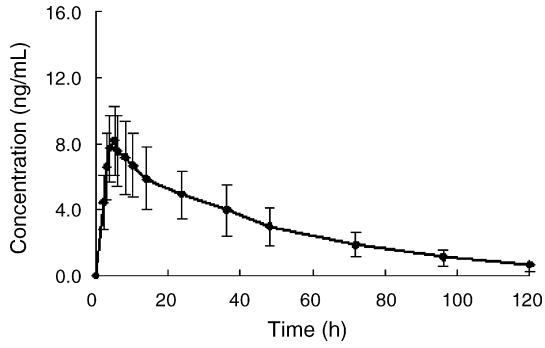

Fig. 4. Mean plasma concentration–time profile of amlodipine after oral administration of amlodipine tablets (containing 10 mg amlodipine) to 20 healthy volunteers (each point represents mean ± S.D.).

8.70 ± 1.96 ng/ml, the time point of maximum plasma concentration (T_{max}) was 5.3 ± 1.7 h, the area under the plasma concentration–time curve from 0 h to the time of the last measurable concentration (AUC_{0-t}) was 348.4 ± 113.3 ng h/ml, the area under the plasma concentration–time curve from 0 h to infinity ($AUC_{0-\infty}$) was 380.5 ± 125.0 ng h/ml, and the half-life of drug elimination during the terminal phase ($t_{1/2}$) was 33.33 ± 7.23 h. These parameters were in agreement with those reported in the literature [10].

4. Conclusion

A sensitive, selective and rapid UPLC–ESI-MS/MS method for the determination of amlodipine in human plasma is described. Compared with the published methods, the sharp peaks produced by UPLC are of particular advantage when coupled to electrospray mass spectrometry, reducing ion suppression and offering superior sensitivity and, hence, lower limits of detection, higher sensitivity with an LLOQ of 0.15 ng/ml, satisfactory selectivity and a short run time of 3.0 min. The method has been successfully applied to a phar-

macokinetic study of amlodipine given in tablet form to healthy volunteers.

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