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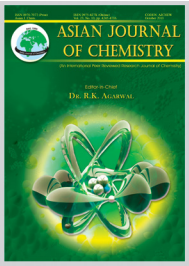

Sensitive Determination and Pharmacokinetic Study of Amlodipine in Human Serum by LC-ESI/MS/MS

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A sensitive LC-ESI/MS/MS method was developed and validated to determine the concentrations of amlodipine in human serum. Amlodipine and the internal standard (IS) omeprazole were extracted from 200 µL of human serum by liquid-liquid extraction, using ethyl acetate as the extraction solvent. Sample analysis was performed by reversed-phase LC-MS/MS with electrospray ionization in positive ion mode, using selected reaction monitoring. The mobile phase composed of methanol and 0.15 % ammonium acetate in water (78 : 22, v/v) at a flow rate of 0.2 mL/min. The linearity ranged from 0.2 to 12.8 ng/mL. The extraction recoveries of amlodipine ranged from 90.68 to 105.27 %. The method was successfully used to pharmacokinetic study of amlodipine after an oral administration of 10 mg amlodipine tablets to 19 healthy volunteers.

Key Words: Amlodipine, LC-ESI/MS/MS, Pharmacokinetic.

INTRODUCTION

Amlodipine besylate (ADB) is a relatively new potent long-acting calcium channel blocking agent[1]. Amlodipine is a third-generation dihydropyridine calcium antagonist, which is used alone or in combination with other medications for treating high blood pressure, certain types of vasospastic angina, cardiac arrhythmias and coronary heart disease[2-4]. Low serum concentration was achieved after oral administration of the drug, thus, sensitive and specific analytical methods for the assay of serum levels are necessary.

Different analytical methods for amlodipine in human plasma, such as high-performance thin layer chromatography densitometry[5], gas chromatography methods involving capillary column and electron capture detection[6,7] or liquid chromatography with fluorescent detection[8] and electrochemical detection[9,10], have been reported. The reported HPLC methods require laborious extraction procedures like solid phase extraction, long run time, high limit of quantification, low recovery and large serum volume. The disadvantage of gas chromatography methods is thermal decomposition of amlodipine at high temperatures. Liquid chromatography tandem mass spectrometric (LC/MS/MS) methods have been also reported[11-16], however, the methods were limited by the low recovery[12, 13], a large serum sample volume[11-13], using much acetonitrile in the mobile phase[13-15] or utilizing solid phase extraction for sample preparation[16]. These methods are not available for most laboratories because of financial reasons.

Here we reported a sensitive and reliable assay to estimate amlodipine in human serum. The approach meets the requirements and provides high accuracy, sensitivity and specificity by liquid-liquid extraction with ethyl acetate by high-performance liquid chromatography tandem mass spectrometry. The proposed method was fully validated and applied to a pharmacokinetic study in healthy volunteers after oral administration of 10 mg tablets of amlodipine .

EXPERIMENTAL

The standards of amlodipine besylate and omeprazole were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, P.R. China). Methanol (HPLC-grade) was purchased from Merck (Darmstadt, Germany). Formic acid, ethyl acetate and ammonium acetate (HPLC-grade) were bought from TEDIA (Fairfield, USA). Distilled water, was prepared from demineralized water. Blank serum was provided by The First Affiliated Hospital of Anhui Medical University (Hefei, China).

A TSQ Quantum Ultra AM triple-stage quadrupole tandem mass spectrometer (Thermo Finnigan, USA), coupled with electrospray ionization (ESI) source, a Finnigan Surveyor liquid chromatography pump and Finnigan Surveyor autosampler, was used for LC-MS/MS analysis. Data

acquisitions were performed with Xcalibur 1.4 software (Thermo Finnigan, USA). The system was operated at ambient temperature 20 ºC.

LC-MS/MS conditions: Chromatographic separation of the analytes from potential interfering materials was performed using a Synergi column (100 × 2.0 mm i.d., 4 um; Phenomenex, USA) with a mixture of methanol and water (containing 0.15 % ammonium acetate) (78:22, v/v) as the isocratic mobile phase, which was pumped at a flow-rate of 0.2 mL/min.

Mass spectrometric detection was performed with selected reaction monitoring (SRM) in positive ion mode. The precursor-fragment ion reaction for amlodipine was m/z 409.01→237.82 and for omeprazole was m/z 346.02→197.74. The product ion spectra of $[M+H]^+$ ions of amlodipine and IS were shown in Fig. 1. (A) and (B), respectively. The optimal parameters were summarized in the following: electrospray voltage 4700 V, capillary temperature 270 ºC. Nitrogen was used as sheath gas and auxiliary gas at the pressures (arbitrary units) of 43 and 3, respectively. Argon was used as collision gas at a pressure of 1.0 mTorr. Collision energies of 22 eV and 15 eV were used for amlodipine and omeprazole, respectively. The scan width for selected reaction monitoring was 0.2 and scan time was 0.5 s. The peak width settings (full width at half maximum, FWHM) for both Q1 and Q3 were 0.7 u.

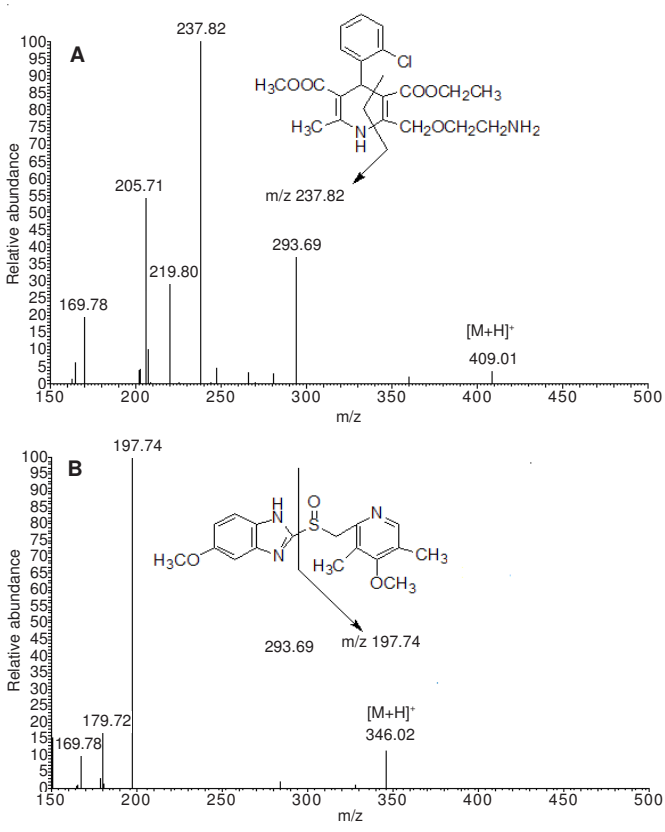

Fig. 1. Product ion spectra of the $[M+H]^+$ ions of (A) AD and (B) omeprazole

Calibration standards and quality control samples: Stock solutions of amlodipine and internal standard omeprazole were prepared in methanol at a concentration of 179 µg/mL and 164 µg/mL, respectively. Calibration curve samples at concentrations of 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8 ng/mL of amlodipine were freshly prepared by spiking blank serum. Omeprazole was also further diluted with methanol to give a concentration of 3.28 ng/mL during all analysis.

The quality control samples were prepared using spiking blank serum at concentrations of 0.4, 1.6, 6.4 ng/mL, representing low-, medium-, high-concentration quality control samples, respectively.

Preparation of serum sample: To a 0.2 mL aliquot of serum in 2 mL clean centrifugal tube, 10 µL omeprazole (3.28 ng/mL) was added. The samples were vortexed for 30 s and 1 mL ethyl acetate was added. The mixture vortex-mixed for 3 min and centrifuged for 10 min at 13,400 g. The upper organic phase (800 µL) was transferred to another centrifugal tube and then evaporated to dryness at 40 ºC under a gentle stream of nitrogen. The residue was reconstituted in 80 µL mobile phase and 10 µL injected into the LC-MS/MS system.

Method validation: The precision, accuracy, linearity, specificity, matrix effect, recovery and stability of the analytical method were confirmed by validation in accordance with the USFDA guidelines[17]. Validations were conducted on three consecutive days. Each validation run consisted of a minimum of one set of calibration standards and five replicates of LLOQ and quality control serum samples at three concentrations. Linear regression was used to obtain calibration curves of the analyte. The results from LLOQ and quality control serum samples in three runs were used to evaluate the precision (RSD) and accuracy (RE) of the developed method. Specificity was evaluated by processing and analyzing six pools of blank human serum.

The extraction recovery of the analyte from the serum was evaluated by comparing the mean detector responses of three replicates of processed quality control samples to the detector responses of mean peak areas of spiked-after-extraction samples with the corresponding concentration.

In order to evaluate the matrix effect, *i.e.* the potential ion suppression or enhancement due to the matrix components, the peak areas of amlodipine obtained from the spike-after-extraction samples were compared with the peak areas from the pure standard solutions at the same concentrations.

Stability tests including three freeze-thaw cycles, long-term stability (-20 ºC for 30 days), short-term stability (20 ºC for 4 h) and 24 h stability (prepared samples at ambient temperature for 24 h) were evaluated by quality control samples in quintuplicate.

Application of the LC-MS/MS method: The LC-MS/MS method was successfully applied to the pharmacokinetic study of amlodipine in healthy human volunteers. A single 10 mg dose of amlodipine was administered orally to 19 healthy volunteers who were advised about the nature and purpose of the study. Blood samples were taken 0, 1.5, 3.5, 5.5, 7.5, 9, 10.5, 12, 15, 24, 48, 72, 120 and 168 h after ingestion. Serum was obtained through centrifugation at 2000 × g for 10 min. Serum specimens were then stored at -20 ºC prior to analysis.

RESULTS AND DISCUSSION

LC/MS/MS conditions: Under the chromatographic conditions described, amlodipine and omeprazole peaks were

well resolved. Endogenous serum components did not give any interfering peaks. Fig. 2 shows typical chromatograms of blank serum in comparision to spiked samples analyzed for a pharmacokinetic study.

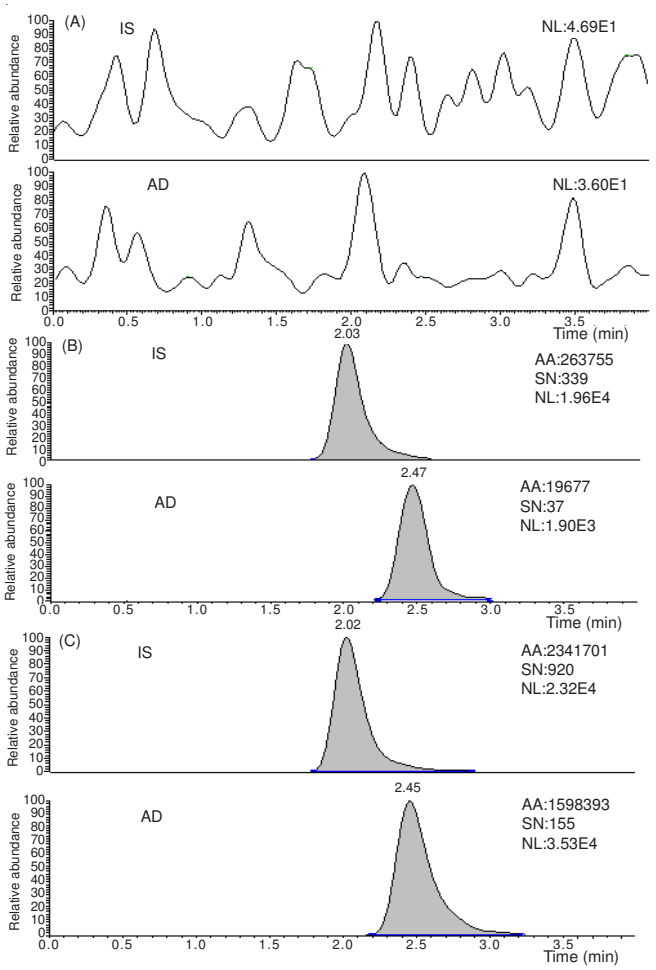

Fig. 2. SRM chromatograms for amlodipine (AD) and omeprazole (IS) in human serum. (A) blank serum; (B) blank serum spiked with amlodipine (0.2 ng/mL) and omeprazole (0.164 ng/mL); (C) a serum sample from a volunteer 1.5 h after an oral dose of 10 mg amlodipine

Method validation: The calibration curve for the determination of amlodipine in serum was linear over the range of 0.2-12.8 ng/mL. A typical standard curve was y = (0.225 ± 0.033) x + (0.019 ± 0.01). Correlation coefficients ranged from 0.998 to 1.0. The lower limit of quantification (LLOQ) was found to be 0.2 ng/mL, at which the calculated accuracy and precision were below 7.9 %. Table-1 summarizes the mean values of accuracy and precision for both intra- and inter-day assays. The assay method demonstrated high accuracy and precision.

The recoveries of amlodipine ranged from 90.68 % to 105.27 % at three quality control concentration levels. In terms of matrix effect, the results ranged from 93.4 to 109.82 % for amlodipine. Thus, ion suppression or enhancement from serum matrix was negligible for this method.

The results from all stability tests presented in Table-2, which demonstrated a good stability of amlodipine over all steps of the determination.

Pharmacokinetics: The method was therefore approved to be applicable for routine analysis. The mean serum concentration-time profile obtained from 19 healthy volunteers after an oral dose of amlodipine was shown in Fig. 3. The values of the main pharmacokinetic parameters were listed as follows: the maximum serum concentration (C_{max}) 8.19 ± 2.80 ng/mL; area under the curve (AUC_{0-t}) 376.8 ± 128.7 ng h/mL; the time to maximum serum concentration (T_{max}) 6.81 ± 5.44 h; half-life ($t_{1/2}$) 44.48 ± 9.60 h.

TABLE-1
PRECISION AND ACCURACY FOR ASSAY OF AMLODIPINE IN HUMAN SERUM

Day	Concentration (ng/mL)		RSD (%)	RE (%)
	Spiked	Found (mean ± SD)		
Day 1	0.4	0.369 ± 0.042	11.38	-7.75
	1.6	1.584 ± 0.177	11.17	-10.00
	6.4	5.991 ± 0.437	7.29	-6.39
Day 2	0.4	0.386 ± 0.027	6.99	-3.50
	1.6	1.546 ± 0.062	4.01	-3.38
	6.4	6.864 ± 0.472	4.20	7.25
Day 3	0.4	0.400 ± 0.038	9.50	0.00
	1.6	1.534 ± 0.157	10.23	-4.13
	6.4	7.176 ± 0.126	1.76	12.13
Inter-day	0.4	0.385 ± 0.016	4.16	-3.75
	1.6	1.555 ± 0.026	1.67	-2.81
	6.4	6.677 ± 0.614	9.20	4.33

TABLE-2
STABILITY FOR ASSAY OF AMLODIPINE IN HUMAN SERUM (n=5)

Storage conditions	Concentration (ng/mL)		RSD (%)	RE (%)
	Spiked	Found (mean ± SD)		
4h-RT	0.4	0.390 ± 0.052	13.33	-2.5
	1.6	1.465 ± 0.183	12.49	-8.44
	6.4	5.648 ± 0.331	5.86	-11.75
24h-RT	0.4	0.396 ± 0.028	7.07	-1
	1.6	1.502 ± 0.074	4.93	-6.13
	6.4	6.638 ± 0.161	2.42	3.72
Three freeze/thaw cycle	0.4	0.402 ± 0.049	12.19	0.5
	1.6	1.725 ± 0.065	3.77	7.81
	6.4	6.735 ± 0.154	2.29	5.23
Long term	0.4	0.374 ± 0.040	10.70	-6.5
	1.6	1.466 ± 0.025	1.71	-8.38
	6.4	6.870 ± 0.164	2.39	7.34

RT = Room temperature; RSD = Relative standard deviation; RE = Relative error.

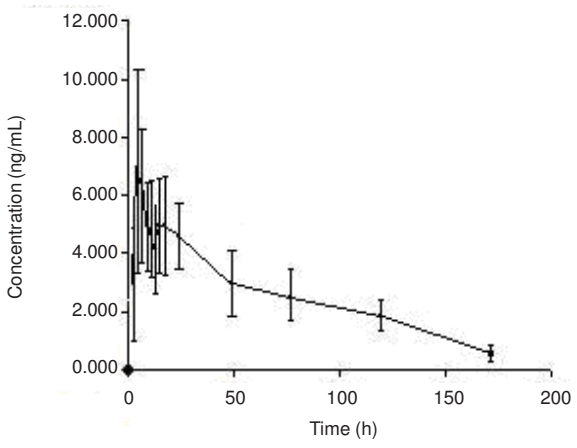

Fig. 3. Mean serum concentration-time profile of amlodipine from 19 healthy volunteers following a single oral dose of 10 mg AD

Conclusion

We have developed and validated a simple HPLC-ESI/MS/MS method for the quantification of amlodipine in serum. This method will permit pharmacokinetic and pharmacodynamic studies of amlodipine in humans.

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