High-Performance Liquid Chromatographic Method for Quantitative Determination of Amlodipine in Human Plasma and Pharmaceutical Dosage Form and its Application to Pharmacokinetic Studies

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Abstract

An accurate, sensitive, and reproducible high-performance liquid chromatographic method for the quantitation of amlodipine besylate in human plasma has been developed and validated. The drug, internal standard, and major metabolite were eluted from a C_{18} hypersil HyPurity column (3 µm, 3.9 mm i.d. × 150 mm) at room temperature with a mobile phase consisting of acetonitrile–potassium dihydrogen phosphate buffer (0.05 M) and acetic acid (62:38:0.1) with the pH adjusted to 3.5 using phosphoric acid. The flow-rate was 1.8 mL/min. The limit of detection was 1.0 ng/mL, and the limit of quantification of amlodipine besylate in plasma was 10 ng/mL. The intra- and inter-day precisions showed coefficients of variation ranging from 5.98–11.4% and from 5.60–11.74%, respectively at three different levels of concentration. The averages of the absolute and relative recoveries were found to be 96.74–98.51% and 95.96–100.71%, respectively. Stability studies showed that amlodipine besylate is stable for at least 2 months in plasma after freezing at –20°C. The method was successfully applied for a pharmacokinetic study and for the determination of commercial amlodipine tablet content.

Introduction

Amlodipine, 2-[(2-aminoethoxy)methyl]-4-(2-chlorophenyl)-1,4-dihydro-6-methyl-3,5-pyridinedicarboxylic acid 3-ethyl 5 methyl ester (Figure 1), is a new third-generation dihydropyridine calcium channel blocking agent, used in the treatment of hypertension and angina pectoris with a slow onset of vasodilatory action (1,2). It is commercially available for clinical uses in 5 and 10 mg tablets. Amlodipine has low plasma concentrations because after oral administration, amlodipine has

a long half-life in humans, ranging from 35 to 45 h due to the large volume of distribution (21 L/kg); moreover, it is highly bound (95%) to plasma proteins (3,4).

Several analytical methods for the analysis of amlodipine in biological samples have been previously reported. In a previously described gas chromatographic method involving a capillary column and electron capture detection, the sensitivity is improved; however, the thermal decomposition of amlodipine at the high temperatures to the pyridine analogue, which is already present in plasma as metabolite, is the major problem (5,6). The sensitivity of published high-performance liquid chromatography (HPLC)–UV methods due to low absorbance of the drug is inadequate for therapeutic drug monitoring (7–9). Although the determination of amlodipine in nanogram level has been achieved by published HPLC–electron capture methods (10,11), they were limited by low recovery (10) and long retention time of the drug and internal standard (11). Amlodipine has been quantified by a high-performance thin layer chromatography procedure which required 2 mL of plasma at a sensitivity of 2 ng/mL (12,13). Also, a very low limit of detection (LOD) and good recoveries were achieved by the
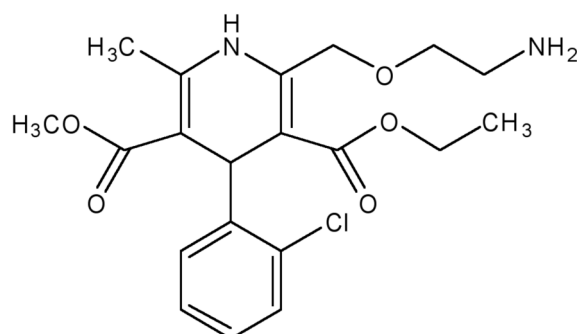

Figure 1. Chemical structure of amlodipine.

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use of LC–tandem mass spectrometry (14–16); however, this procedure is expensive, has very low limits of quantitation (LOQ) (< 0.1 ng/mL), involves highly sophisticated instruments which are not generally available in most routine laboratories, is not easily manageable, and requires highly trained personnel. Packed column supercritical fluid chromatography (17) and fluorescence detection (18,19) have also been reported. Although the sensitivity of these methods is enough to analyze amlodipine in pharmacokinetic studies, sample preparation is long, and the extensive sample clean-up is time-consuming. Therefore, very sensitive and specific analytical methods for the assay of amlodipine plasma levels are necessary.

The present paper describes a quick and simple HPLC method involving a single-step plasma extraction with UV detection for the quantitative determination of amlodipine in human plasma and pharmaceutical dosage in the presence of its major metabolite (20), and the application of the assay method to pharmacokinetic studies.

Experimental

Reagents and materials

All solvents used were of HPLC-grade, while other chemicals and reagents were of spectroquality- or analytical-grade, and were obtained from BDH Laboratories Supplies (BDH Chemical Ltd., Poole, UK). Amlodipine besylate (Batch no. 099MIS-025-00) was kindly supplied by Pfizer Global Research & Development (Pfizer Inc., Groton, CT) and the internal standard, amytryptyline hydrochloride, were purchased from Sigma Aldrich Chemical Company (St. Louis, MO). The major metabolite 2-([4-(2-chlorophenyl)-3-ethoxycarbonyl-5-methoxycarbonyl-6-methyl- 2-pyridyl]methoxy) acetic acid (Batch no. DM-5(I)EXH-007) was a generous gift from Jamjoom Pharma (Jeddah, Saudi Arabia).

Instrumentation and chromatographic conditions

The HPLC system consisted of a Model 515 Waters solvent delivery pump, a Waters autosampler model 717 plus (Waters Inc., Bedford, MA), a Shimadzu variable wavelength model SPD-10AV UV visible detector (Shimadzu Corporation, Kyoto, Japan) governed by a microcomputer running Millennium version 32 software, and vortex mixer (Scientific Industries Inc., Stony Brook, NY). The detector wavelength was set at 220 nm. Chromatographic separation was achieved by isocratic elution with a mobile phase of acetonitrile–potassium dihydrogen phosphate buffer (0.05 M) and acetic acid (62:38:0.1) with the pH adjusted to 3.5 using phosphoric acid. The mobile phase was filtered through a 0.22 µm Millipore filter (Millipore, Bedford, MA) and pumped at a flow rate of 1.8 mL/min at ambient temperature through a stainless steel C_{18} hypersil HyPurity column (3 µm, 3.9 mm i.d. × 150 mm) and a guard column of the same material was used (Thermo Fisher Scientific, Suwannee, GA).

Standardization and calculation

Calibration lines of peak area ratios (peak area analyte/peak area of internal standard) vs. concentration were determined by single-level calibration curve (linear regression equation, $y = Bx + A$), where x: concentration (ng/m:), B: slope, y: peak area ratio, and A: intercept.

Standard solutions

Stock solutions of amlodipine and amytryptyline acid were prepared by dissolving 10 mg (accurately weighed) in methanol in two separate 100-mL volumetric flasks to give standard stock solutions of 100 µg/mL. Further dilutions were made to give the desired concentrations of the drug. All solutions were stored at 4ºC and were stable for at least three weeks.

Plasma extraction

A 20 µL aliquot of internal standard working solution (amytryptyline) was added to 500 µL plasma sample (standard sample, quality control sample, or volunteer sample), and then the samples were extracted by 8 mL chloroform. The extraction procedure was carried out in 50-mL separating funnels. The mixture was shaken for 5 min in a shaker at 700 OSC/min. Before collection of the organic layer in quick-fit test tubes, the mixture was allowed to stand for 3 min. The organic layer was then evaporated under vacuum using a Rotavapor. The residue was reconstituted with 0.5 mL HPLC water, then a 20 µL aliquot sample was injected.

Assay validation

Validation of this procedure was performed in order to evaluate the suitability and accuracy of the proposed method in terms of linearity of the chromatographic response, the accuracy, the precision, sensitivity, specificity, and recovery, as well as the stability.

Applications of the Method

Assay of tablets containing the drug

The method was used to determine the content of a 10 mg amlodipine tablet (Istin) batch no. # Pl 0057/0298 (Pfizer Ltd., Sandwich, UK). One tablet was taken and placed in a 100-mL volumetric flask and completed to 100 mL with methanol. The flask was shaken and placed in an ultrasonic bath for 10 min. One milliliter was taken, placed in a 100-mL volumetric flask, and completed to 100 mL with methanol. Fifty microliters of this solution were directly injected into the chromatographic system.

Pharmacokinetic study

The method was applied to the determination of amlodipine in serum, following oral administration of 10 mg of amlodipine in 12 healthy volunteers. They received a single dose of 10 mg amlodipine after an overnight fasting. Multiple blood samples were taken from each of the subjects before (0 h) and at suitable intervals up to 48 h, and then frozen immediately at –20ºC until assayed. The study protocol was approved by the King Saud University Ethical Committee.

Results and Discussion

A UV scan of a solution of 10 µg/mL of amlodipine was performed to determine the optimum wavelength for detection. The maximum absorption was obtained at λ = 220 nm. The mobile phase used was optimized for rapid, sharp peaks with essentially no interference peaks from endogenous plasma and metabolite and provided optimum resolution of the amlodipine metabolite and internal standard. The typical chromatogram of a blood sample (blank) is shown in Figure 2A. The retention times for the metabolite, amlodipine, and internal standard were 2.97, 3.97, and 7.71 min, respectively. Figure 2B shows a chromatogram of a plasma sample spiked with the drug, the internal standard, and the major metabolite. Figure 2C represents the chromatogram of a plasma sample obtained at 13 h after a single oral dose of amlodipine from a healthy volunteer.
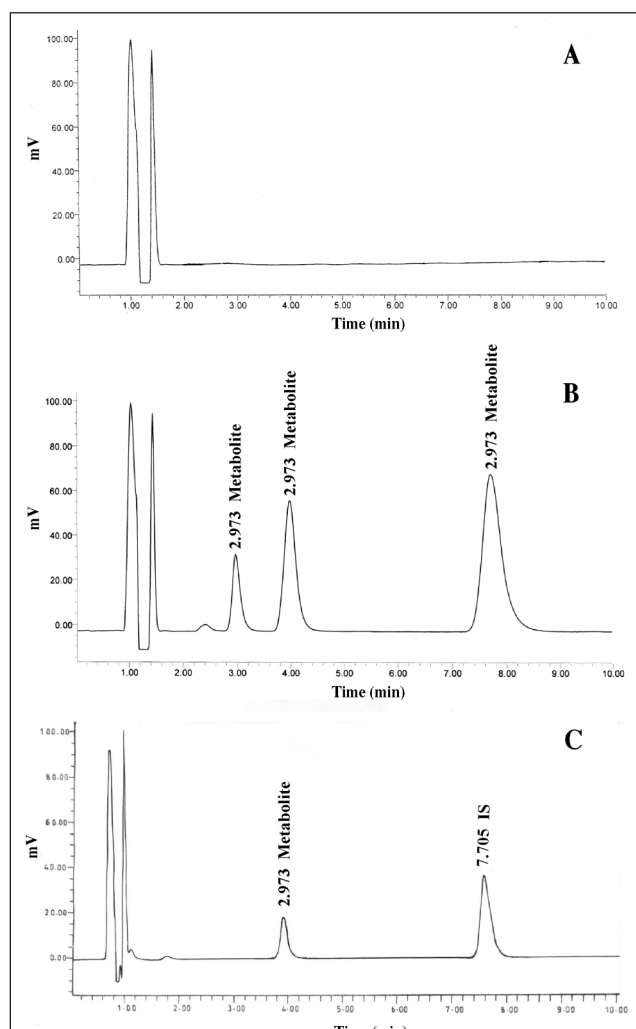

Figure 2. Typical HPLC chromatogram of a blank human plasma (A); chromatogram of an extract of human plasma spiked with 50 ng/mL of amlodipine, internal standard, and metabolite (B); plasma sample obtained at 13 h after oral administration of 10 mg of amlodipine from a healthy volunteer with 50 ng/mL of the internal standard (C).

Method validation
Quantification and linearity

The quantification of the chromatogram was performed by using peak-area ratios of the drug to the internal standard. The plasma standard curves were prepared over the range of 10–1000 ng/mL. Standard plots were analyzed in triplicate over a four-week period to determine the variability of the slopes and intercepts. The linear regression analysis of these data gave the slope, intercept, and correlation coefficient for standard curves constructed in plasma.

The plasma concentrations of amlodipine were found to be linear in the range of 10–1000 ng/mL at constant internal

Table I. Intra- and Inter-Day Precision of Amlodipine in Plasma (mean ± SD, n = 6)

	Intra-day*			Inter-day†		
Added conc. (ng/mL)	Measured conc. (ng/mL)	Bias‡ (%)		Added conc. (ng/mL)	Measured conc. (ng/mL)	Bias‡ (%)
60				**60**		
Mean	62.88	4.80		Mean	64.04	6.73
SD	7.19			SD	7.52	
CV %	11.4			CV %	11.74	
400				**400**		
Mean	413.98	3.50		Mean	443.28	10.82
SD	36.98			SD	40.73	
CV %	8.93			CV %	9.19	
800				**800**		
Mean	862.39	7.80		Mean	895.95	11.99
SD	51.03			SD	50.13	
CV %	5.98			CV %	5.60	

* Mean values represent six different plasma samples for each concentration.
† Inter-day reproducibility was determined from six different runs over a two-month period for the three concentrations.
‡ Bias = 100 × (measured concentration—added concentration) / added concentration.

Table II. Extraction Recovery of the Internal Standard from Spiked Plasma Samples and Absolute and Relative Recovery of Amlodipine

Concentration (ng/mL)	Absolute recovery (%)	Relative recovery (%) Mean ± SD
60	97.14	98.97 ± 1.85
400	96.74	100.71 ± 1.20
800	98.51	95.96 ± 3.86

Internal standard	Nominal concentration (ng/mL)	Measured concentration (Mean ± SD) (ng/mL)	RSD* (%)	Recovery (%)
	50.0	48.73 ± 0.53	1.09	97.50

* % RSD is the absolute value of the CV expressed as a percentage = (SD of X) × 100 / (average of X).

standard concentrations and resulted in the following regression equation:

$$y = 0.2009x - 0.05 \ (r^2 = 0.999) \ \text{(plasma)}$$

The result showed little day-to-day variability of slope and intercept as well as good linearity ($r^2 = 0.999$) over the range studied. The coefficient of variation (CV) ranged from 5.72% to 10.87%. The determination coefficient (r^2) obtained for the regression line demonstrates the linearity of the standard curves over the range studied and the excellent reproducibility of the assay method. The lower LOQ for this method attained with plasma samples was 10 ng/mL of amlodipine.

Sensitivity

The LOD, which is defined as the lowest concentration of the analyte, which can be detected but necessarily quantitated, was 1.0 ng/mL.

Precision and accuracy

The intra-day precision was evaluated by replicate analysis of pooled plasma samples containing amlodipine at three different concentrations (low, medium, and high). The intra-day precision showed a CV% ranging from 5.98% to 11.4% (Table I). The inter-day precision was similarly evaluated over a two-week period. The inter-day CV% ranged from 5.60% to 11.74% (Table I). The repeatability CVs were relatively low and did not exceed the 12% limit.

Recovery

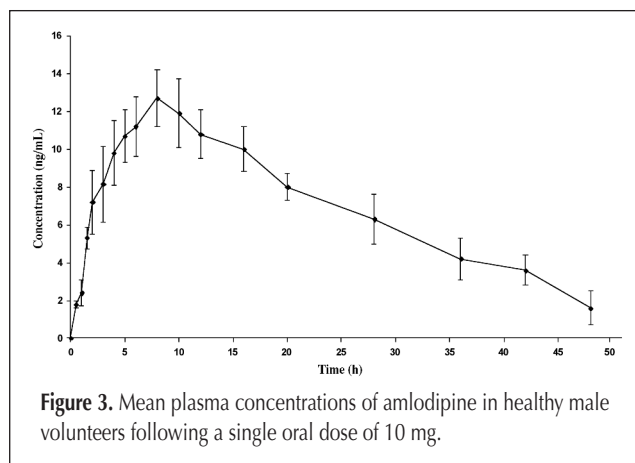The absolute recovery of amlodipine was determined by

Figure 3. Mean plasma concentrations of amlodipine in healthy male volunteers following a single oral dose of 10 mg.

Table III. Effect of Storage and Stability of Amlodipine in Plasma

Added concentration (ng/mL)	% Recovered	
	Month 1	Month 2
60	95.9	96.8
400	93.5	100.3
800	96.0	93.0

comparing the peak area of the drug obtained in plasma by the peak obtained in methanol of the same concentration. The relative recovery of the drug was obtained by the drug eluted from plasma to the actual added concentration. The results of the recovery studies are shown in Table II. The average of the absolute and relative recoveries was found to be 97.46% and 98.54%, respectively. The extraction recovery of the internal standard was 97.90% at the concentration used in the method (Table II).

Stability

Stability studies of plasma spiked with amlodipine (60, 400, and 800 ng/mL) were performed over a two-month period (Table III). Plasma samples were stored in the freezer at –20ºC until analysis. The results demonstrate that amlodipine can be stored frozen in plasma for two months without degradation (Table III).

Applications of the assay method

The validated method was applied to the determination of the drug content in commercially-available 10 mg amlodipine tablets (Istin). The average content of ten tablets was determined and found to be 10.05 mg ± 0.01 and ranged from 9.80–10.01 mg. The proposed assay was statistically compared with that of previously reported methods as well as the manufacturer's data by student's t-test and F-test at a 95% confidence level (10,21–23). The results indicated that there was no significant difference between the proposed method and other results regarding accuracy and precision.

The method was also applied for the determination of amlodipine in serum following oral administration of 10 mg of the drug to healthy volunteers. Figure 3 shows a typical serum concentration-time profile for amlodipine. The mean-plasma concentration-time curve of an amlodipine–plasma concentration reached a maximum of 8.0 ± 0.21 h after dosing with a level of 12.9 ± 0.9 ng/mL (Figure 3). These results are in agreement with previous reports (9,22,24). Although the sensitivity of the previously reported method (22,24) is enough for the analysis of amlodipine in pharmacokinetic studies, the sample preparation is long, and extensive sample clean-up in their methods is time-consuming. The developed technique is more selective; it has a simple sample extraction; the volume of serum for determination is greatly reduced; the retention times for both the drug and the internal standard were short; and it did not require the use of a more tedious and sophisticated analytical technique (22,24).

Conclusion

The HPLC method developed provides a quick, simple, reproducible, and sensitive assay for amlodipine in plasma, and it could be valuable in many applications, especially in content determination of pharmaceutical dosage forms and for pharmacokinetic studies after therapeutic doses. The advantages of the method are its short time of analysis and improved resolution.

Acknowledgments

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