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Short communication

Determination of amlodipine in human plasma by high-performance liquid chromatography with fluorescence detection

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Abstract

A sensitive and specific HPLC method has been developed for the assay of amlodipine in human plasma. The assay involves derivatization with 4-chloro-7-nitrobenzofurazan (NBD-Cl), solid-phase extraction on a silica column and isocratic reversed-phase chromatography with fluorescence detection. Nortriptyline hydrochloride was used as an internal standard. The assay was linear over the concentration range of 0.25–18.00 ng/ml. Both of the within-day and day-to-day reproducibility and accuracy were less than 11.80% and 12.00%, respectively. The plasma profile following a single administration of 10 mg amlodipine to a healthy volunteer was presented.

Keywords: Amlodipine; 4-chloro-7-nitrobenzofurazan

1. Introduction

Amlodipine, 2-[(2-aminoethoxy) methyl]-4-(2-chlorophenyl)-3-ethoxycarbonyl-5-methoxycarbonyl-6-methyl-1,4-dihydropyridine, is a calcium channel blocking agent of the dihydropyridine derivative which is used in the treatment of hypertension and angina [1]. It has low plasma concentrations because after oral administration, amlodipine has a long elimination half-life in humans ranging from 35 to 45 h due to the large volume of distribution (21 l/kg); and moreover it is highly bound (>95%) to plasma proteins [2]. Therefore, very sensitive and specific analytical methods for the assay of amlodipine plasma levels are necessary. Previously described gas chromatographic (GC) methods [3–5] involving the use of capillary column and electron capture detection have good sensitivity. However, the major disadvantage of GC methods is thermal decomposition of amlodipine at the high temperatures to the pyridine analogue which is presented already in plasma as metabolite. High-performance liquid chromatographic (HPLC) methods with ultraviolet [6] and electrochemical [7,8] detection have also been reported for the determination of amlodipine in human plasma. Although these assays are sufficiently sensitive, the drug has been measured by utilizing either a large plasma sample volume [6] or extraction procedures with a low recovery [7]. In the HPLC method described by Josefsson et al. [8], sample preparation has been considerably simplified by using solid-phase extraction. Yasuda et al. [9] have reported a liquid chromatography tandem mass spectrometric (LC–MS–MS) method for the determination of amlodipine in serum and it has been

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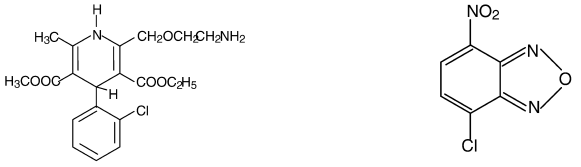

Amlodipine NBD-Cl

Fig. 1. Chemical structures of amlodipine and NBD-Cl.

modified by Marzo et al. [10]. The only disadvantage of these methods that have very low limits of quantitation (<0.1 ng/ml) is that an expensive LC–MS tandem system is used. Otherwise amlodipine has been estimated by high-performance thin layer chromatography–densitometry which requires 2 ml of human plasma sample [11]. The sensitivity of this method is 2 ng/ml.

In the present study, a selective and sensitive HPLC method with fluorescence detection for the assay of amlodipine in human plasma is reported. The method involves derivatization with 4-chloro-7-nitrobenzofurazan (NBD-Cl) which is a specific reagent for primary and secondary aliphatic amines (Fig. 1). A solid-phase extraction procedure was used on silica gel columns to separate the fluorescent NBD–amlodipine derivative from the endogenous amino acid derivatives. The method was tested for applicability in pharmacokinetic studies by assaying plasma samples of a healthy volunteer after the therapeutic doses of amlodipine.

2. Experimental

2.1. Materials and reagents

Amlodipine besylate and internal standard (I.S.), nortriptyline hydrochloride were kindly supplied by Pfizer (Istanbul, Turkey) and Mustafa Nevzat (Istanbul, Turkey), respectively. Methanol, ethyl acetate, *n*-heptane, isopropanol, hydrochloric acid, potassium chloride, sodium hydroxide, anhydrous sodium sulphate and boric acid were purchased from Merck (Darmstadt, Germany). All reagents used were of analytical grade except methanol which was HPLC grade. Water was doubly distilled. For solid-phase extraction, home-made small columns (25 mm×6 mm, I.D.) were prepared by filling silica gel of 0.063–0.200 mm particle size (Merck) into the pipette tip.

2.2. Solutions

Amlodipine besylate (equivalent to about 10 mg of free base) was dissolved with 2 ml of ethanol and diluted to 100 ml with water. This was diluted twice with water to give a stock solution of 0.1 μg/ml. A plasma standard at 25 ng/ml was prepared by spiking drug-free plasma with the stock solution of amlodipine. Subsequent serial dilution of this solution provided plasma calibration samples of 0.25, 0.75, 1.5, 2.5, 3.5 ng/ml and 3.0, 6.0, 9.0, 12.0, 15.0, 18.0 ng/ml.

The aqueous stock I.S. solution of nortriptyline was prepared at a concentration of 50 μg/ml and appropriate dilutions were made to obtain working solution of 50 ng/ml.

The stock solutions were stored at 4°C and were stable for a month. NBD-Cl solution (6 mg/ml) was prepared freshly in methanol.

Borate buffer was prepared by dissolving 0.620 g of boric acid and 0.750 g of potassium chloride in 100 ml of water. The pH was adjusted to 8.5 with 0.1 *M* sodium hydroxide solution and the volume was made up to 200 ml with water.

2.3. Instrumentation

The HPLC analysis was carried out on a system consisted of a Model 6000 A solvent-delivery system, equipped with a Rheodyne injection valve with a 20 μl loop (Waters, Milford, MA, USA). The column eluate was monitored by a Model 470 fluorescence detector (Waters) at an excitation wavelength of 459 nm and an emission wavelength of 528 nm. The detector was connected to a strip-chart recorder (Linear 355). Chromatographic separation was achieved isocratically on a 10 μm Bondapak C_{18} column (300 mm×3.9 mm, I.D.) fitted with a guard column (20 mm×3.9 mm, I.D.) packed with the same material of 5 μm particle size (Waters). The mobile phase, methanol–water (80:20) was filtered through a 0.50 μm filter (Millipore, Bedford, MA, USA), degassed and delivered at a flow-rate 0.8 ml/min at ambient temperature.

2.4. Sample preparation

Venous blood samples (2–3 ml) were collected into citrated tubes. Following centrifugation at 4500 *g* for 10 min, resultant plasma was separated and stored at −20°C until assayed.

A 0.5 ml of plasma sample was extracted with 5 ml of *n*-heptane–isopropanol (99:1) after addition 0.1 ml of I.S. working solution and 0.5 ml of 0.1 N NaOH solution. After mixing for 2 min on a vortex mixer and centrifugation for 10 min at 4500 *g*, 4.5 ml of organic phase was evaporated under nitrogen at 45°C.

The dried extracts were reconstituted for derivatization by adding 0.1 ml of borate buffer; then the mixture was derivatized by using the method [12] that the optimum conditions of the reaction have been reported. A 0.1 ml of NBD-Cl solution was added and the mixture was kept at 70°C for 30 min. After cooling, 0.1 ml of 0.1 N HCl was added and the contents were extracted with 3×1.5 ml of ethyl acetate. A 4 ml aliquot of the extract was evaporated to about 0.5 ml after drying on anhydrous sodium sulphate, then passed through a solid-phase extraction column and eluted with ethyl acetate to obtain a final volume of 4 ml. After evaporation the residue was dissolved in 200 μl of mobile phase and then injected into the HPLC system.

2.5. Linearity

Calibration curves were constructed by analysing a series of plasma calibration samples spiked with amlodipine to obtain concentrations ranging from 0.25 to 3.50 ng/ml and 3.00 to 18.00 ng/ml. The same concentration of the I.S. at 50 ng/ml was used for both calibration curves. The chromatograms were evaluated on the basis of amlodipine/I.S. ratios of the peak areas.

2.6. Extraction recovery

Recovery studies were performed by analysing plasma samples spiked with amlodipine at concentrations of 0.25, 3.00 and 18.00 ng/ml. Four replicate samples for each concentration were derivatized, extracted on silica gel and chromatographed. To determine the recovery, aqueous amlodipine solutions at the same concentrations were analysed by using the same procedure except solid-phase extraction and the results were compared with each other.

Extraction recovery of the I.S. was determined as described above independently of amlodipine at the concentration of 50 ng/ml.

2.7. Assay validation

The within-day and day-to-day precision and accuracy were determined by analysing plasma samples spiked with amlodipine at concentrations of 0.25, 1.50, 6.00, 15.00 and 18.00 ng/ml on four separate occasions. Determinations were performed with six replicates on the same day, as well as on separate days.

2.8. Applicability

After oral administration of 10 mg of amlodipine to a healthy 28-year-old woman volunteer on an empty stomach, venous blood samples of 2–3 ml were drawn in citrated tubes at 4, 6, 8, 10, 12, 13, 14, 30, 37, 61, 85 and 109 h. The blood was processed to plasma as described above and samples were stored at −20°C until analysis.

3. Results and discussion

Amlodipine was chromatographically separated from endogenous compounds of plasma using reversed-phase HPLC with fluorescence detection. Derivatization with NBD-Cl was carried out to enhance sensitivity and specificity.

Typical chromatograms of the blank human plasma and plasma sample spiked with amlodipine and I.S. are shown in Fig. 2A and B, respectively. Fig. 2C and D represent the chromatograms of plasma samples obtained at 12 and 85 h after a single oral dose of amlodipine from a healthy volunteer, respectively. The retention times of amlodipine and nortriptyline–NBD derivatives were 7 and 19 min, respectively, and the run time was 21 min. The chromatogram of blank plasma showed no interfering peaks having the same retention times as amlodipine or internal standard derivatives.

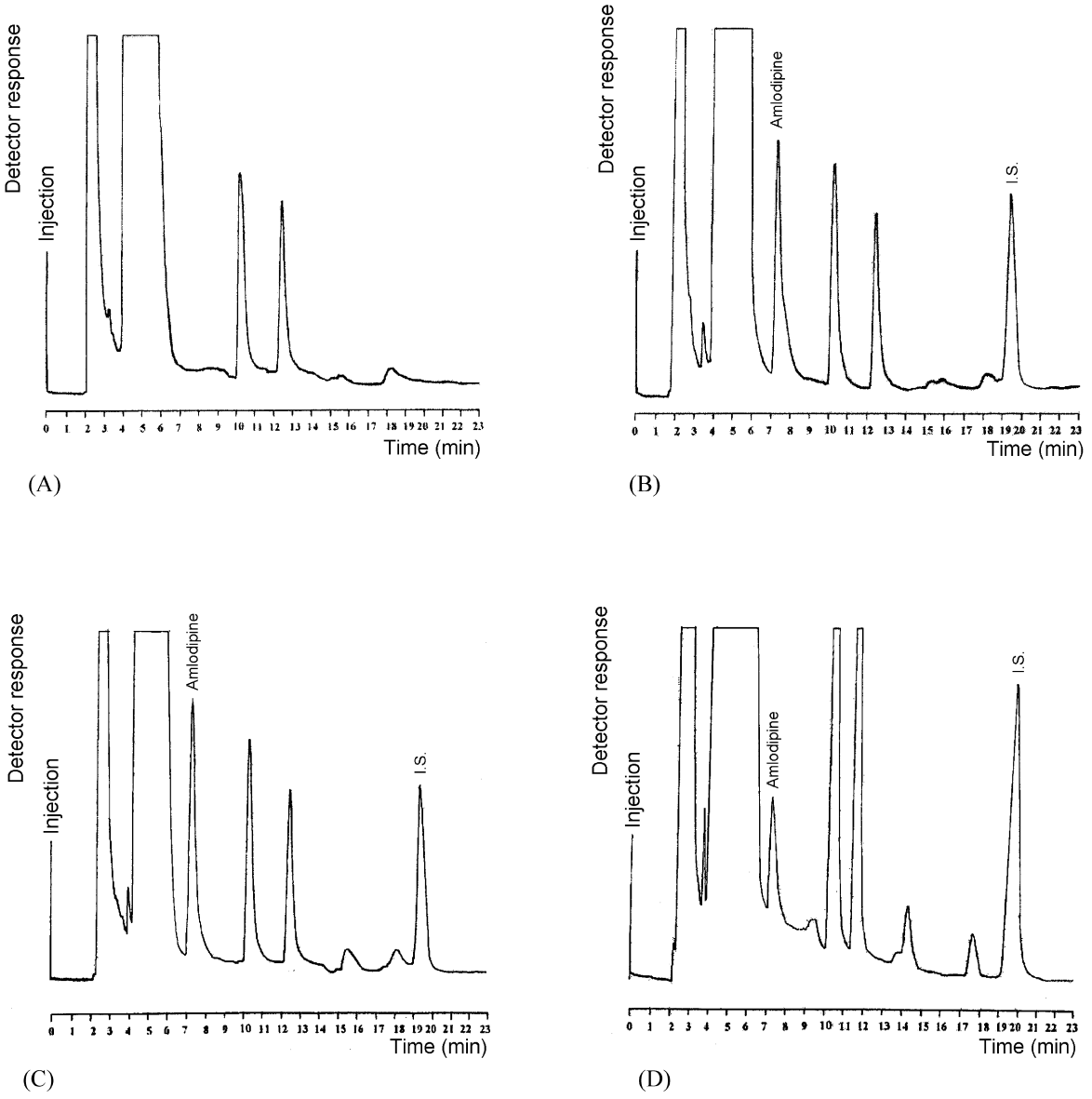

Fig. 2. HPLC chromatograms of (A) a blank human plasma (B) a human plasma spiked with 8 ng/ml of amlodipine and 50 ng/ml of the I.S., (C) and (D) plasma samples obtained at 12 and 85 h after oral administration of 10 mg of amlodipine from a healthy volunteer with 50 ng/ml of the I.S., respectively.

A solid-phase extraction procedure seemed to be necessary after derivatization to reduce the interfering products and hence to minimize background signal.

Linear detector response for the peak-area ratios of amlodipine to I.S. was observed in two concentration ranges between 0.25 and 3.50 and 3.00 and 18.00 ng/ml plasma with a correlation coefficients of 0.9985 and 0.9996, respectively. The linearity was sufficient in covering the range of concentrations expected to be found in plasma after oral administration of 10 mg of amlodipine. Under the experimental

Table 1
Extraction recovery of amlodipine and the internal standard from spiked plasma samples ($n=4$)

Drug	Nominal concentration (ng/ml)	Measured concentration (mean±SD) (ng/ml)	RSD (%)	Recovery (%)
Amlodipine	0.25	0.18±0.021	11.54	72.00
	3.00	2.62±0.159	6.05	87.33
	18.00	16.84±0.330	1.96	93.56
Internal standard	50.00	47.76±0.593	1.24	95.52

conditions used, the lower limit of detection (LOD) was 0.125 ng/ml at a signal-to-noise ratio of 3. The lower limit of quantitation (LOQ) was found to be 0.25 ng/ml.

The extraction recoveries of amlodipine were in the range 72.00–93.56% at three different concentrations and that of the I.S. was 95.52% at the concentration used in the method (Table 1).

The results of the assay validation study are presented in Table 2. The within-day and day-to-day reproducibilities expressed as relative standard deviation (RSD) were found to be 1.11–10.74% and 2.63–11.80%, respectively, indicating good precision. The accuracy of the method expressed as relative mean error (RME) was below 12.00% which was shown to be satisfactory. The precision and the accuracy were 10.74% and 8.00%, respectively, at the LOQ.

Concomitant medications such as diltiazem, digoxine, amiodarone, enalapril, dipyridamole, pindolol and hydrochloridethiazide did not interfere with the method.

The stability of the extracts was verified over a 72 h period. Additionally the stability of amlodipine prolonged storage at −20°C in plasma was verified after 6 months.

To test the applicability of the presented HPLC method in pharmacokinetic studies a pilot experiment was performed with a healthy volunteer. In this study, plasma concentrations were calculated from the regression equations of the calibration curves. Fig. 3 shows the plasma concentration–time profile of amlodipine after a single 10 mg oral administration. A maximum plasma concentration of 10.60 ng/ml (C_{max}) was reached at 12 h (t_{max}) the elimination half-life of the drug ($t_{1/2}$) and area under the curve (AUC) were found to be 40.7 h and 258.68 ng.h/ml, respectively. These pharmacokinetic parameters are in good agreement with that found previously [2].

Table 2
Within-day and day-to-day precision and accuracy of amlodipine in plasma[a]

Nominal concentration (ng/ml)	Measured concentration (mean±SD) (ng/ml)	RSD (%)	RME (%)
Within-day			
0.25	0.27±0.029	10.74	+8.00
1.50	1.58±0.127	8.04	+5.33
6.00	5.73±0.211	3.68	−4.50
15.00	14.77±0.245	1.66	−1.53
18.00	18.27±0.203	1.11	+1.50
Day-to-day			
0.25	0.28±0.033	11.80	+12.00
1.50	1.36±0.139	10.22	−9.33
6.00	6.22±0.365	5.87	+3.67
15.00	14.58±0.383	2.63	−2.80
18.00	17.16±0.521	3.03	−4.67

[a] Results of the six replicates of amlodipine obtained on four occasions.

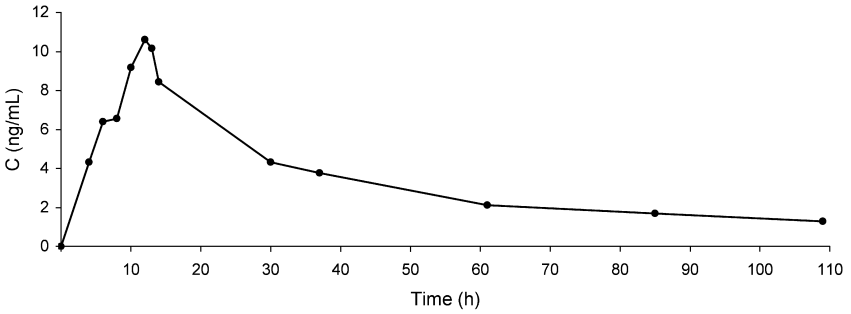

Fig. 3. Plasma concentration–time profile of amlodipine in a healthy volunteer after a single oral administration of 10 mg amlodipine.

The proposed method offers some advantages over earlier methods. The extraction recovery is better (72.00–93.56%) than those of the studies reported by Beresford et al. [3] and Shimooka et al. [7], in which the recoveries are 60 and 50.2%, respectively. The present method requires as little as 0.5 ml of plasma whereas the sample volume has varied between 1 to 5 ml in previous methods [3–11]. On the other hand, the mobile phase in the present method consists methanol–water instead of buffered systems used in previously reported HPLC methods [6–11]. Therefore flushing of the column after analysis is not required. Besides, the detector used in the present study is commonly available in a laboratory and not expensive as the MS-tandem system [9,10].

The proposed HPLC method with fluorescence detection is sufficiently sensitive and reliable to measure amlodipine at concentration as low as 0.25 ng/ml in plasma. In addition the method showed high selectivity, precision and accuracy for the use in bioavailability and pharmacokinetic studies of amlodipine.

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