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Analysis of amlodipine in serum by a sensitive high-performance liquid chromatographic method with amperometric detection

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Abstract: An analytical method for a new calcium channel blocking agent, amlodipine, has been developed using high-performance liquid chromatography with electrochemical detection. No compound modification is required for detection and the calibration curve in spiked sera is linear and reproducible over the range 0.2–2.0 ng ml^{-1}. The method has been applied successfully to pharmacokinetics studies in rats and also can be used for other dihydropyridine compounds such as nifedipine and nicardipine.

Keywords: *Amlodipine; calcium channel antagonist; reversed-phase liquid chromatography; amperometric detection.*

Introduction

Amlodipine [1, 2], a new calcium channel blocking agent of the dihydropyridine family, lowers blood pressure in hypertensive patients with coronary artery disease at doses as low as 10 mg [3]. Usually, dihydropyridine drugs are rapidly oxidized enzymatically to pyridine metabolites after oral dosing. Therefore, the determination of unchanged drug concentration in serum requires an assay with a sensitivity capable of measuring levels below 1 ng ml^{-1}.

Several analytical methods for quantifying dihydropyridine calcium antagonists in biological fluids have been reported, such as packed column or capillary gas chromatography (GC) with electron-capture detection [4–7], GC with electron-impact mass spectrometry (EI–MS) [8–9], and high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection [6, 7, 10, 11].

However, in GC methods the thermal instability of the drugs under GC conditions represents a serious problem, since pyridine analogues are formed in non-reproducible amounts, many of which are already present in plasma as metabolites [6, 7, 9]. Furthermore, the GC method described for amlodipine [12] employed a dropping needle injector which precluded automated assay. In the HPLC–UV method the sensitivity is inadequate owing to the low absorbance of the analytes.

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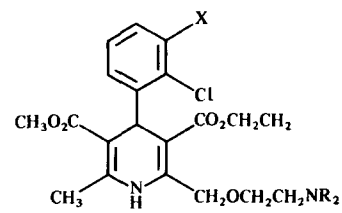
This paper describes a sensitive and selective HPLC–amperometric method for direct quantification of a dihydropyridine drug, amlodipine, in the concentration range 0.1–2.0 ng ml^{-1} in human serum.

The amperometric assay method for dihydropyridine drugs is based on measuring the electric current following electrochemical oxidation to pyridine analogues in the detector cell.

Experimental

Reagents and materials

Amlodipine besylate [3-ethyl-5-methyl-2-(2-aminoethoxymethyl)-4-(2-chlorophenyl)-1,4-dihydro-6-methyl-3,5-pyridinedicarboxylate besylate (**I**), its *N,N*-dimethyl analogue (**II**) and 2,3-dichlorophenyl analogue (**III**), and nifedipine were supplied by Central Research, Pfizer Ltd (Sandwich, UK). Nicardipine (hydrochloride salt) was purchased from Sigma Chemical Company (St. Louis, MO, USA).



(**I**) X = H, R = H
(**II**) X = H, R = CH_3
(**III**) X = Cl, R = H

Boric acid, citric acid, sodium dihydrogen phosphate, sodium ethylenediaminetetraacetate (EDTA), acetonitrile and diethyl ether were obtained from Wako Pure Chemical Industries (Osaka, Japan), PIC B-8 solution containing sodium octane sulphonate from Millipore Corporation (Milford, MA, USA) were all analytical reagent grade.

Diethyl ether was used after shaking with 0.05 M potassium permanganate for 5 min followed by washing with distilled water, 1.0 M sodium hydroxide and 0.2 M citric acid prior to glass distillation. The solutions of 0.2 M borate buffer (pH 10) and 0.2 M citric acid were extracted by distilled ether prior to use.

The standard solutions of amlodipine were prepared by dissolving the besylate salt in distilled water and diluting to 0.2–2.0 ng/0.1 ml as free base. The *N,N*-dimethyl analogue was used as internal standard (IS) and the 2,3-dichlorophenyl analogue (DCPA, **III**) was added to minimize the adsorption of amlodipine to glass and to binding sites in the injection port and on the column. These compounds were dissolved in water and diluted to 50 and 500 ng ml^{-1}, respectively.

Sample preparation

Into a 10-ml centrifuge tube, 1 ml of serum, 0.1 ml of IS solution and 0.1 ml solution of **III** were added, the mixture made alkaline with 2 ml of 0.2 M borate buffer solution (pH 10), and extracted with 6 ml of diethyl ether on an auto-shaker for 5 min. After centrifugation, the solvent was decanted into another 10 ml centrifuge tube, 2 ml of 0.2 M borate buffer solution (pH 10) was added and washing was conducted as above. The resultant ether fraction was shaken for 5 min after 0.1 ml of 0.2 M citric acid and 50 μl of methanol had been added. Following centrifugation, the organic solvent layer was aspirated and discarded. Aliquots (80 μl) of the acidic aqueous fraction were

assayed by HPLC–amperometry. Samples at this stage could be held for at least 3 days when stored in the dark at 4°C, as demonstrated by the same peak height ratio of amlodipine to IS measured on days 1 and 3. In the same way, amlodipine in serum was stable for at least 4 months when stored at −20°C.

HPLC–amperometry

An analytical liquid chromatograph (TRI ROTAR model, Japan Spectroscopic Co. Ltd, Tokyo, Japan) equipped with a 4.6 × 150 mm column packed with octadecyl-bonded silica, particle size 5 μm (Cosmosil 5 C_{18}-P Nakarai Chemicals, Kyoto, Japan) and an amperometric detector (E-502 model, Irica Instruments, Kyoto, Japan). A mobile phase of 0.05 M phosphate buffer solution (pH 3.1)–acetonitrile (65:35, v/v) containing sodium octane sulphonate and EDTA at final concentrations of 0.005 M and 5 mg l^{-1}, respectively, was used at a flow rate 1 ml min^{-1} after filtration and helium sparging. The detector, equipped with glassy carbon as working electrode and Ag/AgCl as reference electrode, was set at a potential +1.0 V. The signal was monitored by Shimadzu Chromatopac C-R3A integrator at a sensitivity of 0.37 nA mV^{-1} using a chart speed of 3 mm min^{-1}. Calibration curves were prepared by determining the peak height responses from known amounts of amlodipine and IS added to control serum, and fitted to the equation $y = ax + b$ by least-squares regression. The concentrations in the unknown samples were subsequently calculated using the calibration curve.

Assay linearity was demonstrated over the 0.2–2 ng ml^{-1} serum concentration range by HPLC of standard samples (0.2–2 ng/0.1 ml) prepared by direct dilution with 0.2 M citric acid and spiked samples after processing according to the sample preparation method. The amount of IS was held constant at 5 ng/sample.

The assay sensitivity and specificity to amlodipine in human serum was assessed by conducting assays of serum samples in six healthy male volunteers given an oral dose of amlodipine in the form of a 2.5 mg tablet. Serum samples were stored at −20°C until analysed.

Results and Discussion

Amperometric detector response of amlodipine at various applied potentials is shown in Fig. 1. The highest response was achieved at more than +1.15 V. However, in view of the ratio of signal to noise level (S/N), 1.0 V was chosen. Lower noise level, reduction of solvent peak tailing and better sensitivity were also achieved by helium sparging of the mobile phase and by adding a small amount of EDTA to the mobile phase.

A linear detection response for peak height ratio was observed between 0.2–2.0 ng/0.1 ml in standard samples prepared by diluting amlodipine with 0.2 M citric acid, directly ($y = 0.40x + 0.01$; correlation coefficient, 0.9999) and the standard curve was parallel to calibration curves obtained from fortified sera ($y = 0.39x - 0.01$; correlation coefficient, 0.9993). This result suggests that amlodipine and IS have the same distribution coefficient in the extraction systems for the assay. Assay recoveries for amlodipine and IS, determined by comparing the peak height response from amlodipine (0.2–2 ng ml^{-1}) and IS (5 ng ml^{-1}) with processed spiked sera, were 50.2 ± 3.4% ($n = 12$) and 58.3 ± 6.3% ($n = 12$), respectively. At the last step of sample preparation, the low recoveries of amlodipine and IS are mainly attributed to their low distribution to the aqueous layer (0.2 M citric acid) containing methanol from the ether layer. Reproducibility was calculated by normalizing the observed ratios over the assay

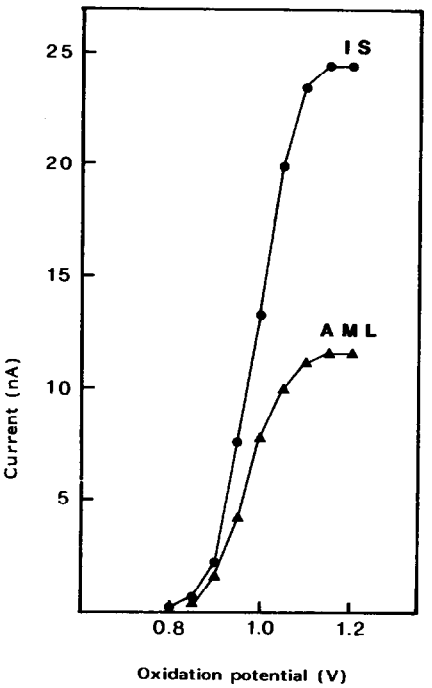


Figure 1
Voltammograms obtained after injecting 80 ng of amlodipine (AML) and 160 ng of the internal standard (IS) at various applied potentials.

Table 1
Calibration curve prepared from control sera fortified with amlodipine (0.2–2.0 ng ml^{-1}) and IS (5.0 ng ml^{-1})

Fortification level (ng ml^{-1})	Number of samples	Mean of peak height ratio ± standard deviation
2.0	3	0.765 ± 0.061
1.0	3	0.390 ± 0.045
0.4	3	0.131 ± 0.006
0.2	3	0.076 ± 0.002

range (Table 1) to 1 ng ml^{-1}. A relative standard deviation (RSD) of 10.2% was found ($n = 12$).

Chromatograms of amlodipine are shown in Fig. 2. Elution is in the order amlodipine, IS and the dichlorophenyl analogue (III).

The sensitivity for this assay, which is about 0.1 ng ml^{-1} in view of the S/N ratio of 5 for 0.2 ng ml^{-1}, allows the measurement of serum levels of amlodipine in patients given clinical doses.

The serum concentrations in human male volunteers after a 2.5 mg dose were investigated using the above assay method and are shown in Fig. 3. Mean peak serum level (C_{max}), the time at peak (T_{max}), the area under the serum concentration curve (AUC) and the half life of serum concentration ($T_{½}$) were 1.64 ng ml^{-1}, 7.3 h, 68.1 ng h ml^{-1} and 33.3 h, respectively. Faulkner *et al.* [13] have reported pharmacokinetics in human volunteers following a 10 mg oral dose whose plasma levels had been determined

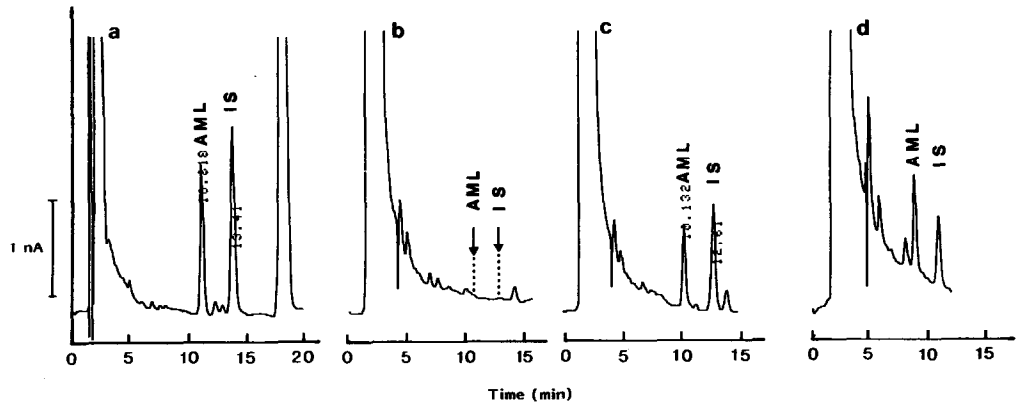


Figure 2
Chromatograms showing the separation of amlodipine (AML) and the internal standard (IS) and dichlorophenyl analogue (DCPA, III) (a), control serum (b), control serum fortified with 2 ng ml^{-1} AML and 5 ng ml^{-1} (IS) (c) and serum 8 h after a 2.5 mg dose of AML with 2.5 ng of IS (d).

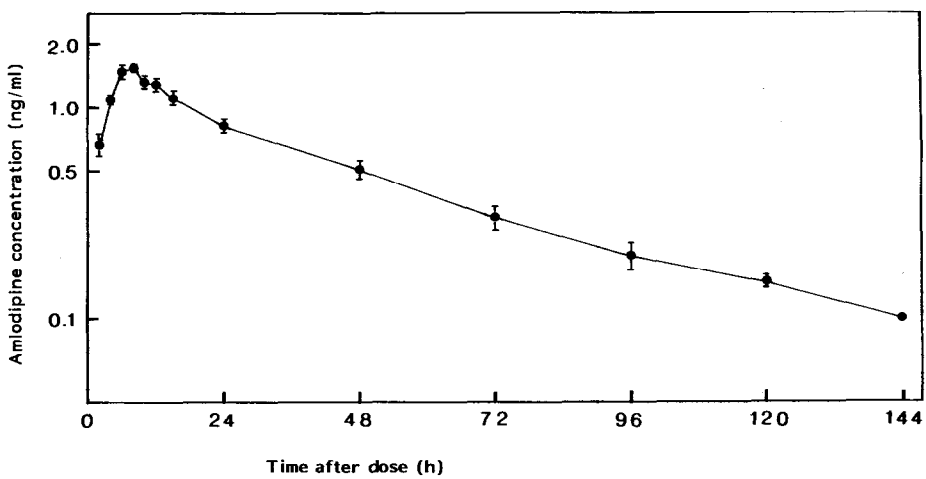


Figure 3
Serum concentrations of amlodipine in healthy male volunteers following a single oral dose of 2.5 mg. Vertical bars represent standard errors of the mean, $n = 6$ ($n = 5$ at 120 h and $n = 3$ at 144 h after dose when some serum levels were not detected, i.e. <0.1 ng ml^{-1}).

by capillary GC–EC method (C_{max} 5.9 ng ml^{-1}, T_{max} 7.6 h, AUC 238 ng h ml^{-1}, $T_{½}$ 35.7 h). Results that are in good agreement with the current data.

In the present HPLC system, amlodipine, nifedipine, and nicardipine as dihydropyridine calcium antagonists are well separated and could be determined with comparable sensitivity (Fig. 4).

Conclusion

The method described provides a sensitive and specific assay for amlodipine in serum and is suitable for pharmacokinetic studies after therapeutic doses. HPLC with

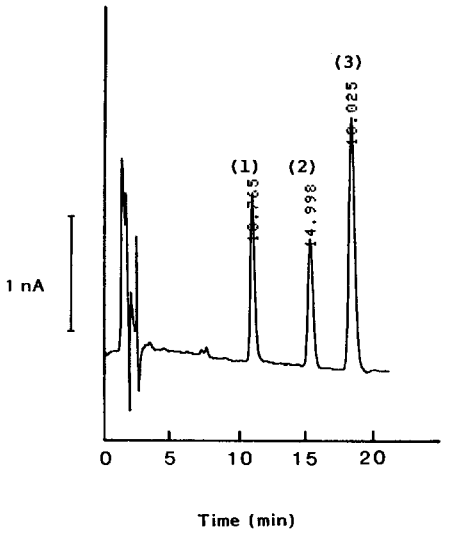


Figure 4
Chromatogram showing the separation and sensitivity of amlodipine (1), nicardipine (2) and nifedipine (3); 1.6 ng of each compound.

amperometric detection may be useful for analysing other dihydropyridine calcium antagonists with the advantage of no interference from pyridine metabolites which are not detected by amperometry under oxidative conditions. An autosampler can be used for sample injection to provide a high sample throughput.

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References

[1] A. P. Beresford, M. J. Humphrey and D. A. Stopher, *Br. J. Pharmac.* **85**, 333 (1985).
[2] J. E. Arrowsmith, S. F. Campbell, P. E. Cross, J. K. Stubbs, R. A. Burges, D. G. Gardiner and K. J. Blackburn, *J. Med. Chem.* **29**, 1696–1702 (1986).
[3] M. A. Frais, B. Silke, S. P. Verma, S. Sharma, G. Reynolds, N. Jackson and S. H. Taylor, *Herz* **11**(6), 351 (1986).
[4] S. R. Hamann and R. G. McAllister Jr, *Clin. Chem.* **29**, 158–160 (1983).
[5] L. J. Lesko, A. K. Miller, R. L. Yeager and D. C. Chatterji, *J. Chromatogr. Sci.* **21**, 415–419 (1983).
[6] J. Dokladalove, J. A. Tykal, S. J. Coco, P. E. Durkee, G. T. Quercia and J. J. Korst, *J. Chromatogr.* **231**, 451–458 (1982).
[7] G. J. Krol, A. J. Noe, S. C. Yeh and K. D. Raemsch, *J. Chromatogr.* **305**, 105–118 (1984).
[8] S. Higuchi and Y. Shiobara, *Biomed. Mass Spectrom.* **5**, 220–223 (1978).
[9] S. Higuchi and S. Kawamura, *J. Chromatogr.* **223**, 341–349 (1981).
[10] P. Pietta, A. Riva and P. Biondi, *J. Chromatogr.* **210**, 516–521 (1981).
[11] T. Sadanaga, K. Hikida, K. Tameto, Y. Matsushima and Y. Ohkura, *Chem. Pharm. Bull.* **30**, 3807–3809 (1982).
[12] A. P. Beresford, P. V. Macrae, D. A. Stopher and B. A. Wood, *J. Chromatogr.* **420**, 178–183 (1987).
[13] J. K. Faulkner, D. McGibney, L. F. Chasseaud, J. L. Perry and I. W. Taylor, *Br. J. Clin. Pharmac.* **22**, 21–25 (1986)

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