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Detection and determination of total amlodipine by high-performance thin-layer chromatography: a useful technique for pharmacokinetic studies

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Abstract

A novel analytical method for determination of the total plasma levels (free and protein bound) of the calcium channel blocking agent amlodipine has been developed using a high-performance thin-layer chromatographic (HPTLC) procedure. Detection and quantitation were performed without internal standards. In previously described methods for the estimation of amlodipine by gas chromatography and high-performance liquid chromatography, only the free levels in plasma and serum were quantified at 7% of the total amlodipine level, with the remaining 93% bound to plasma protein and tissue. The present method employs proteolysis of the plasma proteins by incubating plasma for 2 h in pepsin solution. After proteolysis amlodipine is extracted and a known amount of the extract is spotted on precoated silica-gel 60 F_{254} plates using a Camag Linomat IV autosampler. Amlodipine was quantified using a dual-wavelength TLC scanner. The method provides a direct estimate of the total amlodipine present in plasma.

1. Introduction

Amlodipine, 2 - [(2 - aminoethoxy)methyl] - 4 - (2 - chlorophenyl) - 3 - ethoxycarboxyl - 5 - methoxycarbonyl-6-methyl-1,4-dihydropyridine (Fig. 1), is a calcium channel blocking agent of the dihydropyridine family [1,2], which is used to lower blood pressure in hypertensive patients.

Several analytical methods for quantifying dihydropyridine calcium antagonists in biological fluids have been reported, e.g. high-performance liquid chromatography (HPLC) with amperometric detection [3], HPLC with ultraviolet (UV) detection [4–7], packed-column or capillary gas chromatography (GC) with electron-capture detection [8,9] and GC with electron-

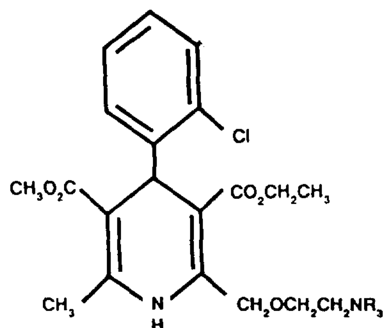

Fig. 1. Chemical structure of amlodipine.

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impact mass spectrometry (EI-MS) [10,11]. Enantiomers of amlodipine were detected by GC after derivatisation [12].

In the HPLC-amperometric method the detection of amlodipine was performed without compound modification and a low concentration range of 0.2 to 2.0 ng ml^{-1} was achieved by using an internal standard and the recovery of drug was 50.2 ± 3.4% [3]. However, in the HPLC-UV method the sensitivity is inadequate due to the low absorbance of the analytes and in the GC methods the thermal instability of the drug under GC conditions presents problems as many pyridine analogues are formed in non-reproducible amounts, many of which are present already in plasma as metabolites [4,5,11].

Dihydropyridine calcium channel blocking agents, the class to which amlodipine belongs, usually are rapidly oxidized enzymatically to pyridine metabolites after oral dosing [3]. Therefore, determination of the level of unchanged drug in serum requires an assay with high sensitivity. This paper describes a reliable and selective high-performance thin-layer chromatographic (HPTLC) method which enables the determination of the total plasma concentration of amlodipine (both free and protein bound) when the drug was given in therapeutic doses of 10 mg to healthy volunteers. The method was also used to obtain comparative pharmacokinetic information about the drug in healthy volunteers for two marketed tablet preparations.

2. Experimental

2.1. Reagents

Amlodipine besylate was obtained from Promopharma Establishment (Switzerland) and pepsin (0.35%, pH 1.2, 108 U/mg, p.7125 from Sigma, St. Louis, MO, USA) was used for proteolysis. Two marketed preparations of 5-mg amlodipine tablets were used for comparative pharmacokinetic studies. Borate buffer (pH 10.0) and dichloromethane (analytical grade) were used for extraction, and chloroform, methanol and acetic acid (analytical grade) were used for developing TLC plates (Silica gel 60 F_{254}, Art 5554, Dc-Alufolien, Kieselgel 60 F_{254}, E. Merck, Darmstadt, Germany).

2.2. Preparation of standards

A stock solution of amlodipine was prepared in methanol at a concentration of 0.1 mg/ml. Standard solutions were obtained by diluting the stock solution to concentrations ranging from 20 to 200 ng/ml.

2.3. Plasma sample preparation

A 2-ml volume of human plasma (plasma containing a known amount of the drug, drug-free plasma and unknown plasma) was transferred to a 10-ml graduated glass centrifuge tube. Plasma was collected from healthy male human volunteers after giving written consent. All volunteers were maintained on standard diet during experimentation and ideal conditions at our clinical pharmacology unit.

Incubation

All test samples were incubated for 2 h at 36°C with 2 ml of pepsin (proteolytic enzyme) solution. The incubation time of 2 h was found to be optimum for maximum proteolytic action and complete release of amlodipine. Percent recoveries of amlodipine from plasma incubated with pepsin for 1, 2 and 3 h were found to be 84.2, 98.3 and 98.5%, respectively.

Precipitation

After incubation, the total plasma protein was precipitated by the addition of 2 ml of 0.2 *M* borate buffer solution (pH 10), and extracted with 2 ml of dichloromethane by vortex-mixing for 2 min. The tubes were centrifuged at 700 *g* for 10 min. After centrifugation, the supernatant was decanted into another 10-ml centrifuge tube and extracted again with 2 ml of dichloromethane and centrifuged. The combined dichloromethane extracts were evaporated to dryness, in the dark, at 25 ± 2°C.

2.4. Instrumentation and chromatographic conditions

All concentrated and dried extracts obtained by the above precipitation method were redissolved in 100 μl of dichloromethane by vigorous vortex-mixing and 50-μl aliquots of the samples were spotted on TLC plates with the help of a Camag Linomat IV autosampler. Amlodipine (100 ng) reference standard was separately spotted on each TLC plate as external standard. The TLC plates were developed (10 cm) in a glass chamber (25 × 25 × 12 cm), first with chloroform, followed by chloroform–methanol–acetic acid (15:2.5:0.4, v/v) (5 cm) where the spots were clearly separated as concentrated zones. The chambers were saturated with solvent system before development and the TLC plates were dried completely by hot air after development in both the solvent systems. The spots of amlodipine were visualized under UV (λ_{max} 365 nm). It was observed that amlodipine moved at R_f 0.30 and the spots remained stable for 24 h when kept in the dark. Determination of amlodipine was done by scanning the fluorescence of the TLC plates with a Shimadzu dual-wavelength scanner (Model CS 930, Shimadzu, Kyoto, Japan).

2.5. Quantitation

Calibration curves were obtained daily by plotting the area under the peak of amlodipine against the concentrations over the range 0–100 ng. The area under the peaks of unknown samples were compared with calibration curves of standards.

2.6. Method validation

The recovery of amlodipine from plasma was determined by comparing peak areas obtained from plasma spiked with amlodipine at concentrations of 2, 5, 10, 20, 50 and 100 ng/ml with the peak areas obtained from standards. The intra-day precision was completed by analyzing plasma samples in triplicate spiked with amlodipine at 2, 10, 50 and 100 ng on the same day. The inter-day precision was determined by analyzing 2, 10 and 50 ng standards simultaneously with unknown plasma daily for 5 days and also by comparing with the calibration curve. The linearity of the detector response was tested by spotting standards in triplicate for each concentration ranging between 2 and 100 ng.

3. Results

The peak area was observed to be dependent on the amount of standard amlodipine, and a linear relationship ($r = 0.998$) was found between the peak areas of amlodipine at various concentrations over the range 2–100 ng (Fig. 2). The solvent system used for development of the plates produced no interfering peaks in the area under the curve and all other compounds were distinctly separated. The R_f value of amlodipine under the conditions used was found to be 0.30 ± 0.05 and spots were quantified at λ_{max} 365 nm. The accuracy, precision and reliability of the procedure were ascertained by adding known concentrations of drug to drug free plasma and analyzing three samples of each concentration by the method described for extraction (Table 1). The recovery of amlodipine in the extraction procedure from 2 ml of plasma was found to be $98 \pm 3.9\%$ ($n = 5$). The intra-day and inter-day precisions are given in Table 2.

In order to verify the applicability of this

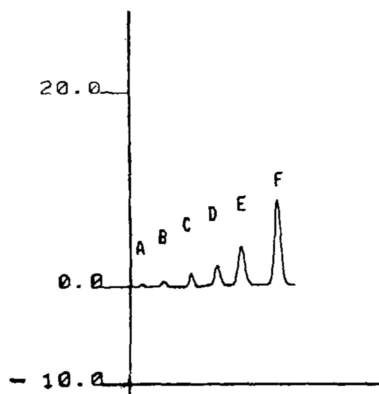

Fig. 2. Linear response of amlodipine. A = 2 ng, B = 5 ng, C = 10 ng, D = 20 ng, E = 50 ng, F = 100 ng.

Table 1
Accuracy and precision of HPTLC method for determination of amlodipine in plasma

Concentration added (ng/ml)	Concentration detected (mean ± S.D., $n = 5$) (ng/ml)	C.V.[a] (%)	Accuracy[b] (%)
2	2.15 ± 0.24	11.16	109.68
5	5.07 ± 0.50	10.00	103.46
10	10.30 ± 1.23	11.94	105.10
20	19.80 ± 0.75	3.80	101.02
50	50.35 ± 2.47	4.92	102.75
100	98.00 ± 3.91	3.99	100.00

[a] Coefficient of variation.
[b] After correction for recovery.

method to different formulations, experiments with two marketed amlodipine tablet preparations were conducted in healthy volunteers at a dose of 2 × 5 mg (therapeutic dose) per volunteer (Fig. 3). Various pharmacokinetic parameters of two samples were analyzed (Table 3) and found to be bioequivalent. The HPTLC method was also used to quantify the amount of amlodipine in two marketed tablet preparations.

4. Discussion

The proposed HPTLC method can measure the total concentration of plasma amlodipine (both free and plasma bound) at a therapeutic dose of 10 mg per oral single-dose administration.

A comparison of the proposed HPTLC method with a previously published HPLC method [3] was performed by using the plasma samples from the recovery studies. Two methods were tried, first, the sample was processed by the HPLC method and subsequently spotted on the TLC plate. Second, the sample was processed by the proposed HPTLC method and then injected onto the HPLC column and eluted with the

Table 2
Precision data of the HPTLC assay for amlodipine

Concentration added (ng)	Peak area[a] (mean ± S.D.)	Coefficient of variation (%)
Intra-day[b] ($n = 5$)		
2	632.33 ± 34.97	5.53
10	3040.90 ± 138.49	4.55
50	14 799.17 ± 738.13	4.99
100	30 814.93 ± 1235.20	4.01
Inter-day[c] ($n = 5$)		
2	672.93 ± 28.61	4.25
10	2974.03 ± 172.79	5.81
50	14 552.20 ± 790.39	5.43

[a] Calculated for total concentration (integrated value).
[b] 1 ml of plasma was spiked at the indicated concentrations.
[c] 2 ml of plasma were spiked at the indicated concentrations.

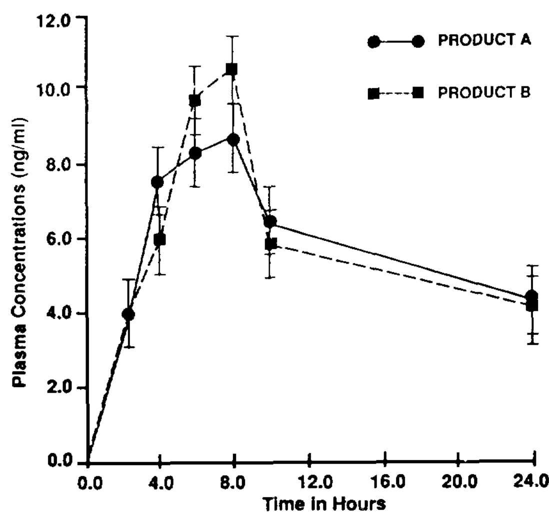

Fig. 3. Plasma concentration after oral administration of 2 × 5 mg of amlodipine from two marketed products determined by HPTLC. Each point represents mean ± S.E. values ($n = 6$, cross-over design).

Table 3
Pharmacokinetic parameters of amlodipine tablets (2 × 5 mg) in human volunteers

Parameter	Product A ($n = 6$)	Product B ($n = 6$)	'P' value (A:B)
C_{max}	10.26 ± 1.01	10.84 ± 0.87	NS
T_{max}	6.00 ± 0.73	7.00 ± 0.45	NS
$AUC_{0\rightarrow 24h}$ (ng/ml/h)	135.84 ± 11.05	131.37 ± 11.51	NS
$K_{el, 0\rightarrow 24h}$ (h^{-1})	0.02655 ± 0.0047	0.02462 ± 0.0046	NS
$T_{1/2}$B (h)	21.135 ± 1.139	22.015 ± 1.120	NS

Data obtained by cross-over design and giving mean ± S.E.M. values. n = No. of volunteers, NS = not significant.

reported solvent system. Neither method succeeded in quantifying the drug. This is because only a very small amount of drug was extracted in the first method, and a large number of interfering peaks and poor separation were observed in the second method. The HPLC-amperometric method [3] reported previously, though more sensitive to detect amlodipine, can be used to detect drug which is only 5–7% available in its free form in plasma.

However, by using pepsin, a proteolytic enzyme used for denaturation of plasma proteins [13,14], and processing by the proposed HPTLC method it is possible to quantify the total levels of amlodipine in plasma (free and protein bound) by spotting and detecting the samples without using an internal standard. A similar HPTLC method for the detection of total plasma astemizole was reported earlier [15]. The proposed method can also be used to accurately determine amlodipine in tablets without interference from the excipients. Unlike the GC method reported for the detection of both enantiomers of amlodipine [12], the proposed HPTLC method can not be used for enantiomer separation as no chiral reagent is used for derivatisation.

5. Conclusions

The proposed HPTLC method for the estimation of amlodipine in plasma has certain advantages over other reported methods; e.g. (1) It gives a clear picture of total drug present after absorption (both free and protein bound amlodipine) and thus has direct clinical relevance; (2) It is economical and faster than previously published methods. On a single plate at least ten to twelve samples can be analyzed in 5–6 h. (3) Unlike earlier methods, this method does not require an internal standard and quantitation can be done using reference drug as external standard. (4) The recovery of the drug is improved compared with the HPLC method (98 ± 3.9%). (5) The method describes a sensitive and specific assay for amlodipine in plasma and is suitable for pharmacokinetic studies after therapeutic doses.

References

[1] A.P. Beresford, M.J. Humphrey and D.A. Stopher, *Br. J. Pharmacol.*, 85 (1985) 333.
[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 (1986) 1696.
[3] K. Shimooka, Y. Sawada and H. Tatematsu, *J. Pharm. Biomed. Anal.*, 7 (1989) 1267.
[4] J. Dokladalove, J.A. Tykal, S.J. Coco, P.E. Durkee, G.T. Quercia and J.J. Korst, *J. Chromatogr.*, 231 (1982) 451.
[5] G.J. Krol, A.J. Noe, S.C. Yeh and K.D. Raemsch, *J. Chromatogr.*, 305 (1984) 105.
[6] P. Pietta, A. Riva and P. Biondi, *J. Chromatogr.*, 210 (1981) 516.
[7] T. Sadanaga, K. Hikida, K. Tameto, Y. Matsushima and Y. Ohkura, *Chem. Pharm. Bull.*, 30 (1982) 3807.
[8] S.R. Hamann and R.G. McAllister, *J. Clin. Chem.*, 29 (1983) 158.
[9] L.J. Lesko, A.K. Miller, R.L. Yeagen and D.C. Chatterji, *J. Chromatogr. Sci.*, 21 (1983) 415.
[10] S. Higuchi and Y. Shiobara, *Biomed. Mass Spectrom.*, 5 (1978) 220.

[11] S. Higuchi and S. Kawamura, *J. Chromatogr.*, 223 (1981) 341.

[12] F. Scharpf, K.D. Riedel, H. Laufen and M. Leitold, *J. Chromatogr. B*, 655 (1994) 225.

[13] D.T. Plummer, *An Introduction to Practical Biochemistry*, Tata-McGraw-Hill Publishing Company, New York, NY, 1985. Ch. 5, p. 128.

[14] S.W. Fox and J.F. Foster, *Introduction to Protein Chemistry*, John Wiley and Sons, New York, NY, 1957, Ch. 22, p.405.

[15] S. Mangalan, R.B. Patel, T.P. Gandhi and B.K. Chakravarthy, *J. Chromatogr.*, 567 (1991) 498.