JOURNAL OF
CHROMATOGRAPHY B:
BIOMEDICAL APPLICATIONS

ELSEVIER

Journal of Chromatography B, 678 (1996) 360–364

Short communication

Automated gas chromatographic assay for amlodipine in plasma and gingival crevicular fluid

S.C. Monkman[a,*], J.S. Ellis[b], S. Cholerton[a], J.M. Thomason[b], R.A. Seymour[b], J.R. Idle[a]

[a]*Pharmacogenetics Research Unit, Department of Pharmacological Sciences, Medical School, University of Newcastle upon Tyne, Framlington Place, Newcastle upon Tyne, NE2 4HH, UK*

[b]*Department of Restorative Dentistry, Dental School, University of Newcastle upon Tyne, Framlington Place, Newcastle upon Tyne, NE2 4BW, UK*

Received 11 September 1995; revised 16 November 1995; accepted 16 November 1995

Abstract

This paper describes an automated capillary gas chromatographic method for the determination of amlodipine in plasma, and in sub-microlitre volumes of gingival crevicular fluid (GCF), in order to assess if amlodipine is present in GCF under conditions of gingival overgrowth, as has been shown for nifedipine, another dihydropyridine drug. Liquid–liquid extraction followed by derivatisation was employed to isolate amlodipine and render it suitable for gas chromatography. Amlodipine was analysed in plasma and GCF of four patients undergoing amlodipine therapy for cardiovascular disorders, three of whom had significant gingival overgrowth. Amlodipine was detected in the plasma of all patients and in massive concentrations in the GCF of those patients with overgrowth, 23- to 290-fold greater than in their plasma. Like nifedipine, amlodipine sequestration into GCF appears to be linked with gingival overgrowth.

Keywords: Amlodipine

1. Introduction

Amlodipine is a second generation dihydropyridine calcium antagonist. It is used as an anti-hypertensive agent [1,2], as therapy for ischaemic heart disease [3] and as an anti-anginal agent [4]. There is a low incidence of "vasodilator" side-effects compared to that reported for other dihydropyridines [5], although there is no reduction of longer-term adverse events such as gingival overgrowth [6] and ankle oedema [7]. In gingival overgrowth, the gingiva is firm, bleeds easily on probing, is usually inflamed and exudes excess gingival crevicular fluid (GCF). GCF is a plasma transudate, formed of the smaller plasma elements, and is modified by the gingiva as it passes from the capillaries to the cavity (crevice) between the teeth and the gums. Nifedipine is sequestrated into GCF in nifedipine-induced gingival overgrowth [8] and this paper reports a method to determine whether amlodipine is similarly sequestrated.

An automated gas chromatographic (GC) assay is described which was used to measure amlodipine in plasma and gingival crevicular fluid of hypertensive patients exhibiting amlodipine-induced gingival overgrowth. Amlodipine has been analysed using

*Corresponding author.

0378-4347/96/$15.00 © 1996 Elsevier Science B.V. All rights reserved
SSDI 0378-4347(95)00526-9

high-performance liquid chromatography [9,10] with fluorescence and amperometric detection, respectively, and by GC with electron-capture detection (GC–ECD) [11,12]. Although these assays report good sensitivity and fair precision, amlodipine was measured in serum or plasma only, using relatively large volumes (≥1 ml). Our requirement was to measure amlodipine both in plasma and in submicrolitre volumes of GCF. Furthermore, the GC methods above used a dropping needle technique and were unsuitable for automated analysis.

2. Experimental

All clinical and analytical procedures took place under yellow lighting to prevent photodegradation of amlodipine and of the other dihydropyridine drugs used.

2.1. Materials

The following compounds were used: *R,S*,-2-[(2-aminoethoxy)methyl]-4-(2-chlorophenyl)-3-ethoxycarbonyl-5-methoxycarbonyl-6-methyl-1,4-dihydropyridine (amlodipine), the gift of Pfizer Research (Sandwich, UK) and 1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridinedicarboxylic acid ethyl methyl ester (nitrendipine), the gift of Bayer (Newbury, UK).

Drug-free plasma was obtained from the Regional Blood Transfusion Service (Tyneside, UK). Methanol, toluene and acetone, of Distol grade, were obtained from Fisons (Loughborough, UK). Nanopure water (Fisons) was used. The derivatizing reagent, trifluoroacetic acid anhydride (TFAA) was from Aldrich (Gillingham, UK) and was diluted to 10% in acetone, as this gave cleaner chromatograms. Triethylamine (TEA) (Sigma, Poole, UK) was prepared fresh for each assay to a concentration of 50 m*M* in toluene, by equilibration with sodium hydroxide (3 *M*) and aqueous TEA in a separating funnel. The organic TEA extract was dried by passing over sodium sulphite. Concentrated ammonia (Merck, Lutterworth, UK) was diluted with water to 5%.

2.2. Glassware

Amber screw-top 4-ml vials with PTFE-lined caps and amber autosampler vials (2 ml) were supplied by Zinsser Analytic (Maidenhead, UK). Tapered amber autosampler vials (600 μl) with grey PTFE-lined crimpcaps (to fit both sizes of autosampler vial), and PTFE sleeves to fit the Hewlett-Packard 7673A autosampler were supplied by Chromacol (London, UK). Glass Pasteur pipettes for sample transfer were from Bilbate (Daventry, UK).

Liquid–liquid extractions were achieved either by vortex-mixing, on a customised multiple-sample vortex-mixer (IKA-Labortechnik, Janke and Kunkel, Germany), or on a rotary wheel (Harvard/LTE, Oldham, UK). Glass gas-tight Hamilton syringes and the centrifugal evaporator (Centrivap) were obtained from V.A. Howe (London, UK). Periopapers and Periotron were from Pro Flo (New York, NY, USA). A positive displacement pipette using glass capillaries (SMI micro/pettor 1200, 20–100 μl) was from Alphalabs (Eastleigh, UK) and a glass gas-tight 701 Hamilton syringe with repeating adaptor (0.5 to 2.0 μl size) and disposable PTFE capillary tips was from Pierce and Warriner (Chester, UK).

2.3. Sample collection

All patients had been recieving amlodipine for treatment of hypertension and/or angina. Three adult patients were referred to the Department of Periodontology for investigation and treatment of their gingival enlargement. A further patient, also medicated with amlodipine, but with no evidence of gingival enlargement, acted as a control.

Venous blood (10 ml) and GCF were collected ca. 1 h after dosage. Venous blood was centrifuged (900 *g*, 10 min) and the plasma removed and frozen at −20°C until analysis.

Crevicular fluid from a previously cleaned, dried and isolated tooth was collected on 2 × 6 mm filter papers (Periopapers) as was described for nifedipine in a previous paper [13]. When a visible amount of fluid was seen (ca. 0.5 μl) the filter paper was removed and the volume measured using a calibrated Periotron 6000 [14]. Two filter paper samples were taken from each site and the plastic handling strips were removed. They were then placed, in pairs, into

a 4-ml amber vial containing phosphate buffer (pH 6.0, 1.0 ml) and gently rocked for 48 h at 4°C. The filter paper was then removed with sterile tweezers and the buffer frozen at −20°C until analysis.

2.4. Preparation of standards

Plasma

Amlodipine besylate was dissolved in methanol to a concentration of 1.282 g/l free base. This was diluted twice into water to give stock solutions of 5 mg/l and 100 μg/l, respectively. From the aqueous 5 mg/l solution, standards of 25 μg/l and 12.5 μg/l in plasma were prepared. From the aqueous 100 μg/l solution, standards of 5.0, 2.5 and 1.25 μg/l in plasma were prepared. Quality control samples were prepared in a similar fashion.

GCF

For each standard, 2 periopapers were spiked with a total of 1 μl of filtered plasma, containing amlodipine besylate at free base concentrations of 3.6, 2.2, 1.4, 0.73, 0.37 and 0.18 mg/l. Quality control samples were prepared in a similar fashion.

2.5. Extraction

Plasma

To 1 ml of plasma standard, or unknown, internal standard (nitrendipine, 500 μg/l, 100 μl), sodium hydroxide (3 *M*, 50 μl), and toluene (2 ml) were added. After rotary mixing (45 min), the tubes were centrifuged (1500 *g*, 10 min). The toluene supernatant was transferred to a fresh vial and TEA (50 m*M* in toluene, 200 μl) and TFAA (10% in acetone, 40 μl) were added. Derivatisation was carried out at 50°C for 20 min followed by cooling. The mixture was cleaned as follows: water (1 ml) was added and the mixture was vortex-mixed for 1 min, and then ammonia (5% aqueous, 1 ml) was added and the mixture was vortex-mixed for 5 min. After centrifugation (1500 *g*, 5 min) the supernatant was transferred to an autosampler vial (2 ml) and an aliquot (0.2 μl) injected on-column into the GC.

GCF

On the day of assay, internal standard (nitrendipine, 26 mg/l, 100 μl), sodium hydroxide (3 *M*, 50 μl) and toluene (2 ml) were added. The mixture was vortex-mixed (15 min) and centrifuged (1500 *g*, 5 min). The supernatant was transferred to a fresh vial and derivatised and cleaned as above, except that the final product was dried by centrifugal evaporation, reconstituted in 100 μl of toluene and transferred to a tapered autosampler vial (600 μl) before an aliquot (0.2 μl) was injected on-column into the GC.

2.6. Gas chromatography

A Hewlett-Packard 5890A Series II gas chromatograph equipped with a ^{63}Ni electron-capture detector (15 mCi) and an HP7673A robotic autosampler was used. A fused-silica pre-column, 7 m × 0.56 mm I.D. × 0.15 m DB1 (JW Scientific, Folsom, CA, USA) was connected, by a universal-fitting glass butt-connector (Chrompak, London, UK), to the analytical fused-silica column, 40 m × 0.25 m I.D. × 0.25 mm DB1 (JW Scientific). The on-column injection port was unheated, the detector was maintained at 300°C, and chromatography was carried out isothermally at 250°C. The carrier gas was hydrogen (32 cm/s, 300 kPa back-pressure) with nitrogen make-up gas (60 ml/min). An HP3396A computing integrator was used to process the chromatographic signals and to drive the autosampler. Under the described conditions, the retention times of amlodipine and nitrendipine were 9.9 and 6.7 min, respectively.

Standards, samples and quality controls were analysed in duplicate and the peak-area ratio of drug to internal standard was calculated by the integrator. Calibration curves were constructed and used to calculate sample concentrations.

3. Results and discussion

Chromatograms of extracts of plasma and GCF are displayed in Fig. 1. Calibration curves were linear, with slopes typically of 0.15 for plasma and 3.42 for GCF. Intercepts were not significantly different from zero. Correlation coefficients were greater than 0.97. The estimated limit of detection was 0.5 μg/l. Recovery of the solvent extraction was 100%. By diluting the TFAA in acetone to 10%, derivatisation

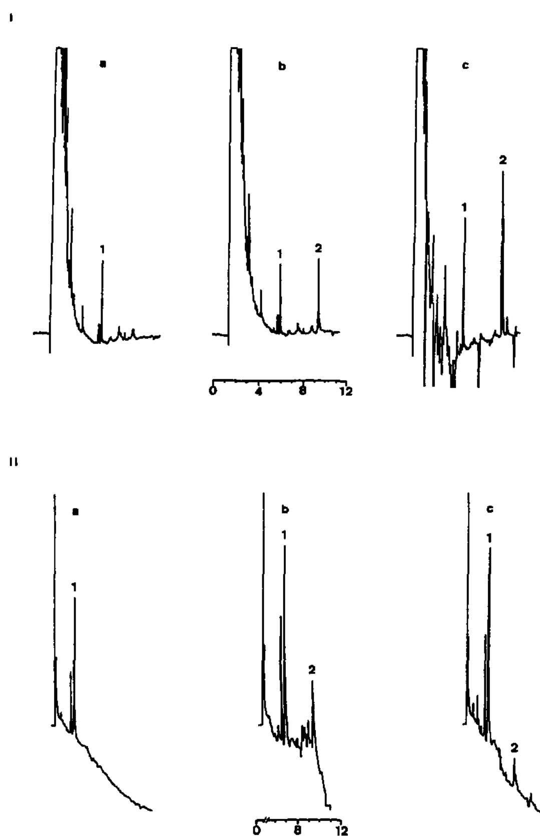

Fig. 1. Chromatograms of extracts of plasma (I) and GCF (II). (I): a = human blank plasma, b = human blank plasma spiked with 9 μg/l amlodipine and c = patient's plasma containing amlodipine at 12.8 μg/l. (II) a = filtered human blank plasma, b = filtered human blank plasma spiked with amlodipine to a concentration of 3 mg/l and c = patient's GCF containing 1.25 mg/l of amlodipine. Peak 1 is the internal standard (nitrendipine) and peak 2 is amlodipine.

efficiency was maximised (see Fig. 2). This dilution also reduced unwanted product formation and hence minimised background signal. It was essential to prepare fresh TEA for each assay and the organic TEA extract was dried by passing over sodium sulphite to improve reaction efficiency. No other commercially available dihydropyridine compound has similar functional groups for derivatisation, therefore, the internal standard used (nitrendipine) was chosen for its extractibility into toluene and its retention time relative to amlodipine.

The assay proved to be sufficiently accurate and robust (Table 1 and Table 2) and was used to assess

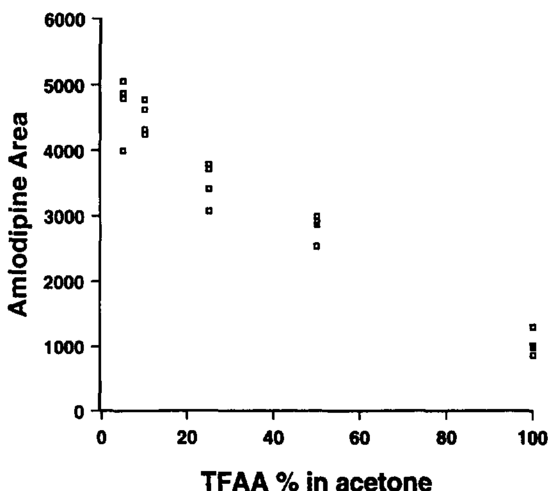

Fig. 2. The effect of dilution of trifluoroacetic acid anhydride (TFAA) with acetone upon the efficiency of derivatisation of amlodipine.

Table 1
Assay reproducibility for the measurement of amlodipine in plasma (n = 6)

QC	Theoretical concentration (μg/l)	Mean measured concentration (μg/l)	C.V. (%)
Intra-assay			
Low	2.25	2.15	7.1
Medium	9.0	7.69	8.6
High	18.0	20.2	6.6
Inter-assay			
Low	2.25	2.38	12.2
Medium	9.0	10.9	6.2
High	18.0	20.1	5.6

Table 2
Assay reproducibility for the measurement of amlodipine in gingival crevicular fluid (n = 6)

QC	Theoretical concentration (mg/l)	Mean measured concentration (mg/l)	C.V. (%)
Intra-assay			
Low	0.80	0.87	7.2
Medium	1.60	1.78	4.9
High	3.20	3.41	2.7
Inter-assay			
Low	0.80	0.89	2.9
Medium	1.60	1.88	5.5
High	3.20	2.99	8.2

Table 3
Dosage and amlodipine concentrations in plasma and GCF of four patients

Patient (status)	Amlodipine dosage (mg/day)	Duration of therapy (months)	GCF concentration (μg/l)	Plasma concentration (μg/l)	GCF/plasma ratio
1(GO)[a]	5	4	4000	13.7	290
2(GO)	5	6	260	11.5	23
3(GO)	10	8	2730	12.8	213
4(NGO)[b]	5	6	ND[c]	8.7	NA[d]

[a] GO = gingival overgrowth.
[b] NGO = no gingival overgrowth.
[c] ND = not detected.
[d] NA = not applicable.

the concentrations of amlodipine in plasma and GCF of four patients receiving amlodipine for various cardiovascular complaints. The data in Table 3 shows that amlodipine is found in the GCF of gingival overgrowth sufferers, yet is not present where there is no overgrowth. Where amlodipine is found in GCF, the concentrations are far greater than in plasma and sequestration can be assumed to occur.

Patient 2 had been withdrawn from amlodipine therapy shortly before the study date, due to the overgrowth, but was challenged with his usual dose (5 mg/day). It is likely therefore, that the lower concentration of amlodipine in this patient's GCF reflected the single dose, whereas as therapy proceeded in patients 1 and 3 there was a gradual build-up of amlodipine within the gingival crevice. Those physicochemical properties of amlodipine responsible for its long plasma half-life could also account for this slow rate of sequestration.

This study has shown that amlodipine can be measured in GCF when it is collected under conditions of gingival overgrowth, and is not found when no overgrowth is present. Further investigation is needed with greater numbers of patients to extend the usefulness of this pilot study.

Acknowledgments

The authors would like to thank Bayer plc for their sponsorship of this project

References

[1] J. Webster, O.J. Robb, T.A. Jeffers, A.K. Scott and J.C. Petrie, J. Cardiovasc. Pharmacol., 12 (S7) (1988) S72.
[2] D.R. Abernethy, J. Gutkowska and L.A. Winterbottom, Clin. Pharmacol. Ther., 480 (1990) 76.
[3] R.A. Burges, Postgrad. Med. J., 67 (S3) (1991) S9.
[4] H. Kishida, N. Hata, I. Kunimi, H. Miyagawa, H. Nishiyama and K. Kotoh, Cardiovasc. Drug Ther., 6 (1992) 481.
[5] A.D. Bremner, P.J. Fell, J. Hosio, I.G.U. Jones, P.A. Saul and S.H. Taylor, J. Hum. Hyperten., 7 (1993) 79.
[6] J.S. Ellis, R.A. Seymour, J.M. Thomason, S.C. Monkman and J.R. Idle, Lancet, 341 (1993) 1102.
[7] H. Osterloh, Am. Heart J., 118 (1989) 1114.
[8] J.S. Ellis, R.A. Seymour, S.C. Monkman and J.R. Idle, Lancet, 339 (1992) 1382.
[9] D.A. Stopher, A.P. Beresford, P.V. Macrae and M.J. Humphrey, J. Cardiovasc. Pharmacol., 12 (S7) (1988) S55.
[10] K. Shimooka, Y. Sawada and H. Tatematsu, J. Pharm. Biomed. Anal., 7 (1989) 1267.
[11] J.K. Faulkener, D. McGibney, L.F. Chasseaud, J.L. Perry and I.W. Taylor, Br. J. Clin. Pharmacol., 22 (1986) 21.
[12] A.P. Beresford, P.V. Macrae, D.A. Stopher and B.A. Wood, J. Chromatogr., 420 (1987) 178.
[13] J.S. Ellis, S.C. Monkman, R.A. Seymour and J.R. Idle, J. Chromatogr., 621 (1993) 95.
[14] M. Bickel and G. Cimansoni, J. Periodont. Res., 19 (1984) 313.