Journal of Chromatography, 420 (1987) 178–183
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3725

Note

Analysis of amlodipine in human plasma by gas chromatography

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(First received December 29th, 1986; revised manuscript received March 16th, 1987)

Amlodipine, *R,S*-2-[(2-aminoethoxy)methyl]-4-(2-chlorophenyl)-3-ethoxycarbonyl-5-methoxycarbonyl-6-methyl-1,4-dihydropyridine, is a new and potent dihydropyridine calcium channel blocker [1,2]. The structure of amlodipine differs from other dihydropyridines in possessing a basic side-chain attached to the 2-position of the dihydropyridine ring (Fig. 1). The pharmacokinetic properties of amlodipine are novel to dihydropyridines in that it has a mean plasma half-life of 36 h [3] and potential for once daily administration. Currently amlodipine is under extensive clinical evaluation for the treatment of angina and hypertension.

A high-performance liquid chromatographic assay similar to that for nifedipine [4] but using fluorescence detection has been used for studies in the dog [5]. However, this method was not sufficiently sensitive for use in the human studies where a detection limit of less than 1 ng/ml was required. This paper describes a method for the determination of amlodipine in human plasma by derivatisation and capillary gas chromatography (GC) with electron-capture detection (ECD) which achieves the required sensitivity.

EXPERIMENTAL

Chemicals and reagents

Amlodipine maleate and the internal standard UK-52,829 fumarate (Fig. 1) were prepared by Pfizer Central Research. Trimethylacetyl chloride (99%, Aldrich Chemicals) was redistilled at 105°C and stored in a glass-stoppered bottle, avoiding contact with plastic. Other chemicals and reagents were of Analar or equivalent quality, except for methyl *tert.*-butyl ether (Fisons, HPLC grade).

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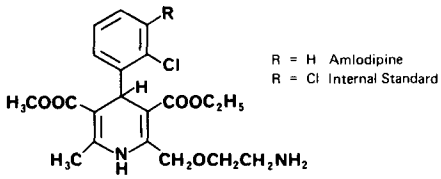

Fig. 1. Structures of amlodipine and internal standard.

Preparation of solutions

Stock solutions of amlodipine and internal standard were prepared in methanol–water (1:1) at a concentration of 10 μg/ml equivalents of base and stored at 4 °C. Stock solutions were stable for several months. These solutions were diluted before use with methanol–water (1:1) to concentrations of 2 or 0.2 μg/ml as required. Borate buffer was prepared by dissolving 12.4 g of boric acid in 900 ml of water, adjusting the pH to 9.0 using 1.0 *M* sodium hydroxide solution, and making up to 1 l with water. Citric acid solution, 0.1 *M*, and potassium carbonate solution, 1.0 *M*, were prepared by dissolving the appropriate weight of compound in water.

Extraction procedure

Plasma, 1.0 ml, after addition of 10 μl of internal standard solution, was basified by the addition of 2.0 ml borate buffer, and extracted with 4.0 ml of methyl *tert.*-butyl ether. After centrifuging, the organic layer was transferred to a second tube and extracted with 2.0 ml of citric acid solution. The organic layer was discarded and the aqueous phase basified by the addition of 1.0 ml of potassium carbonate solution. After mixing using a vortex mixer, 20 μl of trimethylacetyl chloride were added and the solution mixed for 10 min at room temperature using a rotary mixer. The trimethylacetyl derivatives were extracted with 2.0 ml of methyl *tert.*-butyl ether, and the ether layer was evaporated to dryness in a tapered glass tube at 37 °C under a stream of nitrogen. The dry residue was dissolved in 50 μl of methyl *tert.*-butyl ether immediately before analysis. Injection volumes (4 μl) were used for capillary GC.

Instrumentation

A Hewlett-Packard Model 5790 capillary gas chromatograph with an electron-capture detector and Hewlett-Packard Model 3390 integrator were used. The capillary column, 25 m × 0.2 mm I.D., was a Hewlett-Packard ultra-performance column, with cross-linked 5% phenylmethylsilicone stationary phase, 0.33 μm film thickness. Nitrogen (oxygen-free) was used as the carrier gas at a flow-rate of 1.5 ml/min and also as the detector make-up gas at a flow-rate of 30–40 ml/min. A Chrompack solid injector was fitted, maintained at a temperature of 300 °C. The column temperature was programmed from 300 to 320 °C at a rate of 20 °C/min and held at 320 °C for 10 min.

Calibration curves

Two calibration ranges were used: 0.2–2 ng/ml (internal standard at 2 ng/ml) and 2–20 ng/ml (internal standard at 20 ng/ml). Volumes of 1–10 μl of appro-

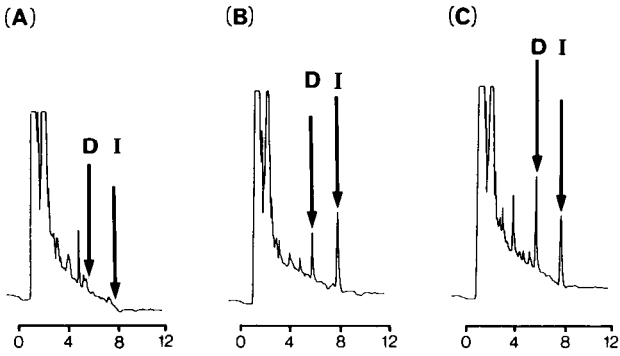

Fig. 2. Typical gas chromatograms showing (A) drug-free control plasma extract, (B) control plasma extract containing 2 ng/ml amlodipine and 2 ng/ml internal standard and (C) plasma extract of a human subject 4 h after oral administration of 20 mg amlodipine. Peaks: D = derivatised amlodipine (retention time 6 min); I = derivatised internal standard (retention time 8 min).

priately diluted amlodipine solution were added to 1 ml of control human plasma and treated as described above. A calibration using six points was prepared for each assay run.

Analysis of results

The peak-area ratio (drug/internal standard) was calculated for all samples. The calibration curve was calculated by unweighted linear regression of peak-area ratio on concentration of calibration samples. The concentration of amlodipine in test samples was calculated using the regression line parameters.

RESULTS AND DISCUSSION

Fig. 2 shows a typical gas chromatogram, at the lowest attenuation used, of a drug-free control plasma extract, a chromatogram of a control plasma extract to which was added 2 ng/ml amlodipine and internal standard, and a chromatogram of an extract of plasma from a human subject dosed with amlodipine and containing 3 ng/ml amlodipine. The retention times of the amlodipine and internal standard derivatives were 6 min and 8 min, respectively. The chromatograms obtained from analysis of control human plasma show no interfering peaks having the same retention times as amlodipine or internal standard derivatives.

The recovery of amlodipine from plasma was determined by analysing control plasma to which ^{14}C-labelled amlodipine had been added. The extraction efficiency was determined at a concentration of 20 ng/ml by measuring the radioactivity of the extracts at each stage of the procedure using liquid scintillation counting. The overall recovery of amlodipine in the assay was 60%. The major loss occurred during the acid extraction stage, with 40% of radioactivity remaining in the organic phase.

It was not possible to check assay recovery below 20 ng/ml using radiolabel due to the low specific activity of [^{14}C]amlodipine (0.067 Bq/ng). Constant recovery over the whole assay range was confirmed by examination of the calibration curve

TABLE I

PRECISION FOR AMLODIPINE IN HUMAN PLASMA

Assay range (ng/ml)	Added concentration (ng/ml)	Found concentration (mean ± S.D., $n=5$) (ng/ml)	Coefficient of variation (%)
0.2–2	0.20	0.20 ± 0.02	11
	2.04	1.96 ± 0.20	10
2–20	2.04	2.47 ± 0.07	3
	20.04	20.4 ± 1.52	7

data. The peak-area ratio of drug to internal standard at 20 ng/ml was ten-fold higher than at 2 ng/ml and correspondingly on the lower concentration range the peak area ratio at 2 ng/ml was ten-fold higher than at 0.2 ng/ml.

Precision

Precision of the method was determined by analysing five identical samples of control plasma containing amlodipine at concentrations corresponding to the top and bottom of each calibration range used (Table I).

Accuracy

The concentration of amlodipine in control plasma samples to which drug had been added at concentrations unknown to the analyst was determined. Table II shows the result of twelve samples analysed. Six of the samples were determined on the lower concentration range and the other six determined on the upper calibration range. The mean deviation ($n=12$) was 2.4% (±0.9 standard error of the mean).

Linearity of calibration

Calibrations obtained by plotting peak-area ratio (drug/internal standard) versus concentration were linear over the ranges 0.2–2 and 2–20 ng/ml. Two calibration ranges were used as the circulating plasma concentrations of amlodipine were found to be over a 100-fold range. The assay of clinical samples was carried

TABLE II

ANALYSIS OF CONTROL PLASMA CONTAINING AMLODIPINE AT CONCENTRATIONS UNKNOWN TO THE ANALYST

Amount added (ng/ml)	Amount found (ng/ml)	Amount added (ng/ml)	Amount found (ng/ml)
0.3	0.31	3.6	3.55
0.4	0.43	4.0	4.19
0.5	0.52	4.2	4.26
0.9	0.87	6.2	6.23
1.5	1.58	6.8	7.00
1.6	1.58	9.5	9.95

out with the operator working with randomised samples and standards to eliminate any bias, the only exception to this being that, in general, samples were sorted into two groups. Samples taken later than 48 h after administration were assayed against the lower calibration range and those samples prior to the 48 h time point assayed on the higher range. This enabled the operator to process all the samples without the need to change attenuations during the analysis.

Calibration data for the assay suggested consistent results with coefficients of correlation greater than 0.999 (4 degrees of freedom) and a relative standard deviation of the mean peak-area ratio of between 1 and 4% over both calibration ranges ($n=4$). The limit of quantitation (the concentration at which the lower 90% confidence limit becomes zero) was less than 1 ng/ml ($n=2$) for the upper range and less than 0.1 ng/ml ($n=2$) for the lower calibration range.

Specificity of the assay

Gas chromatography–mass spectrometry (GC–MS) was used to confirm the specificity of the method and identify any thermal decomposition product since several studies [6,7] have implied that dihydropyridines are heat-labile and may undergo thermal decomposition at the high temperatures used in GC. The electron-impact (EI) mass spectrum of the amlodipine derivative showed two intense ions at m/z 128 (base peak) and m/z 381. The ion at m/z 381 is due to a loss of the chlorophenyl ring and m/z 128 is due to the trimethylacetyl amino ethyl group. By contrast, the decomposition or oxidation product of amlodipine, the pyridine homologue, eluted much earlier from the gas chromatograph (retention time 4.2 min) and accounted for 4% of total ion current. It was essential to develop an assay for the intact dihydropyridine as the pyridine homologue has been shown to be a circulating metabolite by GC–MS, with similar plasma concentration to amlodipine.

An important factor was that the solid injector used in the analysis kept oxidation to a minimum. A standard splitless injection system was tried but a greater degree of oxidation was observed. The solid injector had two other advantages for the analysis of amlodipine compared to conventional injectors. At the low concentrations of amlodipine found in human plasma a larger proportion of the final extract could be applied to the column to increase the response at the detector. The solid injector allows concentration of the sample on the injector needle without the problems encountered when injecting large volumes of solvent on to conventional injectors. Furthermore, high-boiling impurities and pyrolysis products accumulate on the glass needle which can be readily cleaned, rather than coating the walls of the column with subsequent degradation of column performance.

Clinical samples

The procedure described has been used extensively for assay of plasma samples from clinical studies in human volunteers and patients. Fig. 3 shows a typical plasma concentration–time profile for amlodipine over 144 h after a single oral dose of amlodipine (20 mg). The plasma concentration peak at approximately 6 h followed by a trough with a secondary maximum at approximately 12 h has

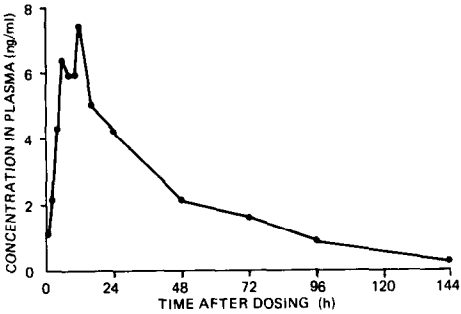

Fig. 3. Typical profile for the plasma concentrations of amlodipine in man. Data from a human subject given a single oral dose of 20 mg.

been seen in several studies. This complex profile has been confirmed using a selected-ion monitoring GC–MS assay for amlodipine.

Amlodipine has a long elimination half-life in man, about 36 h, due to a high volume of distribution, 21 l/kg [3]. The high volume of distribution also results in low plasma concentrations compared to other calcium channel blockers. Low plasma concentrations are not due to poor systemic availability [3,5].

Check for interference

Drugs which may be used together with amlodipine in patients with essential hypertension and angina pectoris were added to drug-free plasma and plasma containing amlodipine at 10 ng/ml. The samples were analysed to check for interference in the amlodipine determination. Six compounds (amiloride, atenolol, doxazosin, hydrochlorthiazide, propanolol and triampterene) were tested at concentrations corresponding approximately to the plasma maxima observed in man. None of the compounds tested affected the amlodipine analysis or gave rise to peaks on the chromatogram.

CONCLUSION

The method has been used by ourselves and several external laboratories for the analysis of many thousands of clinical samples during the development of amlodipine as an agent against angina and essential hypertension in both volunteer studies and in patients.

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