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DETERMINATION OF NILVADIPINE IN HUMAN PLASMA BY CAPILLARY COLUMN GAS CHROMATOGRAPHY—NEGATIVE-ION CHEMICAL-IONIZATION MASS SPECTROMETRY

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SUMMARY

A highly sensitive and specific method for the determination of nilvadipine, a new dihydropyridine calcium antagonist, in human plasma is described. A deuterated analogue of nilvadipine is added to the plasma as an internal standard. The agent and its internal standard are extracted at pH 9 from the plasma into a benzene—*n*-hexane (1:1) mixture. The extract is analysed by fused-silica capillary column gas chromatography—negative-ion chemical-ionization mass spectrometry with methane as the reagent gas. The mass spectrometer is set to monitor the negative molecular ions of the agent and internal standard. Quantitation is possible down to 0.01 ng/ml using 1 ml of plasma. The coefficients of variation of the method are 6.4 and 2.1% at the 0.01 and 0.1 ng/ml levels, respectively. Plasma levels obtained with this method are given for four healthy volunteers who had received a 6-mg oral dose of nilvadipine.

INTRODUCTION

Nilvadipine*, 5-isopropyl 3-methyl 2-cyano-6-methyl-4-(3-nitrophenyl)-1,4-dihydro-3,5-pyridinedicarboxylate (see Fig. 1A) is a new dihydropyridine derivative with calcium antagonistic activity [1—3] developed in our laboratories. Since an oral dose of nilvadipine in man yields a relatively low plasma concentration, an analytical method which can detect nilvadipine in plasma in the 0.05—10 ng/ml concentration range is required.

Several analytical methods for quantifying dihydropyridine calcium antagonists in biological fluids have been reported, such as gas chromatography (GC) with electron-capture detection (ECD) [4—11], GC with electron-impact

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(EI) mass spectrometry (MS) [12, 13], high-performance liquid chromatography (HPLC) [10, 11, 14, 15] and radioreceptor assay [16, 17]. These methods, except the radioreceptor assay, were tried for quantifying nilvadipine in plasma, but none of the methods were sensitive enough.

Negative-ion chemical-ionization (NICI) MS affords high sensitivity for quantitation of compounds containing a group with electron affinity [18—22]. As observed earlier, dihydropyridine compounds are known to decompose during GC separation and in the transfer line between the gas chromatograph and mass spectrometer. Packed column GC—ECD or packed column GC—EI-MS methods involving oxidation of the dihydropyridine compounds to the more stable pyridine analogues prior to chromatography have been used for their determination [4, 7, 11, 12]. However, the pyridine analogues are already present in plasma as metabolites of dihydropyridine compounds [10, 11, 13].

This paper describes an assay method for direct quantification of nilvadipine in the picogram range in human plasma. The method is based on the extraction of a test solution with organic solvent and capillary column GC—NICI mass fragmentography.

EXPERIMENTAL

Materials

Nilvadipine and its deuterium-labelled compound (nilvadipine-d_3, see Fig. 1) were synthesized and supplied by the Department of Organic Chemistry in our laboratories. *n*-Octacosane and *n*-triacontane were purchased from Tokyo Chemical Industry (Tokyo, Japan). Benzene, *n*-hexane and ethyl acetate were of ECD grade, and other reagents and solvents of analytical-reagent grade were used.

Standard solutions of nilvadipine were prepared by dissolving it in ethanol and diluting to appropriate concentrations. Nilvadipine-d_3 was used as an internal standard for the assay. It was dissolved in ethanol and diluted to 2 and 20 ng/ml with ethanol or 1% ethyl acetate in *n*-hexane.

Gas chromatography—mass spectrometry

A Finnigan Model 9610 gas chromatograph (Finnigan-MAT, CA, U.S.A.) was equipped with a 5% phenylmethyl silicone-coated fused-silica capillary column (Ultra No. 2, Hewlett-Packard, PA, U.S.A.), 12.5 m × 0.31 mm I.D., coating thickness 0.52 μm. The column was plumbed directly into the mass spectrometer so that the end of the column was within 2.0 cm of the ion source. The temperature of the column oven was maintained at 180°C for 1 min, followed by temperature programming at 20°C/min up to 250°C, and then at 10°C/min up to 285°C. The injector, interface oven and transfer line were operated at 290°C. Helium was used as the carrier gas at 0.28 kg/cm^2 of inlet pressure. Helium flow through the septum sweep and split valves was stopped ca. 5 s. prior to injection. The sweep and split valves were opened ca. 55 s after injection.

A Finnigan Model 4000 quadrupole mass spectrometer with pulsed positive-ion/negative-ion CI accessory was operated in the CI mode using methane as the reagent gas at an ion source pressure of ca. 0.30 Torr. The emission current

was 0.40 mA, the ionizer temperature 250°C, and the electron energy 70 eV. For selected-ion monitoring (SIM) measurements, a Finnigan Promin with a four-channel recorder (Rikadenki, Tokyo, Japan) was used to set the mass spectrometer to monitor *m/z* 385 (M^- of nilvadipine) and *m/z* 388 (M^- of nilvadipine-d_3). For recording the mass spectra after separation by capillary GC, an INCOS data system was set to scan every 1.0 s from *m/z* 100 to 600.

Extraction procedure

To a 10-ml centrifuge tube were added 1 ml of plasma sample, 0.05 ml of ethanol, 0.05 ml of internal standard ethanol solution, 1 ml of 0.1 *M* borate—hydrochloric acid buffer (pH 9.0) and 4 ml of benzene—*n*-hexane (1:1) mixture. The tube was shaken for 5 min and centrifuged at 1900 *g* for 2 min. A 3-ml volume of the organic phase was transferred into a 10-ml centrifuge tube and evaporated under a stream of nitrogen. The residue was immediately dissolved in 0.5 ml of ethyl acetate after evaporation. When necessary, the solution from this step was stored at −20°C until injected. The solvent was removed by evaporation in a stream of nitrogen before injection. The residue was dissolved in 0.05 ml of 1% ethyl acetate in *n*-hexane. A 1-μl aliquot of the solution was injected into the gas chromatograph.

All glassware used in the extraction procedure had been washed with concentrated sulphuric acid containing Nochromix (Godax Labs., New York, NY, U.S.A.).

Quantitation

The procedure was standardized by analysing the blank plasma to which had been added 0.05 ml of nilvadipine standard solution instead of 0.05 ml of ethanol as in the extraction procedure. Peak height ratios of *m/z* 385 to 388 were used to establish the calibration graph. The calibration graph was fitted to a $y = ax + b$ equation by the least-squares method. The weighting factor in the least-squares method adopted the reciprocals of the observations [19]. The concentrations in the unknown samples were subsequently calculated using the calibration graph.

Recovery

Blank plasma samples containing 0.1 and 1 ng/ml nilvadipine were carried through the above procedure without addition of the internal standard. Nilvadipine-d_3 (50 μl) dissolved in 1% ethyl acetate in *n*-hexane solution was added to the benzene—*n*-hexane extraction residue before injection into the column. Recoveries were calculated by comparing the peak height ratios with those obtained when nilvadipine and nilvadipine-d_3, dissolved in 1% ethyl acetate in *n*-hexane containing blank plasma extract, were processed without the extraction procedure.

Stability

To study whether the storage of the plasma sample affected the plasma concentration of nilvadipine, spiked plasma samples containing 2 ng/ml nilvadipine were frozen at −20°C. The concentrations of nilvadipine in the respective samples frozen for 7, 29, 74, 114 and 227 days were determined.

Clinical study

Four healthy volunteers, fasted overnight, were given an oral dose of nilvadipine in the form of a 6-mg tablet. Plasma samples were obtained from blood collected by venipuncture at designated intervals and stored at −20°C until analysed.

RESULTS

NICI mass spectra of nilvadipine and nilvadipine-d_3 are shown in Fig. 1. The spectra are simple and provide intense M^- peaks. In the spectrum of nilvadipine, the ion of *m/z* 388 was observed with a relative intensity of 0.16% and the ion of *m/z* 382 was not observed. In the spectrum of nilvadipine-d_3, the ion of *m/z* 385 was observed with a relative intensity of 3.2%. This ion is probably due to contamination of the proton analogue of nilvadipine-d_3.

Typical mass fragmentograms obtained from the human plasma samples are shown in Fig. 2. As shown in Fig. 2A—C, the background peaks of the blank human plasma were few and completely separated from the peaks of nilvadipine and nilvadipine-d_3. Fig. 2D shows typical mass fragmentograms of the plasma from a healthy volunteer after a 6-mg oral dose of nilvadipine. There were no interferences at the retention times of nilvadipine and nilvadipine-d_3.

GC retention indices (Kovats indices) of nilvadipine and nilvadipine-d_3 were obtained by total-positive-ion detection using *n*-octacosane and *n*-triacontane as standards [23, 24]. The retention indices obtained from five replicate analyses were 2884.6 ± 1.4 (mean ± S.D.) for nilvadipine and 2881.7 ± 2.1 for nilvadipine-d_3.

Absolute overall recovery from five replicate analyses of nilvadipine-spiked plasma samples were 67.3 ± 5.9% for 0.1 ng/ml and 80.6 ± 1.8% for 1 ng/ml.

Typical calibration graphs for human plasma are shown in Table I. The calibration graphs show good linearity in each range. Assay sensitivity was limited by the amount of internal standard. The response at *m/z* 385 of nilvadipine-d_3

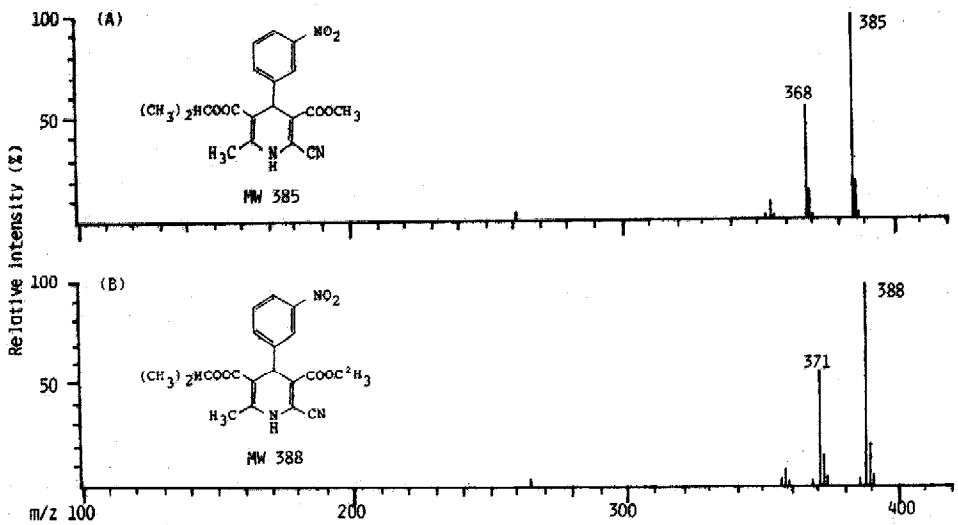

Fig. 1. Methane NICI mass spectra of (A) nilvadipine and (B) nilvadipine-d_3.

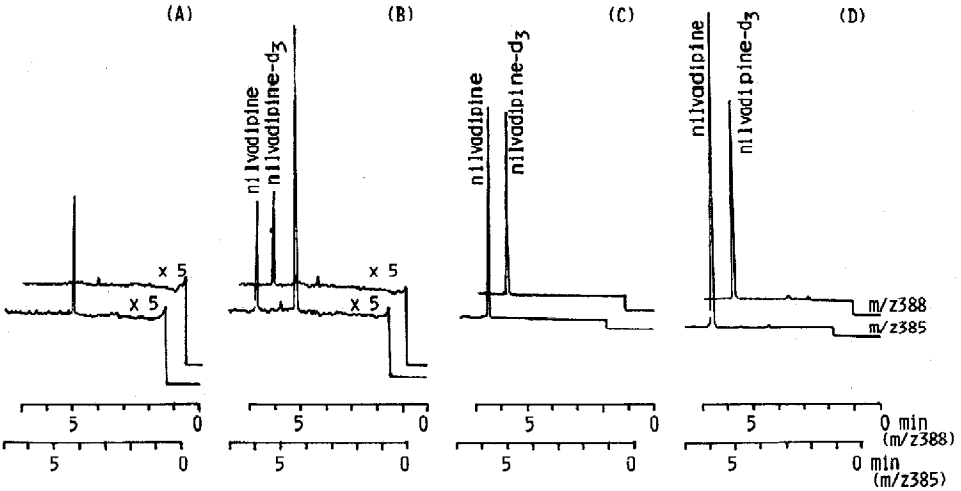

Fig. 2. Human plasma mass fragmentograms of (A) blank plasma, (B) plasma containing 0.1 ng/ml nilvadipine and 0.1 ng/ml nilvadipine-d_3, (C) plasma containing 1 ng/ml nilvadipine and 1 ng/ml nilvadipine-d_3 and (D) plasma collected from a healthy volunteer after a 6-mg oral dose of nilvadipine (calculated concentration of nilvadipine was 1.27 ng/ml). The M^- of nilvadipine (*m/z* 385) and nilvadipine-d_3 (*m/z* 388) were monitored.

TABLE I

TYPICAL CALIBRATION GRAPHS FOR HUMAN PLASMA

Concentration range (ng/ml)	Slope	Intercept	Correlation coefficient
0.01—1.0	10.6810	0.0767	0.9993
0.1 —10	1.0003	0.0495	0.9996

TABLE II

REPRODUCIBILITY OF DETERMINATION FOR NILVADIPINE IN HUMAN PLASMA

n = 5.

Actual concn. (ng/ml)	Mean concn. found (ng/ml)	Percentage of actual concn.	Range (ng/ml)	S.D. (ng/ml)	C.V.* (%)	Concn. range of calibration graph (ng/ml)
0.0100	0.0090	90	0.0082—0.0096	0.0006	6.4	0.01— 1.0
0.0250	0.0251	100	0.0236—0.0264	0.0011	4.5	
0.100	0.102	102	0.100 —0.105	0.002	2.1	
0.100	0.095	95	0.091 —0.100	0.004	3.9	0.1—10.0
1.00	1.02	102	0.99 —1.04	0.02	2.3	

*C.V. = Coefficient of variation.

was about 3% of that at *m/z* 388. Taking twice the response of the blanks as sensitivity criteria, the sensitivities of the assay were estimated to be 0.01 ng/ml with 0.1 ng/ml nilvadipine-d_3 and 0.1 ng/ml with 1 ng/ml. The response at *m/z* 385 of plasma containing only 0.01 ng/ml nilvadipine had a signal-to-noise ratio of 5.

Reproducibility was determined by performing five replicate analyses of spiked plasma samples. The results are given in Table II. This method for the determination of nilvadipine in human plasma thus provides good accuracy and precision even around the lower limits of sensitivity.

The concentrations of nilvadipine in the plasma samples after freezing for 7, 29, 74, 114 and 227 days were unchanged.

Plasma concentrations of the unchanged drug after a 6-mg oral dose of nilvadipine was given to healthy volunteers are shown in Fig. 3. Plasma concentrations of the unchanged drug peaked within 2 h, reached a mean of 5.78 ng/ml, and decreased to a mean of 0.22 ng/ml at 12 h, with a mean apparent half-life of 3.38 h.

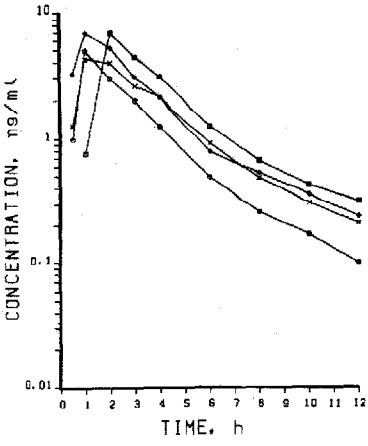

Fig. 3. Plasma concentrations of the unchanged drug in healthy volunteers after a 6-mg oral dose of nilvadipine. (○) Subject 1; (□) subject 2; (◇) subject 3; (×) subject 4.

DISCUSSION

Early studies on methods for quantifying nilvadipine in plasma were carried out by GC—ECD without oxidation to the pyridine analogue prior to chromatography [5, 6, 8, 9]. Oxidation to the pyridine analogue during chromatography was kept to less than 5%, but these methods did not separate the background peaks of plasma from the peak of nilvadipine below the 0.5 ng/ml level. In recent years, NICI has been found to be an excellent ionization technique for MS analysis of trace amounts of compounds containing a group with electron affinity in complex biological matrices [18—21]. The NICI mass spectrum of nilvadipine which has an NO_2 group was simple and provided an intense M^- peak (Fig. 1). This ion is ideal for specific and sensitive quantitative analysis of nilvadipine. Packed-column GC—NICI-MS was applied to determine nilvadipine, but its oxidation in the injection port, column and transfer line was observed. We found that the use of a capillary column connected directly to the ion source prevents the oxidation of nilvadipine in the column and transfer line, and the presence of plasma extract decreases the oxidation of nilvadipine in the injection port. Details of our findings for preventing the oxidation of nilvadipine during chromatography will be published elsewhere [25]. The combination of capillary column GC and NICI-MS made specific

detection of nilvadipine possible down to 10 pg/ml with respect to endogenous compounds co-extracted from plasma with a benzene—*n*-hexane (1:1) mixture. The extensive pretreatment comprising thin-layer chromatography, oxidation to the pyridine analogue and solvent extraction which is described for nicardipine [13] was not necessary. Although the overall recovery from the plasma sample spiked with 0.1 ng/ml nilvadipine was not as good, this method provides good accuracy and precision even at 0.01 ng/ml as shown in Table II. It is assumed that nilvadipine-d_3 added to the plasma prior to the extraction cancels the loss of nilvadipine during the extraction. The occurrence of interfering peaks by contamination was low because of the use of capillary column GC and SIM of *m/z* 385 (M^- of nilvadipine), but one interfering peak occurred by contamination of nilvadipine itself in the analytical procedure. Therefore, washing of glassware was critical to obtain chromatograms without interfering peaks.

The assay was shown to be sufficiently sensitive to quantify nilvadipine in human plasma after a 6-mg oral dose of nilvadipine. It is assumed that the assay is also sufficiently sensitive after oral administration of lower clinical doses (2 or 4 mg) and that it is a useful technique for examining the pharmacokinetics of nilvadipine in human beings. Capillary column GC—NICI-MS used for nilvadipine seems to be suitable for the development of sensitive and specific assays in biological fluids of other dihydropyridine calcium antagonists which have a group with electron affinity [3]. The application of this technique to sensitive and specific assays of these compounds in biological fluids will be discussed elsewhere [25].

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