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SPECIFIC DETERMINATION OF PLASMA NICARDIPINE HYDROCHLORIDE LEVELS BY GAS CHROMATOGRAPHY—MASS SPECTROMETRY

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SUMMARY

A highly specific method for the determination of the plasma level of the potent vasodilator 2-(N-benzyl-N-methylamino)ethyl methyl 2,6-dimethyl-4-(*m*-nitrophenyl)-1,4-dihydropyridine carboxylate hydrochloride (nicardipine hydrochloride) in rats, dogs and humans is described. N-d_3-Methyl derivative was added as an internal standard, then the plasma was extracted with diethyl ether and subjected to thin-layer chromatography (TLC) to remove the pyridine analogue, one of the drug's metabolites. The area corresponding to the unchanged drug was identified with simultaneously run N-d_7-benzyl derivative under UV light. The unchanged drug with a 1,4-dihydropyridine structure was oxidized with nitrous acid to its pyridine analogue, which was stable for gas chromatography, and subjected to mass spectrometry at *m/e* 134 (nicardipine) and *m/e* 137 (N-d_3-methyl derivative). The sensitivity limit was 5 ng ml^{-1}. The ratio of the unchanged drug to the value obtained by the method without TLC separation was 100% for rats and 80% for dogs and humans at almost all times investigated after dosing. These results demonstrate that in these species, the amount of pyridine analogue in plasma was very small compared with that of the parent drug.

INTRODUCTION

Nicardipine hydrochloride, 2-(N-benzyl-N-methylamino)ethyl methyl 2,6-dimethyl-4-(*m*-nitrophenyl)-1,4-dihydropyridine carboxylate hydrochloride, is a new potent vasodilator [1,2], nanogram amounts of which in plasma have been measured by gas—liquid chromatography with electron-capture detection (ECD-GLC) [3] and gas chromatography—mass spectrometry (GC—MS) [4]. In those assays, prior to chromatography, the drug was oxidized with nitrous acid to its pyridine analogue, which is stable for gas chromatography. These

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methods are sufficiently sensitive to determine the plasma concentration of the drug after the oral administration of therapeutic doses to humans. However, when the pyridine analogue is present in the plasma as a metabolite, the values obtained by these methods represents the sum of the unchanged drug and the metabolite. We describe here a highly specific method for determining the unchanged drug by GC—MS after removing the pyridine analogue by thin-layer chromatography (TLC). The plasma concentrations in healthy volunteers were also measured by two previous methods [3, 4], which are the same in principle except for the detector, and the specificities were compared.

EXPERIMENTAL

Chemicals

Nicardipine hydrochloride and two deuterium-labelled compounds (N-d_3-methyl and N-d_7-benzyl derivatives) (Fig. 1) were described in a previous paper [4]. Tablets containing 20 or 30 mg were prepared in our laboratories. Other chemicals and reagents were obtained commercially and were of analytical-reagent grade.

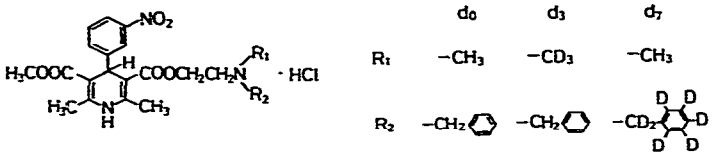

Fig. 1. Structures of nicardipine hydrochloride and its deuterium-labelled compounds.

Gas chromatography—mass spectrometry

A JMS D-300 mass spectrometer (JEOL, Tokyo, Japan) and a Hewlett-Packard 5710A gas chromatograph were used. Separation was effected on a glass column (1.8 m × 2 mm I.D.) packed with 3% OV-1 80—100-mesh Chromosorb W AW DMCS (Nihon Chromato Works, Tokyo, Japan). The column temperature was maintained isothermally at 270°C and the flash heater and separator temperatures were held at 280°C. The flow-rate of the carrier gas (helium) was 30 ml min^{-1}. The ionization potential and trap current were 20 eV and 80 μA, respectively. The entrance and collector slits of the mass spectrometer were adjusted to 0.4 mm. The multiplier voltage supply was set at 2.0—2.3 kV.

Procedure

The ECD-GLC method was as described previously [3]. The GC—MS method has also been reported previously [4]. In the present study, however, the N-d_3-methyl derivative was used as an internal standard (I.S.).

GC—MS combined with TLC (TLC—GC—MS). A plasma sample (1 ml) was introduced into a 10-ml centrifuge tube and 0.5 ml of the internal standard solution (N-d_3-methyl derivative, 200 ng) and 0.5 ml of sodium hydroxide solution (2 *M*) were added, mixed and extracted with two 2-ml volumes of diethyl ether. The organic layer was evaporated to dryness in a water-bath at 45°C. The pyridine analogue of the N-d_7-benzyl derivative in benzene (2 μg,

0.05 ml) was added to the residue as a TLC marker. For TLC (silica gel F_{254}, Merck, Darmstadt, G.F.R.; 0.25 mm thickness) chloroform—acetone—diethylamine (5:4:1) was used as the solvent. The R_F values of nicardipine and the pyridine analogue were 0.57 and 0.64, respectively.

Areas corresponding to unchanged nicardipine and its pyridine analogue were identified with simultaneously run N-d_7-benzyl derivatives and the pyridine analogue of N-d_7-benzyl derivative added to the plasma extract, using UV light (254 nm).

The area corresponding to the unchanged drug was scraped from the plate, introduced into a 10-ml centrifuge tube and 4 ml of hydrochloric acid (0.05 *M*) were added. To the aqueous layer was added 0.3 ml of sodium nitrite solution (0.15 *M*) and the mixture was kept in a water-bath at 45°C for 1 h. This procedure resulted in oxidation of the 1,4-dihydropyridine rings of the labelled and non-labelled drug to pyridine rings. After cooling, the mixture was made alkaline with 0.5 ml of sodium hydroxide solution (2 *M*) and extracted with two 2-ml volumes of benzene. The organic layer was evaporated to dryness under reduced pressure and 50 μl of benzene were added to the residue. A 3-μl aliquot was injected into the gas chromatograph—mass spectrometer. Fragment ions at *m/e* 134 and 137 were used to monitor the pyridine analogues of nicardipine and the internal standard, respectively. The amount of nicardipine hydrochloride in each plasma sample was calculated by measuring the peak-height ratios of the pyridine analogues of the drug and internal standard and comparison with a calibration graph, prepared by subjecting control plasma, to which known amounts of nicardipine hydrochloride (5—200 ng) had been added, to the above procedure and plotting the peak-height ratios of the pyridine analogues of corresponding nicardipine hydrochloride and internal standard against the drug concentration.

Recoveries

Control plasma samples (1 ml) containing 100 ng of nicardipine hydrochloride were carried through the above procedure in the absence of internal standard. The pyridine analogue of the internal standard (200 ng), dissolved in benzene, was added to the benzene extract, the benzene solution was evaporated to dryness under reduced pressure and 50 μl of benzene were added to the residue. A 3-μl aliquot was injected into the gas chromatograph—mass spectrometer. Recoveries were calculated by comparing the peak-height ratios with those obtained when the pyridine analogues of the drug and the internal standard, dissolved in benzene, were processed without the extraction procedure.

Specificity

In the present assay procedure, the pyridine metabolite was removed by TLC prior to GC—MS. To check the specificity of this method, we added 100 ng of nicardipine hydrochloride and 100 ng of its synthesized pyridine derivative to 1-ml plasma samples. These samples were then subjected to the TLC—GC—MS method and the recovery of unchanged drug was calculated.

Animal studies

Male Sprague—Dawley rats (100—130 g) and male beagle dogs (9—11 kg), maintained with free access to food and water, were fasted overnight prior to the oral administration of the drug in aqueous solution. After dosing, the animals were kept in individual metabolism cages. Blood samples, obtained a heparinized syringe from the inferior vena cava of the rats and the antecubital vein of the dogs, were centrifuged at 980 *g* for 15 min. Plasma samples were stored at −20°C until taken for assay.

Human studies

After overnight fasting, four healthy adult males (56—75 kg, age 30—36 years) each received 20 mg of nicardipine hydrochloride in tablet form and another four male subjects (54—64 kg, age 31—37 years) each received a 30-mg tablet. Blood samples were obtained from the cubital vein with heparinized syringes and centrifuged at 980 *g* for 15 min. The plasma samples were stored at −20°C until taken for assay.

Plasma samples from patients

Hypertensive patients recieved 20 mg of nicardipine hydrochloride t.i.d. for several weeks and their physicians submitted the plasma samples for determination of the drug concentration. Residual samples, after analysis by the ECD-GLC method reported previously [3], were used in the present study.

RESULTS AND DISCUSSION

We evaluated nicardipine hydrochloride as a potential therapeutic agent in the treatment of cerebrovascular, hypertensive and angina diseases. This drug, selected from among many 1,4-dihydropyridine derivatives, is water-soluble and is completely absorbed from the gastro-intestinal tract after oral administration [5]. However, in animals and humans, the plasma concentration of the unchanged drug after oral administration was very low owing to its first-pass metabolism [6]. Higuchi and co-workers [3, 4] have reported sensitive methods for the determination of nanogram amounts of nicardipine hydrochloride in plasma and these methods were applied in our earlier studies [6—8]. When nanogram amounts of the drug were injected into the gas chromatograph, the drug was partly oxidized to its pyridine analogue, resulting in two peaks [3]. The difficulties in the assay were overcome by oxidizing 1,4-dihydropyridine to pyridine with nitrous acid before chromatography and the pyridine analogue was detected with an electron-capture detector [3] or by mass spectrometry [4]. Although these methods are sensitive enough to determine drug levels as low as 2—3 ng ml^{-1}, when the pyridine analogue is present in the plasma as a metabolite the values obtained represent the sum of the unchanged drug and the metabolite, whose vasodilative effect is 300 times weaker than that of the parent drug [9]. An experimental study on rats and dogs, in which we used a ^{14}C-labelled compound, revealed that, compared with the unchanged drug, the amount of this metabolite was negligible in the plasma [3]. However, to obtain data on humans, a more specific method is required for determination of the unchanged drug in plasma.

In the present assay, TLC was used to remove the pyridine analogue before the oxidation of dihydropyridine to pyridine analogue with nitrous acid. The amounts of nicardipine and the N-d_3-methyl derivative, added as an internal standard, in plasma were in the nanogram range, so that the area corresponding to the compounds after TLC could not be detected with UV light (254 nm). The area of the unchanged drug was identified with the aid of both simultaneously run N-d_7-benzyl derivative and the pyridine analogue of N-d_7-benzyl derivative, which was added to the plasma extract. R_F values of nicardipine and its pyridine analogue from the plasma extract were slightly different from those of simultaneously run N-d_7-benzyl derivatives because the plasma extracts contained large amounts of endogenous substances. Therefore, the pyridine analogue of the N-d_7-benzyl derivative was added to the plasma extracts as a TLC marker to differentiate the area of the unchanged drug from that of its pyridine analogue. Even if d_7-benzyl derivatives, the amounts of which applied to TLC were large compared with those of nicardipine and the internal standard, slightly contaminated the sample during the procedure, this did not interfere with the assay because the compound does not have fragment ions at *m/e* 134 and 137, which were used to monitor nicardipine and the internal standard in the GC—MS method.

To check the specificity of the assay, we determined the recovery of unchanged drug from plasma containing both unchanged drug and the pyridine analogue. The value obtained of 101.1 ± 2.2% (n = 6) indicates that the method is highly specific.

Chromatograms of human plasma samples, obtained by the TLC—GC—MS method, are shown in Fig. 2. The drug-free control plasma (Fig. 2a) gave no interfering peaks at *m/e* 134 and 137, selected for monitoring pyridines of nicardipine and the N-d_3-methyl derivative, respectively.

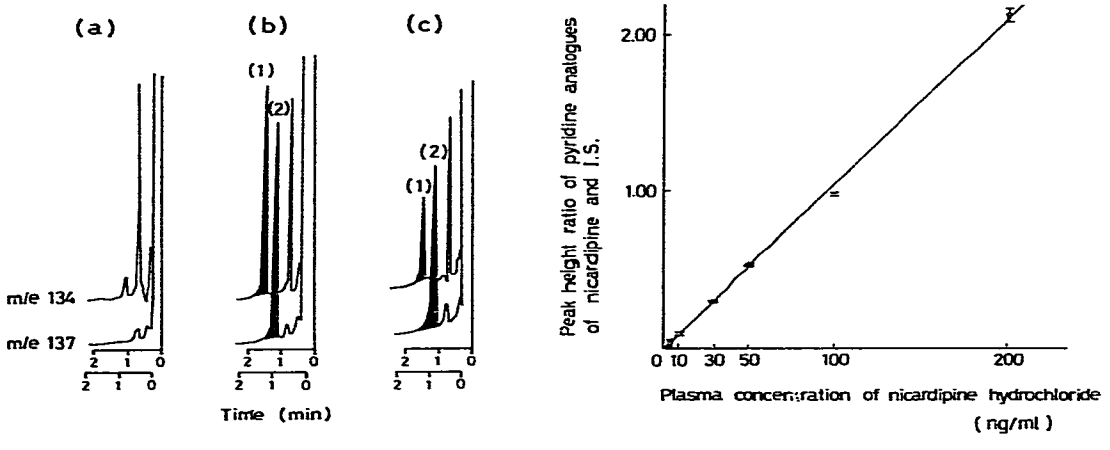

Fig. 2. Chromatograms of human plasma samples obtained by TLC—GC—MS. (a) Control plasma; (b) calibration standard containing nicardipine (1) and the N-d_3-methyl derivative (2) (200 ng ml^{-1} of each); (c) plasma sample obtained at 9.00 a.m. from a hypertensive patient who had received 20 mg of nicardipine hydrochloride t.i.d. for several weeks.

Fig. 3. Calibration graph for determining nicardipine hydrochloride by the TLC—GC—MS method. Each point represents the mean ± standard error from three experiments.

TABLE I

RATIO OF THE PLASMA CONCENTRATION OF THE DRUG OBTAINED BY TLC–GC–MS TO THAT ORTAINED BY GC–MS AFTER THE ORAL ADMINISTRATION OF NICARDIPINE HYDROCHLORIDE TO RATS, DOGS AND HEALTHY HUMANS (%)

Species	Number	Dose	0.5 h	1 h	2 h	4 h	6 h	8 h
Rat	3	5 mg/kg	100 ± 3	87 ± 9	97 ± 10	100 ± 4	—*	—*
Dog	4	5 mg/kg	87 ± 4	95 ± 1	88 ± 6	77 ± 6	84 ± 3	88 ± 1
Man	4	20 mg	95 (n=2)	74 ± 1	87 ± 6	99 ± 3	—*	—*
Man	4	30 mg	82 ± 6 (n=3)	84 ± 6	82 ± 6	109 ± 8 (n=3)	110 ± 7 (n=3)	—*

*The value was below the limit of detection.

The calibration graph obtained from the analysis of animal plasma samples containing various amounts of nicardipine hydrochoride gave a straight line over the concentration range of 5—200 ng ml^{-1} (Fig. 3). The recovery from plasma was 92.5 ± 5.3% ($n = 6$).

This method was sensitive enough to determine plasma levels of the drug as low as 5 ng ml^{-1} when 1-ml plasma samples were used; it was also found to be applicable to the measurement of plasma concentrations occurring after the administration of therapeutic doses to humans.

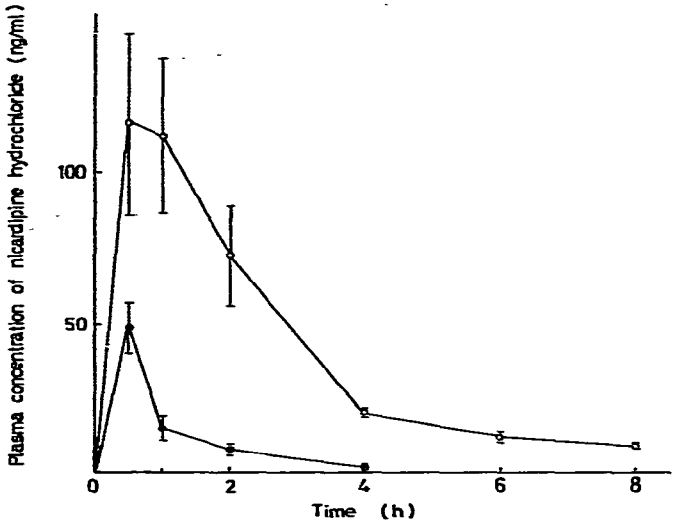

Fig. 4. Plasma concentration of the unchanged drug obtained by TLC—GC—MS after the oral administration of nicardipine hydrochloride (5 mg/kg) to rats (•——•) and dogs (○——○). Each point represents the mean ± standard error from three rat and four dog experiments.

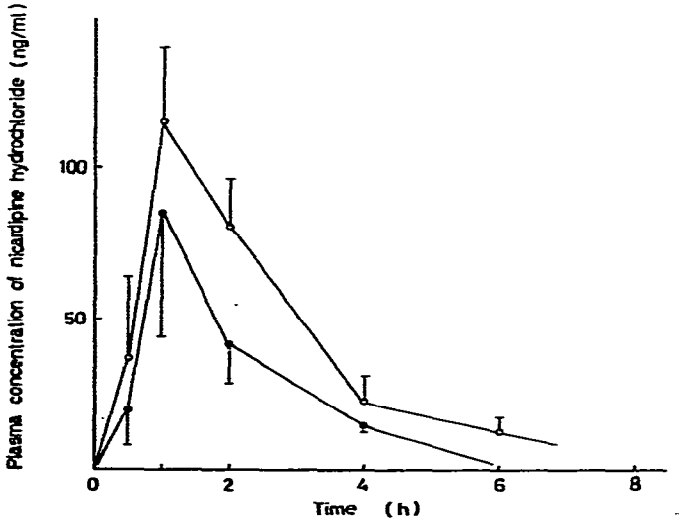

Fig. 5. Plasma concentration of the unchanged drug obtained by TLC—GC—MS after the oral administration of nicardipine hydrochloride [20 mg (•——•) or 30 mg (○——○)] to healthy volunteers. Each point represent the mean ± standard error from four subjects.

The plasma concentrations of the drug after oral administration of nicardipine hydrochloride to rats, dogs and humans were determined by two methods, GC—MS and TLC—GC—MS. The values obtained by the latter method represent the unchanged drug concentration (Figs. 4 and 5), whereas those obtained by the former method represent the sum of the unchanged drug and its pyridine metabolite. There are marked differences in the plasma concentrations among the species examined. This difference is thought to be due to differences in the hepatic metabolizing enzyme activity of the drug [6]. The ratio of the unchanged drug to the value obtained by GC—MS is shown in Table I. This value was about 100% for rats and 80% for dogs and healthy volunteers at almost all times examined after dosing. The value in hypertensive patients was also about 80% (Table II). These results demonstrate that in the species

TABLE II

PLASMA CONCENTRATION OF THE UNCHANGED DRUG OBTAINED BY TLC—GC—MS AND THE RATIO OF THE VALUE TO THAT OBTAINED BY GC—MS IN HYPERTENSIVE PATIENTS

The patients received 20 mg of nicardipine hydrochloride t.i.d. (at 8.00, 13.00 and 18.00) for several weeks.

Time of day	Number of patients	Unchanged drug concentration in plasma (ng/ml)	Ratio of the value obtained by TLC—GC—MS to that obtained by GC—MS (%)
9.00	9	64 ± 22	75 ± 5
13.00	8	14 ± 4	82 ± 6
14.00	11	46 ± 20	85 ± 4
18.00	10	16 ± 4	79 ± 6
19.00	10	40 ± 8	85 ± 3

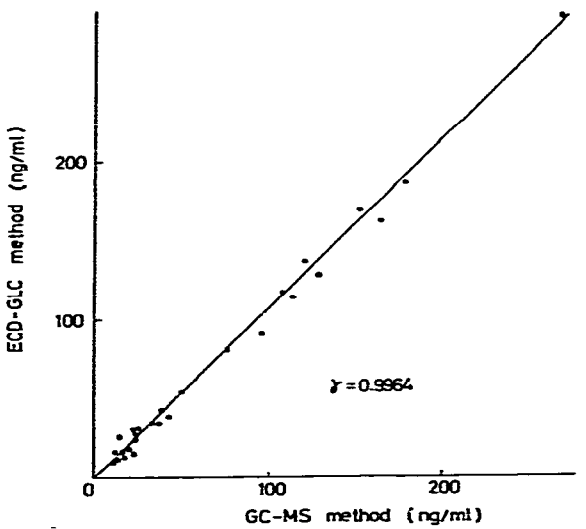

Fig. 6. Correlation between the values obtained by the GC—MS and the ECD—GLC methods.

examined, the amount of plasma pyridine metabolite was very small compared with that of the parent drug.

In the present study the plasma concentrations in healthy volunteers were also measured by ECD-GLC and the values obtained were compared with those obtained by GC—MS. The correlation coefficient (0.9964) indicates that these methods, which are the same in principle except for the detector, have the same specificity (Fig. 6).

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