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Note

Determination of nilvadipine in plasma and urine by capillary column gas chromatography with electron-capture detection

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Nilvadipine, 5-isopropyl 3-methyl 2-cyano-6-methyl-4-(3-nitrophenyl)-1,4-dihydro-3,5-pyridinedicarboxylate (Fig. 1) is a new dihydropyridine derivative with calcium antagonist activity [1–3]. The drug was developed in our laboratories and is currently undergoing clinical evaluation for antihypertensive and antianginal effects.

We have recently shown that capillary column gas chromatography (GC) combined with negative-ion chemical-ionization mass spectrometry (NICIMS) is an excellent technique for developing a highly sensitive and specific assay of dihydropyridine calcium antagonists in biological fluids [4]. The quantitation of nilvadipine in human plasma by this techique was possible down to 0.01 ng/ml using 1 ml of plasma [5, 6]. The pharmacokinetics of nilvadipine in healthy volunteers was studied using GC–NICIMS [7, 8]. This method was highly sensitive and selective, but the procedure was quite costly. Therefore, a more simple and inexpensive analytical method, which could detect higher levels of nilvadipine, was required for pharmacokinetic studies in animals and humans.

This paper describes a simple and relatively sensitive method for the determination of nilvadipine in plasma and urine. The method is based on the extraction of a test solution with organic solvent, followed by capillary column GC with electron-capture detection (ECD).

EXPERIMENTAL

Materials

Nilvadipine, its pyridine analogue and internal standard (Fig. 1) were synthesized and supplied by the Department of Organic Chemistry in our laboratories.

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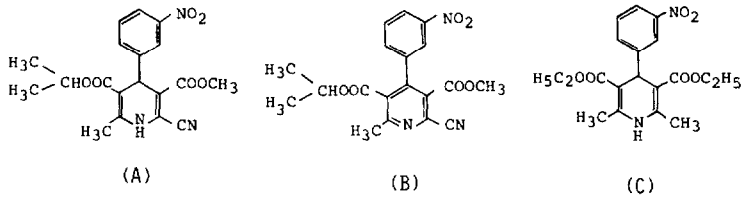

Fig. 1. Chemical structures of (A) nilvadipine, (B) pyridine analogue of nilvadipine and (C) internal standard.

Bis(*p*-nitrophenyl) phosphate (BNPP) was purchased from Nakarai (Kyoto, Japan). Ethyl acetate and *n*-hexane were of ECD grade. The other reagents and solvents were of analytical-reagent grade.

Standard solutions of nilvadipine were prepared by dissolving it in ethanol and diluting to appropriate concentrations. The internal standard was dissolved in ethanol and diluted to 100 and 200 ng/ml with ethanol or toluene.

Gas chromatography

A Hewlett-Packard 5890 gas chromatograph (Hewlett-Packard, PA, U.S.A.) was equipped with a ^{63}Ni electron-capture detector, a Model 7672A automatic sampler (Hewlett-Packard) and a Model 3392A integrator (Hewlett-Packard). A 5% phenylmethyl silicone-coated fused-silica capillary column, 25 m × 0.31 mm I.D., coating thickness 0.52 μm (Ultra No. 2, Hewlett-Packard) was used. The sample was introduced into a split injector (split ratio ca. 17:1) maintained at 290°C. The column oven and detector were operated at 280 and 300°C, respectively. The flow-rate was 1.8 ml/min for helium carrier gas and 35 ml/min for argon–methane (95:5, v/v) make-up gas. Under these conditions, nilvadipine and the internal standard eluted with retention times of 5.8 and 5.3 min, respectively (see Figs. 2 and 3).

Extraction procedure

To a 10-ml centrifuge tube were added 1 ml of plasma or urine 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 *n*-hexane–ethyl acetate (92.5:7.5). The tube was shaken for 5 min and centrifuged at ca. 1900 *g* for 2 min. A 3-ml volume of the organic phase was transferred to a 10-ml centrifuge tube and evaporated under a stream of nitrogen. The residue was immediately dissolved in 0.1 ml of toluene. A 4.5-μl (for plasma) or 2.5-μl (for urine) aliquot of the solution was injected into the gas chromatograph.

Quantitation

The procedure was standardized by analysing the blank plasma or urine 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 nilvadipine to the internal standard were used to establish the calibration graph. The calibration graph was fitted to a $y = ax + b$ equation by the least-squares method. The weight-

ing factor in the least-squares method was taken as the reciprocals of the observation [5, 9]. The concentrations in the unknown samples were subsequently calculated using the calibration graph.

Recovery

Blank plasma and urine samples containing 5 and 10 ng/ml nilvadipine, respectively, were carried through the above procedure without addition of the internal standard. The internal standard, dissolved in toluene, was added to the *n*-hexane–ethyl acetate extraction residue before injection onto the column. Recoveries were calculated by comparing the peak-height ratios with those obtained when nilvadipine and the internal standard, dissolved in toluene containing blank plasma or urine extract, were processed without the extraction procedure.

Stability in plasma

Fresh plasma samples from the B6C3F1 mouse, Sprague–Dawley rat, New Zealand white rabbit, beagle dog and human were spiked with 10 ng/ml nilvadipine and incubated at 4 °C and/or 37 °C for 1 h. Rat plasma containing 10^{-3} *M* BNPP and 10 ng/ml nilvadipine was also incubated at 37 °C for 1 h. The concentrations of nilvadipine in the samples before and after the incubation were determined.

Animal study

Six male beagle dogs weighing 10.2–14.5 kg and 40 male Sprague–Dawley rats weighing 210–240 g were used. Dogs were given an oral dose of 1 mg/kg nilvadipine in the form of HPMC solid dispersion in a gelatin capsule, and rats were given an oral dose of 3.2 mg/kg nilvadipine as polyethylene glycol 400 solution after going without food overnight. Rats were anaesthetized with diethyl ether at predetermined intervals. Blood was collected by cardiac puncture and immediately cooled in an ice-bath and centrifuged at 4 °C. BNPP was added to the separated plasma to give a final concentration of 10^{-3} *M*. Dog blood was collected from a cephalic vein and centrifuged. These plasma samples were stored at −20 °C until analysed.

RESULTS AND DISCUSSION

Separation

Typical chromatograms obtained from the dog and rat plasma samples are shown in Figs. 2 and 3. As shown in Figs. 2A and 3A, the background peaks of the blank dog plasma and rat plasma containing BNPP were completely separated from the peaks of nilvadipine and the internal standard. Figs. 2C and 3C show typical chromatograms of the plasma from a dog and a rat after an oral dose of nilvadipine. There was no interference at the retention times of nilvadipine and internal standard. Although not shown in the figure, there were no interfering peaks for nilvadipine quantitation in the chromatograms obtained from human, mouse, rabbit plasma or human urine.

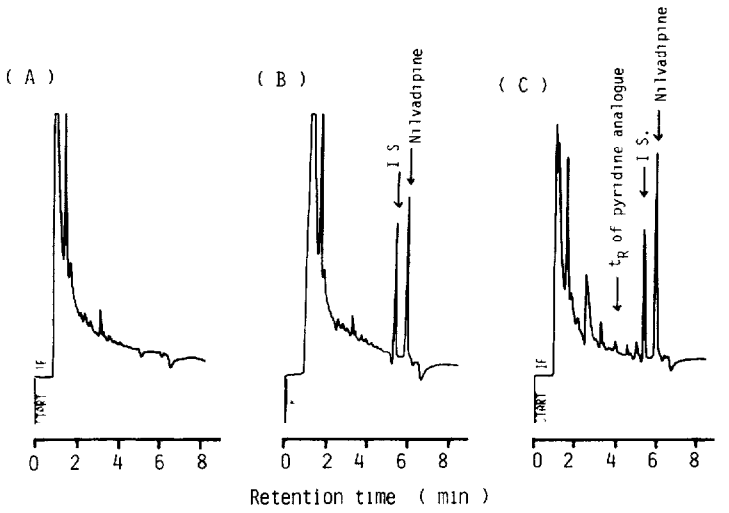

Fig. 2. Chromatograms of (A) blank dog plasma, (B) dog plasma containing 5 ng/ml nilvadipine and 5 ng/ml internal standard and (C) plasma collected from a dog after an oral dose of 1 mg/kg nilvadipine.

Recovery

Absolute overall recovery from four replicate analyses of nilvadipine-spiked sample were 71.5 ± 4.7% (mean ± S.D.) for dog plasma of 5 ng/ml and 83.6 ± 1.0% for human urine of 10 ng/ml.

Calibration graph

Typical calibration graphs for dog plasma and urine are shown in Table I. The calibration graphs show good linearity in each range. The lower limit of sensitiv-

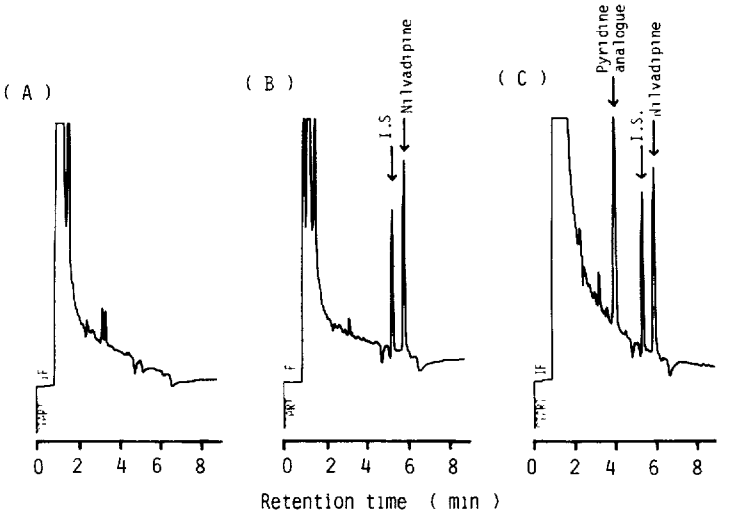

Fig. 3. Chromatograms of (A) blank rat plasma, (B) rat plasma containing 5 ng/ml nilvadipine and 5 ng/ml internal standard and (C) plasma collected from a rat after an oral dose of 3.2 mg/kg nilvadipine. These rat plasma samples contained 10^{-3} *M* BNPP.

TABLE I

TYPICAL CALIBRATION GRAPH FOR DOG PLASMA AND HUMAN URINE

Sample	Concentration range (ng/ml)	Slope	Intercept	Correlation coefficient
Dog plasma	0.1–100	0.20395	0.01502	0.9983
Human urine	0.5–100	0.10806	0.00566	0.9999

ity for dog plasma was 0.1 ng/ml with a signal-to-noise ratio of 2. Although peaks were obtained at lower concentrations in human urine, 0.5 ng/ml was the lower limit of sensitivity when the variation of the background peaks in the urine was taken into account.

Reproducibility

Reproducibility was evaluated by performing five replicate analyses of spiked plasma and urine samples. The results for dog plasma, rat plasma containing BNPP and human urine are given in Table II. The coefficients of variation (C.V.) were less than 5% and the actual concentration of nilvadipine measured by GC ranged from 94 to 104% in all plasma and urine samples except for the lowest concentration in dog plasma. This GC method for determination of nilvadipine in plasma and urine thus provides good accuracy and precision.

TABLE II

REPRODUCIBILITY OF THE DETERMINATION OF NILVADIPINE IN PLASMA AND URINE

Sample	Actual concentration (ng/ml)	Concentration found (ng/ml)		Percentage of actual concentration	Coefficient of variation (%)
		Mean ± S.D.	Range		
Dog plasma	0.25	0.22 ± 0.01	0.21–0.24	88	5.1
	0.5	0.51 ± 0.02	0.48–0.52	102	3.9
	1.0	1.01 ± 0.03	0.97–1.05	101	3.3
	5.0	5.00 ± 0.22	4.66–5.21	100	4.4
Rat plasma*	0.25	0.24 ± 0.01	0.23–0.26	96	4.7
	0.5	0.52 ± 0.01	0.51–0.54	104	2.5
	1.0	1.02 ± 0.02	0.99–1.05	102	2.3
	5.0	4.89 ± 0.09	4.79–4.99	98	1.9
Human urine	0.5	0.47 ± 0.01	0.46–0.48	94	1.9
	1.0	0.98 ± 0.02	0.97–1.01	98	1.7
	2.5	2.53 ± 0.03	2.50–2.59	101	1.4
	10	10.0 ± 0.39	9.72–10.7	100	3.9

*Rat plasma sample containing 10^{-3} *M* BNPP was spiked with nilvadipine.

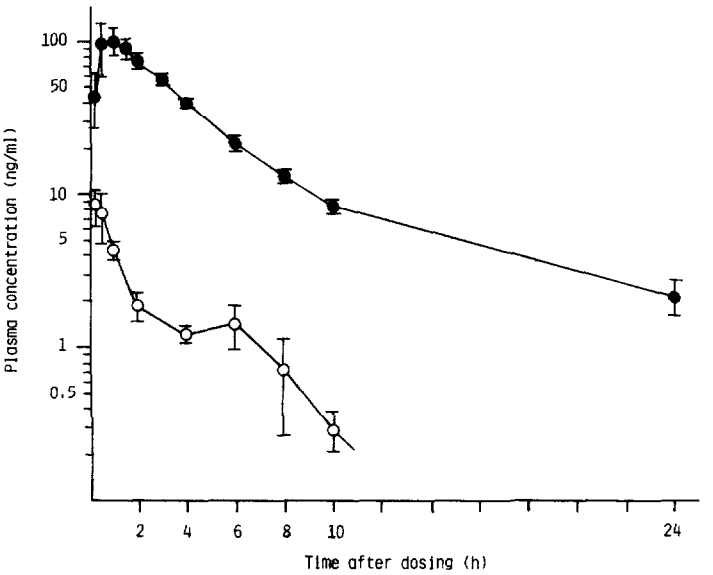

Fig. 4. Mean plasma concentrations of the unchanged drug in dogs (●, 1 mg/kg) and rats (○, 3.2 mg/kg) after an oral dose of nilvadipine. Values are mean ± standard error of the mean for six dogs and five rats.

Stability in plasma

Nilvadipine was stable in mouse, rabbit, dog and human plasma at 37°C up to 1 h, i.e. almost 100% of the spiked nilvadipine remained after incubation. In rat plasma, nilvadipine concentrations after incubation for 1 h at 4°C, room temperature and 37°C were 104, 81 and 43% of the initial concentration, respectively. But the nilvadipine concentrations in rat plasma containing 10^{-3} *M* BNPP, an esterase inhibitor [10], was the same before and after incubation at 37°C for 1 h. These results suggest that nilvadipine in rat plasma is hydrolysed by esterase and that its esterase has species specificity [11].

Plasma concentrations in animals

Plasma concentrations of the unchanged drug in dogs and rats after an oral dose of nilvadipine are shown in Fig. 4. Plasma concentrations of the unchanged drug in dogs peaked within 1 h, reached a mean of 124 ng/ml and decreased with a mean half-life of 6.37 h. In rats, the mean concentration peaked (8.51 ng/ml) at 15 min and decreased with a half-life of 1.75 h. As shown in Figs. 2C and 3C, the concentration of the pyridine analogue of nilvadipine was less than 5% of that of nilvadipine in dog plasma, but it was detected at a level of ca. 300% of nilvadipine in rat plasma. In other dihydropyridine calcium antagonists also, these pyridine analogues are known to be present in plasma as metabolites [12–15].

This method is simple and relatively sensitive, and we have mainly used it for pharmacokinetic studies in animals.

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