Journal of Chromatography, 530 (1990) 438–446
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5391

Note

Simultanous microdetermination of nicardipine and its pyridine metabolite in body fluids by capillary column gas chromatography with electron-capture detection

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(First received March 5th, 1990; revised manuscript received May 8th, 1990)

Nicardipine hydrochloride, (±)-2-(N-benzyl-N-methylamino)ethylmethyl-1,4-dihydro-2,6-dimethyl-4-(*m*-nitrophenyl)-3,5-pyridinedicarboxylate monohydrochloride (I, Fig. 1; subsequently referred to as nicardipine) is a calcium channel-blocking agent with potent oral vasodilating activity. The drug is used for the treatment of angina, hypertension and cerebrovascular disease [1].

Early methods for the determination of nicardipine, including gas chromatography (GC) with either electron-capture detection (ECD) [2] or mass spectro-

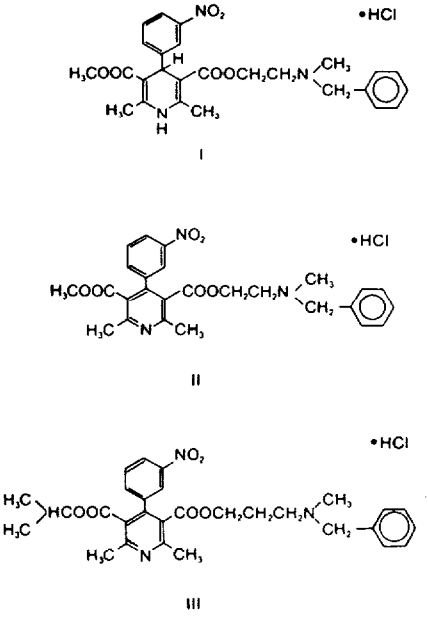

Fig. 1. Structures of nicardipine (I), its pyridine metabolite (II) and the internal standard (III).

0378-4347/90/$03.50

metric (MS) detection [3] are relatively non-specific because they require oxidation of nicardipine prior to GC analysis. This is because when nicardipine is injected onto a packed-column gas chromatograph it undergoes conversion, in varying degrees (up to 16%), into the pyridine analogue (II, Fig. 1) [4]. As the product of this oxidation is the pyridine metabolite of nicardipine [2], the methods measure the total concentration of nicardipine and its pyridine metabolite (II). The individual concentrations of nicardipine and its pyridine metabolite in human plasma have been determined [5] by a method in which nicardipine was separated from its pyridine metabolite by thin-layer chromatography prior to analysis by GC–MS. Subsequently, a high-performance liquid chromatographic method [6] for the determination of nicardipine and its pyridine metabolite in plasma has been reported; however, the limit of detection was 5 ng/ml for both nicardipine and its pyridine metabolite. Later, the same authors [4] developed the simultaneous determination of nicardipine and its pyridine metabolite from 1 ml of plasma by GC–ECD with a limit of detection of 1 ng/ml. However, this method required triple extraction, and there was no reference to body fluids other than plasma.

This paper describes a simple and rapid simultaneous determination for nicardipine and its pyridine metabolite in body fluids, especially in urine, bile and whole blood, using a 100-μl volume.

EXPERIMENTAL

Chemicals and reagents

Nicardipine hydrochloride (I), the pyridine metabolite (II) [(±)-2-(N-benzyl-N-methylamino)ethylmethyl-2,6-dimethyl-4-(*m*-nitrophenyl)-3,5-pyridinedicarboxylate monohydrochloride] and (±)-2-(N-benzyl-N-methylamino)propylisopropyl-2,6-dimethyl-4-(*m*-nitrophenyl)-3,5-pyridinedicarboxylate monohydrochloride (III, Fig. 1) as an internal standard were obtained from Takeshima Pharmaceutical (Tokyo, Japan). All other reagents used were pesticide quality (Wako, Osaka, Japan).

Gas chromatography

A Hewlett-Packard Model 5890 gas chromatograph was equipped with a ^{63}Ni electron-capture detector and a cool on-column injector. GC was performed on a 15 m × 0.32 mm I.D. cross-linked polysiloxane (5% diphenyl and 95% dimethyl) fused-silica column (DB-5 column from J & W Scientific, Rancho Cordova, CA, U.S.A.) with a film thickness of 0.1 μm. Hydrogen was used as carrier gas at a flow-rate of 7 ml/min and nitrogen as make-up gas at a flow-rate of 60 ml/min. The oven was maintained at 260°C for 5 min, programmed from 260 to 300°C at 50°C/min and maintained at this final temperature for 5 min. This procedure was used to "burn-off" the residual extract. The detector temperature was maintained at 300°C. Chromatograms were recorded and peak-height ratios were calculated using a recording integrator (Hewlett-Packard Model 3392A).

At the injection site, a 1-m length of precolumn should be used and be removed after 50 injections. This procedure, which results in more reproducible standard curves, removes non-volatile extract components that accumulate and eventually cause the peaks to broaden and the sensitivity to decrease.

Standard curves and sample preparation

The two calibration standars were prepared as follows: separate primary stock solutions (1 mg/ml) were prepared in methanol for I, II and III. The final stock solutions of I and II (100 and 1000 ng/ml each) were prepared diluting the primary standards in methanol. The final stock solution of III (internal standard) (1000 ng/ml) was prepared in equal volumes of 0.1 *M* sodium acetate solution and 0.1 *M* hydrochloric acid. For the calibration standard, different volumes of the stock solution were evaporated under nitrogen and reconstituted in 100 μl of drug-free body fluids (except for whole blood: 50 μl of whole blood plus 50 μl of distilled water) to give final concentrations of 0.5, 1, 2.5, 5, 7.5, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 ng per 0.1 ml for both I and II. A 30-μl volume of internal standard solution (30 ng) was added to each calibration standard or sample (in 100 mm × 15 mm silanized test-tubes with screw caps), which was then alkalized by mixing with 1 ml of saturated borate solution and 200 μl of 2.0 *M* sodium hydroxide solution. These were extracted with 4 ml of ethyl acetate–hexane (1.25:2.75) by mixing for 5 min. Each sample was then centrifuged for 10 min at 3000 rpm. The organic extract was evaporated (*ca.* 10 min) under nitrogen to a final volume of *ca.* 1 ml at ambient temperature. The extracts were then transferred to 1-ml vials and evaporated to dryness under nitrogen. After the solvent had been completely removed, 50 μl of toluene were added to each vial and the vials were vortexed. A 1.0–1.5 μl aliquot of each extract was immediately injected into the gas chromatograph via a 10-μl gas-tight syringe, which was modified to hold a 110 mm × 0.21 mm I.D. fused-silica capillary "needle".

Recovery

The recovery was determined by extracting body fluids (urine, bile and plasma) prepared by spiking drug-free body fluid samples with known amounts of the drug. After extraction, the internal standard dissolved in methanol was added. Peak-height ratios of drugs to the internal standard were compared with unextracted standards. The unextracted standards were prepared by the addition of stock solutions of drugs and the internal standard to give a concentration equivalent to that of the extracted standards.

RESULTS AND DISCUSSION

Chromatograms

Chromatograms of extracts from rat plasma (A, E) human urine (B, F), rat bile (C, G) and rat whole blood (D, H) are shown in Fig. 2. No interfering peaks

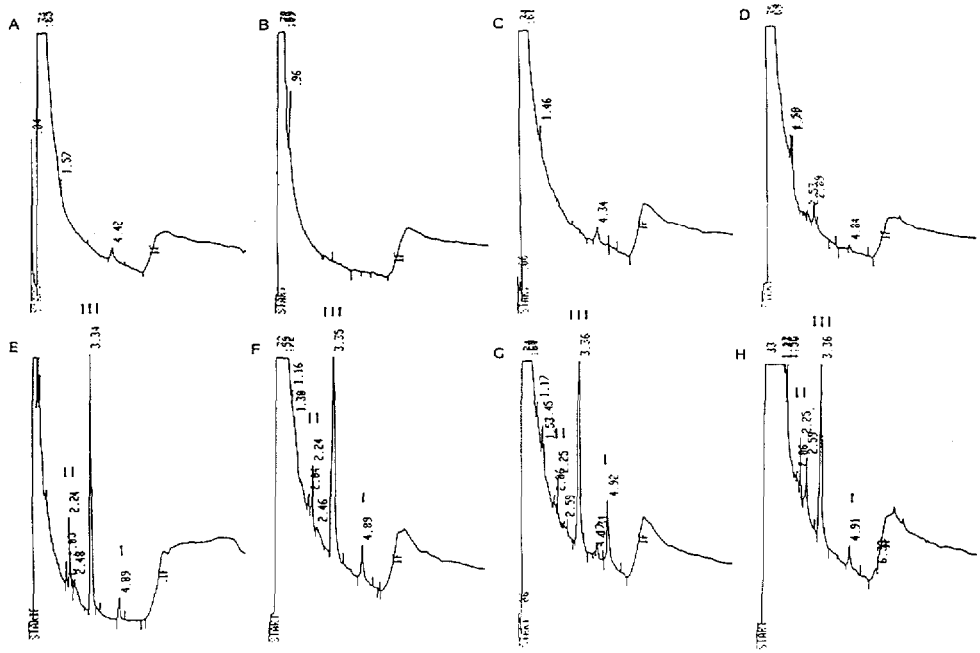

Fig. 2. Chromatograms of blank rat plasma (A), blank human urine (B), blank rat bile (C) and blank rat whole blood (D), and chromatograms of rat plasma (E), human urine (F), rat bile (G) and rat whole blood (H) spiked with 1 ng nicardipine (I) and its pyridine metabolite (II) each, and internal standard (III; 30 ng).

of endogenous substances appeared in the chromatograms of the blank samples (A, B, C and D in Fig. 2), and the peak shapes of I, II and III were excellent. The retention times of I, II and III were 4.90, 2.24 and 3.35 min, respectively. There were also no interfering peaks of endogenous components in the blank samples of rat urine, human plasma and human whole blood (not shown). When I was injected onto a bonded-phase fuse-silica capillary column (10 m × 0.32 mm I.D.; film thickness 0.25 μm) using an injector operating in the split mode (265°C at the injector and 245°C at the oven), less then 2% conversion to the pyridine metabolite (II) was observed [4]. In the present method, 3–4% conversion into II was observed, indicating no problem in the routine assay for I and II.

Recovery

The recoveries of I and II using this extraction method are listed in Table I. There were good recoveries from rat plasma, human urine and rat bile, and the percentages of extractable I and II were close to or above 90% at both low (1 ng per 0.1 ml) and high (25 ng per 0.1 ml) concentrations. The difference in recovery was minimal between human and rat urine, and between human and rat plasma.

These good recoveries of I and II with low coefficients of variation (C.V. less

TABLE I

RECOVERY FROM RAT PLASMA, HUMAN URINE AND RAT BILE

Spiked concentration (ng/0.1 ml)	Recovery (%)	Coefficient of variation (%)
Rat plasma		
Nicardipine		
1	91.5 ± 4.10	4.48
5	93.5 ± 3.77	4.03
25	100.1 ± 4.39	4.39
Pyridine metabolite		
1	98.1 ± 7.91	8.06
5	98.5 ± 6.13	6.22
25	95.8 ± 3.17	3.31
Human urine		
Nicardipine		
1	98.8 ± 3.32	3.36
25	98.2 ± 4.94	5.03
Pyridine metabolite		
1	91.3 ± 6.75	7.39
25	96.5 ± 4.35	4.51
Rat bile		
Nicardipine		
1	93.5 ± 3.82	4.09
25	93.4 ± 3.89	4.16
Pyridine metabolite		
1	89.5 ± 8.47	9.46
25	93.3 ± 3.16	3.39

than 9.5%) provide evidence of the good reproducibility of the method. The recovery of III was 98.0 ± 1.08 (mean ± S.D., $n = 3$), determined as unextracted nicardipine as an internal standard.

Standard curves

Standard curves for I and II are non-linear but reproducible over the concentration range 0.5–100 ng per 0.1 ml. Representative standard curves of I and II from the rat plasma (A), human urine (B) and rat bile (C) are shown in Fig. 3. The standard curves were obtained by fitting the data to a second-order polynomial with equal weight on each datum. The curves from the urine and bile were almost the same as that from the plasma. The standard curve from the whole blood with a concentration range from 0.5 to 50 ng per 0.05 ml was the same as that from the plasma. Non-linearity in the standard curves is well in accord with the results of Wu *et al.* [4], and may be due to the ratio of the flow-rates for the carrier and

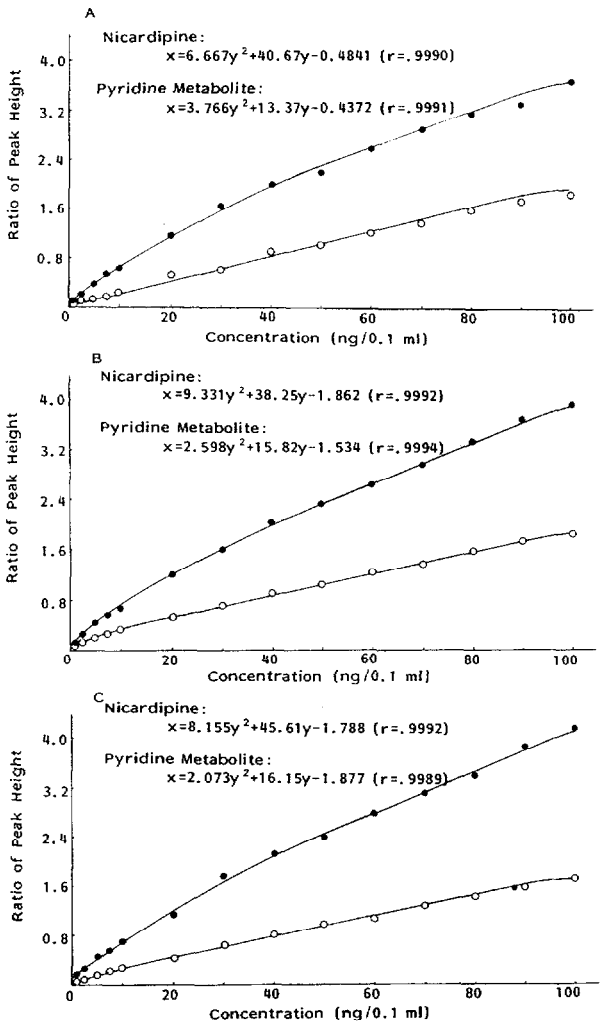

Fig. 3. Representative standard curves of nicardipine (○) and its pyridine metabolite (●) spiked into 0.1 ml of blank rat plasma (A), blank human urine (B) and blank rat bile (C).

make-up gases or to the narrow linear range of the electron-capture detector, because non-linearity was also observed when known concentrations of drugs in toluene were injected, and because the values of recovery were almost the same for both low (1 ng per 0.1 ml) and high (25 ng per 0.1 ml) concentrations (Table I).

The higher ratio of II to the internal standard than I is due to the greater electron capture (some 2.5 times more than I), because the pyridine metabolite (II) has a greater π-electron density than I or the dihydropyridine derivative [4].

TABLE II

WITHIN-DAY PRECISION AND ACCURACY FROM RAT PLASMA

Spiked concentration (ng/0.1 ml)	Calculated concentration (mean ± S.D., $n=6$) (ng/0.1 ml)	Coefficient of variation (%)	Error (%)
Nicardipine			
1	1.03 ± 0.03	2.91	+3.00
5	4.87 ± 0.13	2.67	−2.60
25	24.5 ± 0.51	2.08	−2.00
50	52.3 ± 1.49	2.85	+4.60
Pyridine metabolite			
1	1.02 ± 0.04	3.92	+2.00
5	5.01 ± 0.11	2.20	+0.20
25	24.9 ± 0.63	2.53	−0.40
50	50.6 ± 1.55	3.06	+1.20

Precision and accuracy for within-day and between-day assays

The within-day precision and accuracy of this method were checked by analysis of six replicate samples to which known amounts of I and II were added to rat plasma (Table II). C.V. values for I and II at concentrations of 1, 5, 25 and 50 ng per 0.1 ml were all 4% or less. Calculated concentrations for all samples were within 4.6% of the actual spiked concentrations.

TABLE III

BETWEEN-DAY PRECISION AND ACCURACY FROM RAT PLASMA

Spiked concentration (ng/0.1 ml)	Calculated concentration (mean ± S.D., $n=6$) (ng/0.1 ml)	Coefficient of variation (%)	Error (%)
Nicardipine			
1	1.01 ± 0.04	3.96	+1.00
5	4.94 ± 0.21	4.25	−1.20
25	25.0 ± 0.65	2.60	0.00
50	50.5 ± 1.49	2.95	+1.00
Pyridine metabolite			
1	1.00 ± 0.04	4.00	0.00
5	5.02 ± 0.21	4.18	+0.40
25	24.5 ± 0.84	3.43	−2.00
50	50.1 ± 0.87	1.74	+0.20

The between-day precision and accuracy was checked by analysis of four concentrations of I and II (1, 5, 25 and 50 ng per 0.1 ml) over six days (Table III). C.V. values were less than 4.3% for all concentrations. Calculated concentrations for all samples were within 2% of the actual spiked concentrations.

Because the precision and accuracy for within-day and between-day assays were excellent, specimens could be analysed using only one standard to check colomn conditions for a daily analysis.

Pharmacokinetic studies

This assay method was applied to determine simultaneously the plasma levels of I and II after intravenous and hepato-portal administration in male rats (Fig. 4). There was a considerable amount of pyridine metabolite in the plasma after hepato-portal administration. The dose-normalized area under the curve (AUC) on a per milligram basis of nicardipine was 15.8 ± 2.05 and $3.14 \pm 1.23 \times 10^3$ ng min/ml (mean $\pm$ S.D., $n = 6$) for intravenous and hepato-portal administration, respectively. The calculated hepatic first-pass effect of nicardipine was 80%.

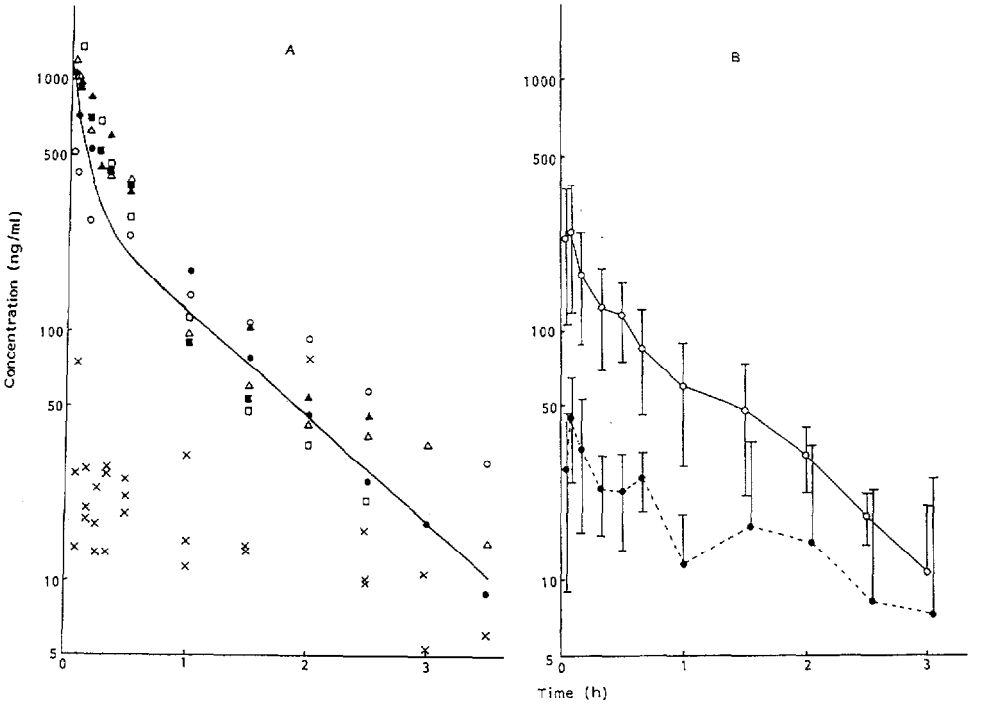

Fig. 4. Time courses of nicardipine (○) and its pyridine metabolite (●) in plasma of male rats after (A) intravenous injection of 2.0 mg/kg nicardipine and (B) hepato-portal infusion of 4.0 mg/kg nicardipine for 5 min (mean $\pm$ S.D., $n = 6$). Each point plotted in (A) is for an individual animal, because the sampling schedules differed slightly for each rat (ten samples per rat). The concentrations of the pyridine metabolite are designated by crosses.

The fractions of the dose of nicardipine recovered from the urine and bile during 7 h after intravenous injection of 2.0 mg/kg nicardipine to male rats were 0.004 ± 0.009 and 0.073 ± 0.030 (mean $\pm$ S.D., $n = 6$), respectively. The fraction of the dose of pyridine metabolite recovered from the bile was 0.013 ± 0.008; however, there was not a detectable concentrations in the urine. Very low excretions of nicardipine and its pyridine metabolite into the urine and bile were observed, in accord with that reported by Higuchi *et al.* [7]; the bile concentrations for nicardipine ranged from 67 to 158 ng/ml and those for pyridine metabolite ranged from 6 to 31 ng/ml.

The present assay method using 100 μl of body fluid (50 μl for whole blood) is simple and rapid compared with that of Wu *et al.* [4], in which it is necessary to use a laborious acid–base partitioning step in the extraction procedure (*i.e.* triple extraction) in order to remove endogenous components because a 1-ml plasma sample is used. Furthermore, the limit of detection of the present method is as low as 5 ng/ml. Therefore, the present method is considered to be sufficiently sensitive and reliable both for detailed pharmacokinetic studies in small animals and for clinical use (therapeutic drug monitoring) in patients.

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