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CAPILLARY COLUMN GAS CHROMATOGRAPHIC METHOD USING ELECTRON-CAPTURE DETECTION FOR THE SIMULTANEOUS DETERMINATION OF NICARDIPINE AND ITS PYRIDINE METABOLITE II IN PLASMA

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SUMMARY

A rapid, specific and direct method based on capillary column gas chromatography with electron-capture detection is described for the simultaneous determination of nicardipine, a new calcium antagonist, and its pyridine metabolite II in human plasma. In this method, the nicardipine, its pyridine metabolite II and internal standard are extracted from the plasma and then partially purified by acid–base partitioning prior to the final injection onto the capillary column gas chromatograph for quantification by means of an electron-capture detector. The quantification limit of the method is 1 ng/ml of plasma for both nicardipine and its pyridine metabolite II. The coefficients of variation for nicardipine and the pyridine metabolite II at concentrations of 1–50 ng/ml are $<7\%$ and $<9\%$ ($n=4$), respectively. The method has been validated against a previously developed high-performance liquid chromatographic method (sensitivity 5 ng/ml).

INTRODUCTION

Nicardipine hydrochloride, (±)2-(N-benzyl-N-methylamino)ethylmethyl-1,4-dihydro-2,6-dimethyl-4-(*m*-nitrophenyl)-3,5-pyridinedicarboxylate monohydrochloride (I, Fig. 1) is an orally active calcium antagonist with potent vasodilating activity. The therapeutic efficacy of this compound for the treatment of angina, hypertension and cerebrovascular disease is under investigation.

Nicardipine hydrochloride, which is effective at relatively low doses, undergoes extensive first-pass metabolism to produce several metabolites [1]. Therefore, a sensitive and specific method is required for its determination in human plasma. Various methods have been reported for the determination of nicardipine in plasma. The early methods that were developed used gas chromatography (GC) with packed columns and electron-capture detection (ECD) [2]. The ECD tech-

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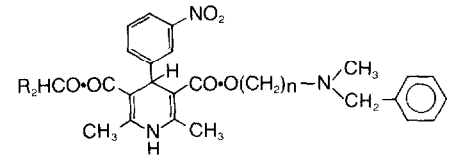
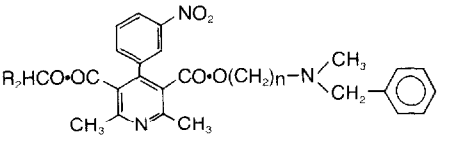


I R = H, n = 2
III R = CH_3, n = 3



II R = H n = 2
IV R = CH_3, n = 3

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

nique was employed because of its ability to provide high sensitivity for detection of compounds with structures related to nicardipine. However, when nicardipine is injected onto a packed column gas chromatograph it undergoes conversion, in varying degrees (up to 16%), to the pyridine analogue II (Fig. 1). Therefore, in these early methods the nicardipine was intentionally oxidized to II prior to GC analysis. In addition to circumventing problems associated with the oxidation of nicardipine during GC analysis, the chemical oxidation of nicardipine to the pyridine analogue II prior to analysis also provided better sensitivity owing to the greater electron-capture capacity and superior chromatographic properties of II compared to those of nicardipine. The sensitivity of these methods was 1 ng of nicardipine per ml of plasma. However, since II is a metabolite of nicardipine, the methods in which nicardipine is oxidized to II prior to analysis determine the combined concentration of nicardipine and metabolite II and as such are relatively non-specific. Pharmacokinetic analysis of data generated using these methods can be misleading in view of the fact that the vasodilative activity of the pyridine metabolite II is only 1/300 that of the parent drug. A thin-layer chromatographic (TLC)–GC–mass spectrometric (MS) method for determination of the individual concentrations of nicardipine and its pyridine metabolite II has been reported [3].

However, this method which has a sensitivity of 2 ng of nicardipine per ml of plasma is too laborious for use in the routine analysis of large numbers of samples. We recently reported a high-performance liquid chromatographic (HPLC) method for determination of the individual concentrations of nicardipine and the pyridine metabolite II [4]. This method, although suitable for routine application, has a sensitivity of only 5 ng/ml and is not sufficiently sensitive for many pharmacokinetic studies. In order to achieve the required sensitivity (1 ng/ml) it is necessary to use ECD. In an attempt to achieve the required sensitivity and avoid the oxidative degradation that occurs on packed columns, we investigated

the use of a fused-silica capillary column in conjunction with the split injection technique. Using this approach, nicardipine was found to undergo $\leq 2\%$ conversion to the pyridine analogue II and a sensitivity of ≥ 1 ng per ml of plasma was achieved as required for pharmacokinetic studies. The capillary GC method, its validation and its application to a pharmacokinetic study are described here.

EXPERIMENTAL

Chemicals and reagents

Nicardipine hydrochloride (I) was from Syntex Research (Palo Alto, CA, U.S.A.). 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-1,4-dihydro-2,6-dimethyl-4-(*m*-nitrophenyl)-3,5-pyridinedicarboxylate monohydrochloride (III, see Fig. 1) were obtained from Yamanouchi Pharmaceutical (Tokyo, Japan).

Toluene was HPLC grade (Fisher Scientific, Fair Lawn, NJ, U.S.A.), diethyl ether was distilled in glass (Burdick & Jackson Labs., Muskegon, MI, U.S.A.) and all other chemicals were of reagent grade. Acid buffer was prepared by mixing equal volumes of 0.1 *M* sodium acetate solution and 0.1 *M* hydrochloric acid.

Gas chromatography

A Hewlett-Packard Model 5880 gas chromatograph equipped with an electron-capture detector was used. GC was performed on a 10 m×0.32 mm I.D. cross-linked methylsilicone fused-silica column (DB-1 column from J & W Scientific, Rancho Cordova, CA, U.S.A.) having a film thickness of 0.25 μm. The gas chromatograph was equipped with a Hewlett-Packard split injection port capillary system. The injector lines contained ca. 100 mg of 3% SP 2100 which was obtained from Supelco (Bellefonte, PA, U.S.A.). A column flow-rate (helium) of 2.8 ml/min, a split ratio of 1:44 and a make-up gas flow-rate (argon–methane, 95:5) of 33 ml/min was used. The oven was maintained at 245°C, the injector at 265°C and the detector at 300°C. Injections were made by means of a Hewlett-Packard 7672 autosampler.

Preparation of internal standard

The internal standard, (±)2-(N-benzyl-N-methylamino)propylisopropyl-2,6-dimethyl-4-(*m*-nitrophenyl)-3,5-pyridinedicarboxylate (IV, see Fig. 1) was prepared by oxidation of III. The procedure was as follows. Transfer 2 ml of a solution of III in ethanol (10 μg/ml) to a 15-ml culture tube. Evaporate the solution to dryness and dissolve the residue in 2 ml of 0.1 *M* hydrochloric acid. Add 0.3 ml of a 1% solution of sodium nitrite (freshly prepared). Cap the tube and incubate for 1 h at 45°C. Cool the reaction mixture and add 500 μl of 2.0 *M* sodium hydroxide. Extract the pyridine analogue IV with diethyl ether (4 ml). Repeat the extraction and combine the organic extracts. Evaporate the organic solvent and reconstitute the residue in 2 ml of ethanol to give a solution of IV containing approximately 10 μg/ml.

Standard solutions

Stock solutions of nicardipine hydrochloride (I) and the monohydrochloride of its pyridine metabolite II at a concentration of 1 mg/ml were prepared in ethanol. The working solution of internal standard IV contained 20 ng of IV per 500 μl of 1% ethanolic acid buffer. Spiking solutions containing 1, 2, 5, 10, 20, 35 and 50 ng of the hydrochloride salts of nicardipine (I) or its pyridine metabolite II per 500 μl of 4% ethanolic acid buffer were prepared by serial dilution of the stock solutions.

Sample preparation for the determination of nicardipine and its pyridine metabolite II

Human plasma (0.1–1.0 ml) was transferred into 15-ml culture tubes (16×25 mm) fitted with Polytef-lined screw caps. The volume was adjusted to 1 ml by the addition of blank human plasma. Ethanolic acid buffer (4%, 1 ml), 500 μl of internal standard solution (IV, 20 ng) and 200 μl of 2.0 *M* sodium hydroxide were added to the sample. The mixture was agitated on a vortex mixer for 15 s. The mixture was extracted with 4 ml of diethyl ether by shaking on a mechanical shaker for 5 min and then centrifuging for 5 min at 2750 *g*. The organic layer was transferred to another 15-ml tube. To the organic phase were added 2 ml of 0.1 *M* hydrochloric acid and the mixture was shaken for 5 min. The mixture was centrifuged and the organic layer was removed and discarded by using a vacuum aspirator. The aqueous fraction was made alkaline by the addition of 200 μl of 2.0 *M* sodium hydroxide. The mixture was extracted with 4 ml of diethyl ether as described previously.

After centrifugation, the organic layer was transferred to another 15-ml culture tube and the solution was evaporated to dryness under a stream of nitrogen in a water bath (45°C). The residue was immediately dissolved in 50 μl of toluene and the solution was transferred to an autosampler vial. Of this final solution, 1.7 μl were injected onto the gas chromatograph for analysis.

Quantification

Concentrations of nicardipine and the pyridine metabolite II in unknown samples of plasma were calculated by reference to their corresponding calibration curves, which were generated using 1-ml samples of blank plasma spiked with nicardipine hydrochloride and the monohydrochloride of the pyridine metabolite II at concentrations of 1, 2, 5, 10, 20, 35 and 50 ng/ml. The samples of spiked plasma were processed as described above except for the addition of the ethanolic acid buffer (4%, 1 ml).

Calibration curves were obtained by plotting the ratio of the peak height for the analyte to that of the internal standard (y) against the quantity of analyte added (x) and fitting the data to a second order polynomial of the form $x=a+by+cy^2$ using least-squares analysis.

RESULTS AND DISCUSSION

Under the capillary column GC conditions employed, the pyridine analogue II, the internal standard and nicardipine are well separated from each other and

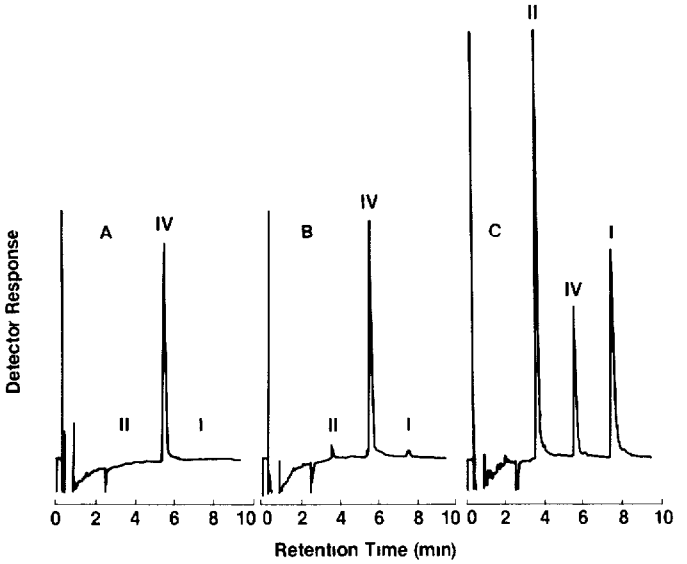


Fig. 2. GC tracings for 1 ml of blank human plasma spiked with internal standard (IV; 20 ng) and with both nicardipine hydrochloride (I) and pyridine metabolite II hydrochloride (II) each at the following concentrations: (A) 0 ng, (B) 1 ng and (C) 50 ng.

have retention times of 3.64, 5.60 and 7.55 min, respectively. A typical example of the chromatogram obtained for blank plasma processed by this method is shown in Fig. 2A. Clearly there are no endogenous components that are co-eluted with nicardipine or the pyridine metabolite II. Representative chromatograms for blank plasma spiked with 1 or 50 ng of nicardipine hydrochloride and the pyridine metabolite II hydrochloride, and 20 ng of internal standard and processed by this method are shown in Fig. 2B and C, respectively. When nicardipine is analyzed by packed-column GC, it undergoes significant oxidation to II (up to 15.5%). Two sharp peaks corresponding to nicardipine and the pyridine metabolite II are observed when nicardipine is injected onto a packed column. This indicates that

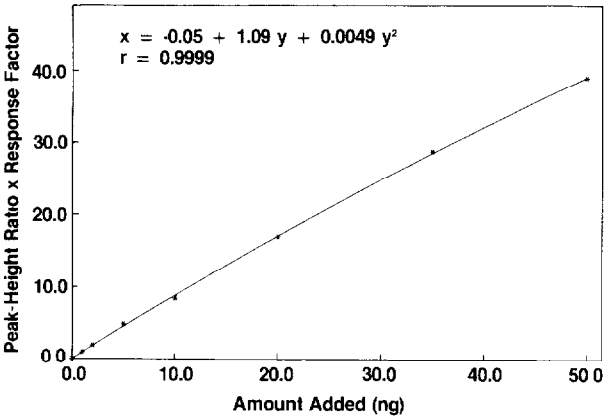


Fig. 3. Typical calibration curve for nicardipine hydrochloride spiked into 1 ml of blank human plasma.

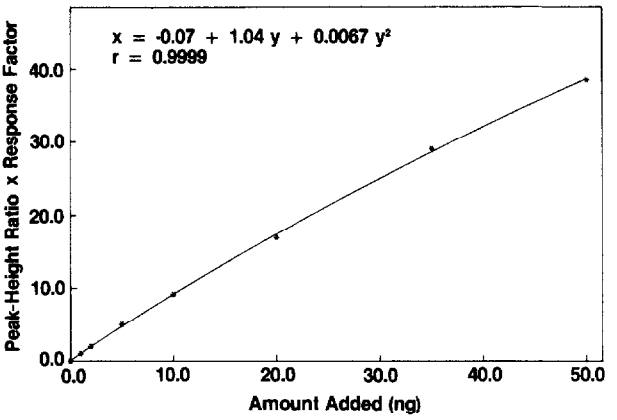


Fig. 4. Typical calibration curve for pyridine metabolite II hydrochloride spiked into 1 ml of blank human plasma.

the decomposition occurs in the injector. When nicardipine is injected onto a bonded-phase fused-silica capillary column using an injector operating in the split mode, $<2\%$ conversion to the pyridine metabolite II is observed. The difference in the extent of conversion probably is the result of the different injection techniques employed. In the capillary injector, the flow through the injector is much higher than with the packed-column injector. Consequently, the length of time that the sample resides in the capillary injector is much less than that for the packed-column injector, with the result that the extent of decomposition is reduced.

To assess the extent of conversion of nicardipine to the pyridine analogue II during the extraction procedure and the capillary column GC analysis, samples of plasma spiked only with nicardipine were analyzed by this procedure. The data obtained show that, overall, less than 3% ($2.5 \pm 0.4\%$, $n=7$) conversion occurs.

TABLE I

ACCURACY OF THE METHOD FOR THE DETERMINATION OF NICARDIPINE IN SAMPLES OF SPIKED PLASMA

Total of four samples for each level of spiking.

Concentration added (ng/ml)	Concentration found (mean ± S.D.) (ng/ml)	Coefficient of variation (%)
1	1.0 ± 0.0	1.4
2	2.1 ± 0.1	4.5
5	5.0 ± 0.3	6.7
10	9.9 ± 0.3	3.3
20	20.0 ± 0.1	0.3
35	35.1 ± 0.3	0.7
50	50.0 ± 0.1	0.3

TABLE II

ACCURACY OF THE METHOD FOR THE DETERMINATION OF PYRIDINE METABOLITE II IN SAMPLES OF SPIKED PLASMA

Total of four samples for each level of spiking.

Concentration added (ng/ml)	Concentration found (mean ± S.D.) (ng/ml)	Coefficient of variation (%)
1	1.1 ± 0.1	8.9
2	2.1 ± 0.1	4.6
5	5.2 ± 0.2	3.4
10	10.0 ± 0.1	1.0
20	19.7 ± 0.8	4.3
35	35.4 ± 1.1	3.1
50	49.8 ± 0.5	0.9

This extent of conversion is comparable with that observed ($1.3 \pm 0.8\%$, $n=5$) during the HPLC method reported previously [4].

To obtain chromatograms of the type shown in Fig. 2A for blank plasma, it is necessary to use the acid–base partitioning step in the extraction procedure in order to remove endogenous components. During routine use of this method, it was found that after approximately ten injections endogenous components in the

TABLE III

COMPARISON OF THE CONCENTRATIONS OF NICARDIPINE AND ITS METABOLITE II IN PLASMA AS DETERMINED BY THE CAPILLARY COLUMN GC-ECD METHOD AND BY AN HPLC METHOD

Sample	Concentration (ng/ml)			
	Nicardipine		Metabolite II	
	GC-ECD (y)	HPLC (x)	GC-ECD (y)	HPLC (x)
1	19.0	20.5	5.5	5.6
2	31.7	32.4	5.3	4.8
3	29.7	29.2	4.4	4.2
4	69.1	72.7	16.2	14.1
5	124.9	126.4	41.9	35.2
6	86.2	84.4	32.5	25.6
7	32.9	32.4	10.1	8.5
8	17.5	15.9	5.3	4.7
	$y=0.9835\,x+0.5745$ ($r=0.9991$)		$y=1.2377\,x-0.7387$ ($r=0.9986$)	

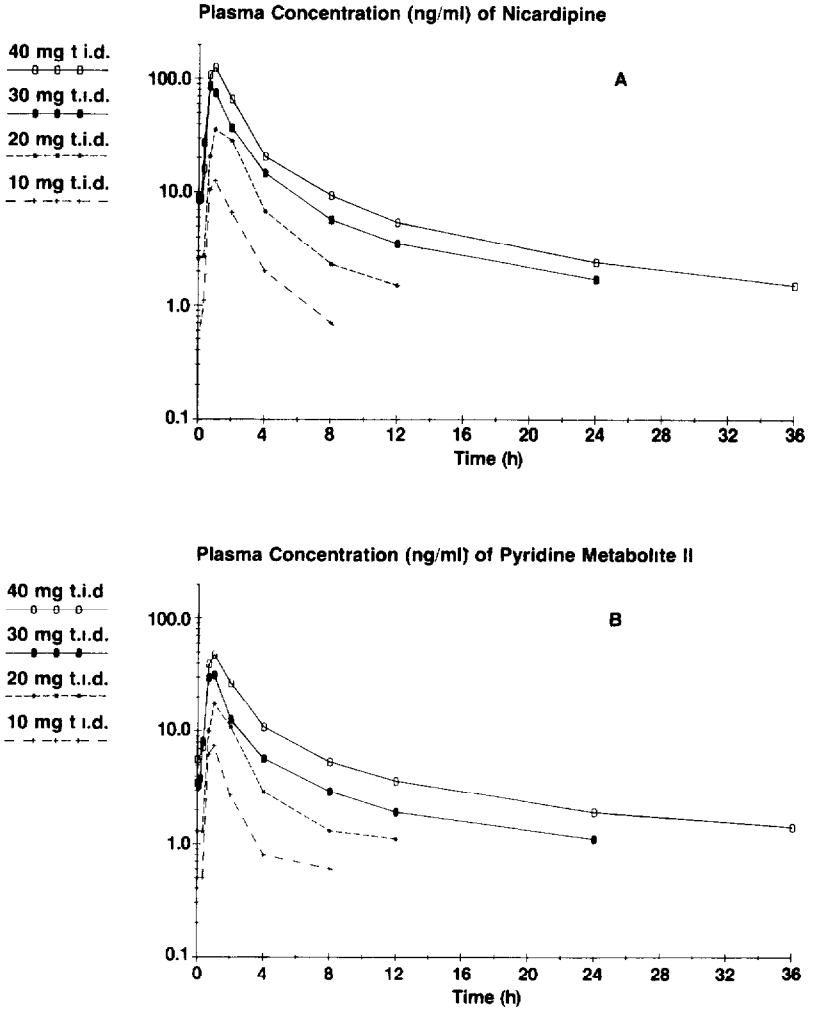


Fig. 5. Mean plasma concentration–time profiles for nicardipine (A) and for pyridine metabolite II (B) during multiple oral administration of nicardipine hydrochloride (10, 20, 30 or 40 mg three times a day, t.i.d.). Each point is the mean of values for plasma collected from six subjects following a single dose of nicardipine hydrochloride on the morning of the fourth day of each dosing regimen

plasma which have long retention times start to be eluted. These components can interfere with the quantification of nicardipine and pyridine metabolite II. In order to avoid this problem, the GC column is heated automatically to 285 °C for 30 min after every ten injections.

Calibration curves for nicardipine hydrochloride and for pyridine metabolite II hydrochloride are non-linear but reproducible over the concentration range 1–50 ng/ml. Typical calibration curves for nicardipine and for pyridine metabolite II are shown in Figs. 3 and 4, respectively. Data for the recovery of nicardipine and pyridine metabolite II from samples of plasma fortified with these compounds in the range 1–50 ng/ml are shown in Tables I and II, respectively. The low coefficients of variation ($<9\%$ at all levels) provide evidence of the reproducibility of the method.

Fig. 6. Typical chromatogram for 1 ml of plasma collected from a subject during a multiple-dose study. For peak identification, see legend to Fig. 2.

The capillary column GC–ECD method described here was validated against the HPLC method reported previously [4]. Both methods were applied to samples of plasma obtained from subjects treated orally with nicardipine hydrochloride. The data obtained by these two methods are shown in Table III. The overall agreement between the results obtained for the concentrations of nicardipine and for the pyridine metabolite II by the two methods is good.

Mean plasma concentration–time profiles for nicardipine and metabolite II for six subjects following oral administration of a single dose of nicardipine hydrochloride (10, 20, 30 or 40 mg) on day 4 of a multiple-dose study (10, 20, 30 or 40 mg, three times a day) are presented in Fig. 5A and B, respectively. A typical chromatogram for 1 ml of plasma taken from a subject during this study is shown in Fig. 6.

In conclusion, the capillary column GC–ECD method described here is simple, specific and sensitive. The method can be partially automated and can be used for the routine analysis of nicardipine and the pyridine metabolite II in large numbers of samples from studies in which subjects receive 10–40 mg of nicardipine hydrochloride. The method is five times more sensitive than the previously reported HPLC method [4] and is the method of choice for the analysis of samples from pharmacokinetic studies of nicardipine hydrochloride.

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