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DETERMINATION OF NICORANDIL IN PLASMA USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH PHOTOCONDUCTIVITY AND ULTRAVIOLET DETECTION

APPLICATION TO PRE-CLINICAL PHARMACOKINETICS IN BEAGLE DOGS

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SUMMARY

A sensitive and precise method for the determination of nicorandil, a new anti-anginal medication, is reported. The method involves solid-phase extraction of the drug and internal standard using Bond-Elut® C_{18} extraction columns, reversed-phase high-performance liquid chromatography on a Zorbax®-Phenyl column and detection with photoconductivity and ultraviolet detection in series. Photoconductivity, performed with the Tracor 965 photoconductivity detector, provided a limit of detection of 2 ng/ml in plasma (between-day coefficient of variation of 15%) but the linear range of response was limited to only about 100 ng/ml. Ultraviolet detection in series with the photoconductivity detector extended the linear range of the analytical system to 1000 ng/ml (coefficient of variation 4.4%). The utility of the method is demonstrated in a dog pharmacokinetic study in which a 5-mg intravenous dose was compared to a 10-mg oral solution dose in six beagle dogs. The oral solution was absorbed rapidly, achieving an average maximum concentration of 857 ng/ml in 11.2 min. The absolute bioavailability of nicorandil in dogs in this study was determined to be 84.2%

INTRODUCTION

Nicorandil, N-[2-(nitrooxy)ethyl]-3-pyridinecarboxamide, is a potent vasodilator currently being developed for the treatment of angina pectoris and congestive heart failure by The Upjohn Company. The structures of nicorandil

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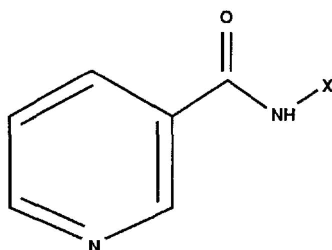
and the internal standard, N-[2(nitrooxy)propyl]-3-pyridinecarboxamide, hereafter referred to as I, are provided in Fig. 1. Reliable quantification of nicorandil at concentrations of 1 ng/ml in blood plasma is required to determine the time course of this drug in clinical trials. A previously reported method for nicorandil in blood plasma and urine relied on high-performance liquid chromatography (HPLC) with ultraviolet (UV) absorbance detection at 254 nm [1]. While suitable for early animal investigations employing large doses of the drug, this method was not sufficiently sensitive for reliable determinations of plasma concentrations below 50 ng/ml which are frequently encountered in clinical studies. We decided to investigate HPLC detection methods which would exploit the nitrate functionality of this compound to achieve our assay goals.

Nicorandil contains an alkyl nitrate ester functionality which is photochemically reactive, readily forming the free alcohol and nitrous acid in aqueous solution. HPLC detection techniques which can exploit this principle include photochemical–electrochemical detection [2,3], in which photolysis products are determined electrochemically, and photoconductivity detection, in which products are detected by their effect on the electrical conductivity of the chromatographic eluent. A commercially available instrument for photoconductivity is available from Tracor Instruments, the Model 965 photoconductivity detector [4]. This instrument has already been successfully applied to the determination of nitrate esters in biological fluids [5] and a variety of pesticide residues in food and feed products [6–10]. For these reasons we decided to develop a highly sensitive method for nicorandil in blood plasma using HPLC with photoconductivity detection.

This report will first describe the nature of the response of the photoconductivity detector to nicorandil. We will then describe the procedure developed for the determination of nicorandil in plasma samples and demonstrate the



X = $(CH_2)_2ONO_2$, Nicorandil

= $(CH_2)_3ONO_2$, Internal standard (I)

Fig. 1. Structures of nicorandil and the *n*-propyl homologue used as an internal standard.

utility of this method in an investigation of the pharmacokinetics of nicorandil in beagle dogs.

EXPERIMENTAL

Materials

All solvents used in sample preparation and chromatographic mobile phase were of HPLC quality (B&J, Muskegon, MI, U.S.A.). Water was obtained by purifying the laboratory-deionized supply with a commercial purification system (Milli-Q reagent water system, Millipore, Bedford, MA, U.S.A.). Successful operation of the photoconductivity detector requires high-quality deionized water. Authentic samples of nicorandil and the *n*-propyl homologue were provided by Chugai Pharmaceutical (Tokyo, Japan).

Extraction of plasma samples

Drug and internal standard (I) were extracted from plasma samples in a two-step solid-phase extraction procedure employing C_{18} bonded-phase columns (Bond-Elut®, Analytichem International, Harbor City, CA, U.S.A.). Briefly, a 1-ml plasma aliquot was mixed with 50 μl of the internal standard solution in water (2 μg/ml), and then drawn through a cartridge which had previously been conditioned by washing with two column volumes of methanol followed by two column volumes of water. The cartridge was then washed twice with 1 ml of water and then eluted with 100% acetonitrile. The extracted sample was reduced to a volume of less than 200 μl by evaporation under a stream of nitrogen. This residue was taken up in 1 ml of water and applied to a second column of the same type. After loading the sample and washing twice with 1 ml water, the column was completely dried by applying a vacuum for 10–15 min. In the absence of plasma proteins, nicorandil and the internal standard interact strongly with the C_{18} column's residual silanol groups. Under water-free conditions, acetonitrile acts as a weak solvent for these compounds [11]. Once dried, 2 ml of acetonitrile were passed through the column to remove weakly retained plasma constituents. Nicorandil and the internal standard were subsequently eluted with 1 ml of methanol–water (95:5, v/v). The extracts were evaporated to dryness under nitrogen gas, dissolved in 200 μl of mobile phase and transferred to autoinjector vials.

Standard and fortified control sample preparation

A stock solution of nicorandil was prepared by dissolving 10 mg of nicorandil in 1 ml of methanol and then diluting to 100 ml in a volumetric flask. Working solutions were prepared by serial dilution of this stock solution. The working solutions were stored frozen for daily use. On each assay day, fortified plasma standards from 2 to 1000 ng/ml were prepared by adding 50 μl of a working solution to 1 ml of drug-free plasma.

Aqueous standards of nicorandil, used to define the linear range of the method, were prepared by simply diluting aliquots of the working solutions with mobile phase to 200 µl after adding the internal standard.

The internal standard was prepared by dissolving 2 mg of I in 1 ml of methanol and then diluting to 1 l in a volumetric flask. A 50-µl aliquot provided 100 ng of the internal standard.

Chromatographic system

Samples were injected by an autosampler equipped with a 200-µl injection loop and a refrigerated sample compartment (ISS-100, Perkin-Elmer, Norwalk, CT, U.S.A.). Refrigeration of extracted samples at 5°C was required in order to insure stability for more than 12 h. It was determined that an injection volume of 160 µl out of the final volume of 200 µl was the maximum reliable injection volume, accounting for losses of sample during transfer to vials.

Separations of nicorandil and the internal standard from the plasma constituents was achieved on a 5 µm particle size, 15 cm×4.6 mm I.D. phenyl-bonded phase column (Zorbax® Phenyl, Dupont Instruments, Wilmington, DE, U.S.A.). A 1.5 cm×3.2 mm I.D. guard column containing phenyl bonded phase preceded the analytical column (Brownlee Labs., Santa Clara, CA, U.S.A.). The chromatographic mobile phase was acetonitrile–isopropyl alcohol–water (12:2:86, v/v), delivered at 1 ml/min (pressure of 7.6–10.3 MPa) by a dual-piston reciprocating pump (LDC Constametric III, LDC, Riviera

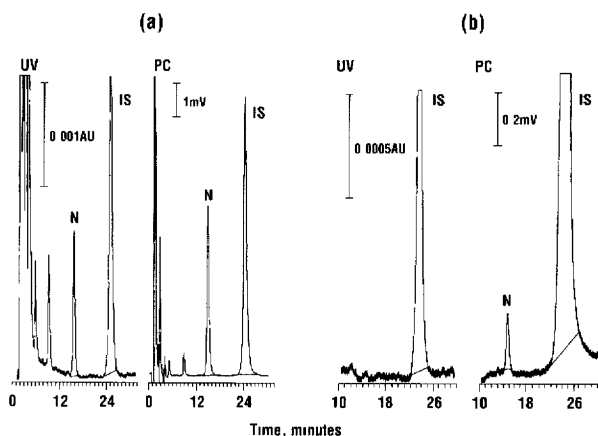


Fig 2. Representative chromatograms of plasma samples with UV absorption (254 nm) and photoconductivity detection, demonstrating the enhanced specificity and sensitivity achieved with photoconductivity detection. Peaks: N = nicorandil; IS = internal standard. (a) 55 ng/ml sample; (b) 2.7 ng/ml sample in the region of quantified peaks only. Chromatographic conditions: Zorbax-Phenyl, 5 µm particle size, column (15 cm×4.6 mm I.D.) with a mobile phase of acetonitrile–isopropyl alcohol–water (12·2 86, v/v) at 1 ml/min. The photoconductivity detector was operated at a range setting of 10× and attenuation of 20×.

Beach, FL, U.S.A.). The column effluent was directed first to a UV detector equipped with a 12-μl flow cell, a low-pressure mercury lamp and operated at 254 nm (LDC UV-1203, LDC). The effluent from the UV detector was then routed to the photoconductivity detector, which was operated without the ion-exchange system provided by Tracor for the conditioning of the mobile phase. In our hands the conditioning system introduced air bubbles which caused severe detector noise and instability. With the availability of high-quality, high-resistivity deionized water, the detector could be operated without this conditioning system.

Under these conditions, the UV detector elution time for nicorandil was 15 min while the internal standard eluted in 24 min. Because of the delay incurred by the photolysis chamber in the photoconductivity detector, an additional 0.5 min was required for peaks to appear on the second detector. Although photoconductivity detection provided a nitrate ester-selective response, large positive and negative peaks early in the chromatogram required that the retention time for nicorandil be delayed until a stable baseline could be assured. The signals from both detectors were monitored simultaneously with a strip-chart recorder and a minicomputer-based data system which utilized an in-house developed software system and a Harris 1000 computer. Representative chromatograms are shown in Fig. 2.

RESULTS AND DISCUSSION

UV and photoconductivity response characterization

The sensitivity and linear range of the UV and photoconductivity detectors were compared by injecting aqueous standards composed of 2–1000 ng of nicorandil and 80 ng of the internal standard solution (amounts injected on-column). The heights of the eluting peaks were measured and peak-height ratios calculated.

Whereas UV detection was linear to 1 μg/ml, the photoconductivity detector response was not. Linear calibration of the photoconductivity detector only extended to 100 ng/ml. With regard to sensitivity, the photoconductivity detector achieved twice the signal-to-noise ratio for low-concentration samples compared to UV detection. For determinations of nicorandil in blood plasma the increased sensitivity and the nitrate ester selectivity of the photoconductivity detector's response was considered to be a significant advantage. We therefore decided to employ both detectors in series for nicorandil determinations, depending on the photoconductivity detector for concentrations below 100 ng/ml and the UV detector for concentrations of 100 ng/ml to 1 μg/ml.

Validation and application

The method was applied to the analysis of plasma nicorandil levels obtained in a dog pharmacokinetic study. A 10-mg amount of nicorandil was administered orally as a solution (2 mg/ml) and 5 mg were administered intravenously to six beagle dogs (three males and three females). Blood samples were obtained at −10, 2, 5, 10, 15, 20, 30 and 45 min and 1, 1.5, 2, 3, 4, 6 and 8 h.) The blood samples were centrifuged at 1200 *g* for 20 min to obtain plasma which was stored at −20°C until analysis. In addition to these samples, control samples were prepared at 20.3 and 811 ng/ml by fortifying drug-free dog plasma. These were analyzed throughout the study at intervals to provide accuracy and precision data.

Plasma samples for each subject were determined on individual assay days, and calibration curves were prepared daily in the biological fluid. Calibration

TABLE I

SUMMARY OF CALIBRATION DATA FOR THE PHOTOCONDUCTIVITY AND UV DETECTOR

Response factors are calculated as peak-height ratio/standard concentration.

Standard concentration (ng/ml)	n	Response factor (mean ± S.D.)	Coefficient of variation (%)
Photoconductivity detector			
1.99	13	5.02 ± 0.76	15.2
4.97	13	5.20 ± 0.51	9.9
9.94	13	5.10 ± 0.45	8.8
24.9	13	5.10 ± 0.35	6.9
49.7	13	5.13 ± 0.39	7.6
99.4	13	5.26 ± 0.35	6.7
199	–	–	–
398	–	–	–
597	–	–	–
809	–	–	–
1011	–	–	–
Ultraviolet absorption (254 nm) detector			
9.94	12	3.31 ± 0.51	15.2
24.9	12	3.25 ± 0.22	6.7
49.7	13	3.39 ± 0.18	5.2
99.4	13	3.42 ± 0.13	3.8
199	14	3.56 ± 0.21	5.9
398	14	3.60 ± 0.17	4.7
597	14	3.59 ± 0.15	4.3
809	14	3.71 ± 0.18	4.9
1011	11	3.66 ± 0.19	5.2

data are summarized in Tables I and II. Linear correlation coefficients were all at least 0.999 for both detectors. In the routine application of this method to the analysis of plasma samples, the photoconductivity detector provided reliable detection down to concentrations of approximately 2 ng/ml. The inter-assay precision of calibration standards determined at 2 ng/ml by photoconductivity detection was 15%, while intra-assay variation of the response fac-

TABLE II

STANDARD CURVE REPRODUCIBILITY FOR NICORANDIL USING PHOTOCONDUCTIVITY AND ULTRAVIOLET ABSORPTION (254 nm) DETECTION

Standard curve No.	Slope ($\times 10^{-3}$)	Intercept
Photoconductivity detector		
1	5.01	−0.0062
2	4.60	−0.0008
3	5.84	−0.0267
4	5.48	−0.0240
5	4.97	−0.0042
6	5.77	−0.0040
7	5.55	−0.0025
8	5.52	−0.0012
9	4.88	−0.0011
10	5.15	−0.0009
11	5.55	−0.0015
12	5.35	−0.0059
13	5.46	0.0011
Mean	5.32	
C.V.(%)	6.7	
UV detector		
1	3.67	−0.062
2	3.36	−0.098
3	3.59	−0.010
4	3.49	−0.009
5	3.75	−0.038
6	3.58	−0.003
7	3.65	−0.005
8	3.63	−0.015
9	3.77	−0.030
10	3.86	−0.036
11	4.09	−0.033
12	3.74	−0.068
13	3.52	−0.028
Mean	3.67	
C.V. (%)	4.8	

TABLE III

SUMMARY OF LOW CONTROL (20.3 ng/ml) SAMPLE DATA

For each run, $n=3$.

Run No.	UV detection		Photoconductivity detection	
	Mean ± S.D. (ng/ml)	C.V. (%)	Mean ± S.D. (ng/ml)	C.V. (%)
1	18.2 ± 0.450	2.5	17.7 ± 0.850	4.8
2	18.8 ± 2 24	11.9	18.5 ± 2.42	13.1
3	20.2 ± 3.23	16.0	18.2 ± 2.08	11.5
4	19.5 ± 1.42	7.3	18.0 ± 0.340	1.9
5	17.2 ± 0.638	3.7	18.9 ± 1.94	10.3
6	18.8 ± 0.694	3.7	19.0 ± 1.62	8.6
7	19.6 ± 0.704	3.6	20.2 ± 0.83	4.1
8	19.0 ± 0.829	4.4	18.2 ± 1.40	7.7
9	16.7 ± 1.41	8.4	18.9 ± 0.77	4.1
10	17.2 ± 0.574	3.3	20.1 ± 0.776	3.9
11	17.3 ± 0.544	3.2	19.4 ± 1 20	6.2
12	13.8 ± 0.450	3.3	18.5 ± 0.368	2.0
13	17.2 ± 1.20	7.0	19.7 ± 0.953	4.8
Overall	18.1 ± 2.03	11.2	18.9 ± 1.56	8.3

TABLE IV

SUMMARY OF HIGH CONTROL (811 ng/ml) SAMPLE DATA

Ultraviolet absorption (254 nm) detector only. For each run, $n=3$.

Run No.	Mean ± S.D. (ng/ml)	C.V. (%)
1	756 ± 1.70	0.22
2	809 ± 3.00	0.37
3	774 ± 38.4	5.0
4	814 ± 1.70	0.21
5	798 ± 2.16	0.27
6	813 ± 17.9	2.2
7	830 ± 10.2	1.2
8	803 ± 19.4	2.4
9	775 ± 10.2	1.3
10	784 ± 15.7	2.0
11	717 ± 31.8	4.4
12	783 ± 11.6	1.5
13	824 ± 8.04	0.98
Overall	790 ± 34.8	4.4

tors was typically only 5–6%. Again, the photoconductivity calibration range was limited to 1–100 ng/ml.

The UV detector provided reliable detection to 10 ng/ml, with an inter-assay precision of 14%. Intra-assay precision, based on the reproducibility of response factors, was typically 4–5%, and linearity of response was observed up to the highest calibration standard, 1 μg/ml.

Two control samples (20.3 and 811 ng/ml) were assayed in triplicate on each assay day, and the results of these assays are provided in Tables III and IV. These quality control samples provide additional information regarding accuracy and precision, as well as information regarding the stability of the assay throughout the study. Overall precision of the photoconductivity detector for the low control sample, expressed as a coefficient of variation (C.V.), was 8.3% compared to 11.2% for UV detection. Precision of the method for the high control was 4.4% (UV detection only).

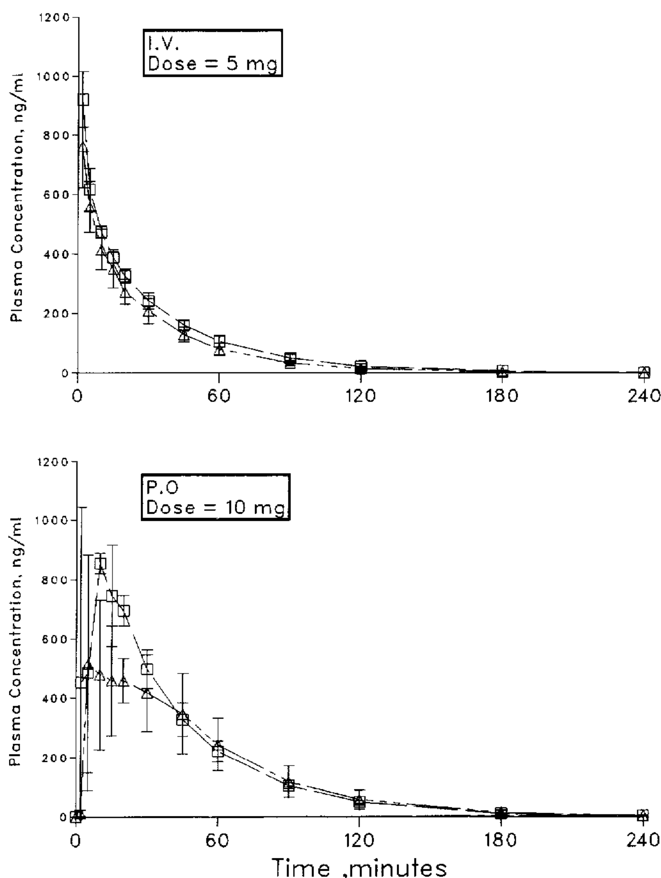


Fig 3 Mean (± S.D) nicorandil plasma concentration versus time profiles for male (□) and female (△) beagle dogs.

With regard to accuracy, the control samples indicated a negative bias for both detectors, with the effect more pronounced for the low control sample. Since linearity is well established for the method, the negative bias is most likely due to sample preparation error – either in the preparation of the control samples themselves or an intrinsic volume error in sampling. There was no trend in either the accuracy or precision of measurement for control samples throughout the study, indicating acceptable stability of the method.

Results of the dog pharmacokinetic study are shown graphically in Fig. 3. The mean plasma concentration for each sex is plotted along with error bars corresponding to one standard deviation. Differences attributable to gender were not observed. In the dog, analysis by the method of residuals [12] showed that the elimination of nicorandil appeared to conform to a single-compartment model with first-order elimination kinetics. The elimination half-life of 22.4–31.5 min and systemic clearance of 16.2–28.8 ml/min/kg is in good agreement with previously published results [1]. The oral solution was, in most cases, rapidly absorbed, and the absolute bioavailability of nicorandil in the dog was determined to be 84.2%.

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