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Note

Rapid and simple determination of nicorandil in rat plasma using a solid-phase extraction column

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Nicorandil, N-(2-hydroxyethyl)nicotinamide nitrate (ester), is a potent vasodilator that does not effect myocardial contractility and oxygen consumption and is used clinically for the treatment of angina pectoris [1–3]. It is also intended to use nicorandil concurrently with other drugs. The correlation between the coronary vasodilating action of nicorandil and blood concentration is apparent, despite an inter-individual variation in patients [4]. Hence monitoring of the blood concentration is needed, in particular to study changes due to the mutual action of drugs that effect drug metabolizing enzymes in the liver and other factors.

The published methods for the determination of plasma nicorandil in rats [5,6] have a number of drawbacks. Rats have to be subjected to laparotomy one by one to obtain plasma. Many rats are consequently required for the time-course of plasma concentration of the drug to be observed. Also, much time is required during the analysis to condense and dry the extracts obtained using ethyl acetate.

Accordingly, for the investigation of the pharmacokinetics of nicorandil, we

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have developed a method for the determination of nicorandil in rat plasma. It requires only small plasma samples and uses a solid-phase extraction column for easy pretreatment.

EXPERIMENTAL

Drugs

Nicorandil and N-(2-hydroxypropyl)nicotinamide nitrate (ester) were used as test drug and internal standard (I.S.), respectively. They were donated by Chugai Pharmaceutical (Japan).

Animals

Twenty-two male Sprague–Dawley rats (250–310 g) were housed three or four to a plastic walled cage (26×36×25 cm), and had unlimited access to food and water except for 12 h before and during the experiment. The animals were maintained on a 12-h light–dark cycle (light on from 08:00 to 20:00). The ambient temperature and humidity were kept at 22–24°C and ca. 60%, respectively.

Apparatus for determining the plasma drug level

For plasma separation from the blood sample, a hematocrit centrifuge (Compur M 1100, Miles-Sankyo, Japan) was used. A spectrophotometer (Type 228, Hitachi) was used for selecting the absorption wavelength to detect nicorandil in plasma. The drug plasma concentrations were determined by high-performance liquid chromatography (HPLC) (Pump-type M45J, Japan Waters Assoc.) with a UV detector (Type 440, Japan Waters Assoc.), and were calculated using a data module (Type 730, Japan Waters Assoc.). A stainless-steel column packed with octadecyl silica (μBondapak C_{18}, 300 mm×3.9 mm I.D., 10 μm particle size, Japan Waters Assoc.) was maintained at room temperature (22±2°C). Samples were injected with an automatic sample processor (Type WISP 7010B, Japan Waters Assoc.). The mobile phase was methanol–0.05 *M* $(NH_4)_2HPO_4$, pH 8.4 (35:65, v/v) and the flow-rate was 1.2 ml/min.

Detection wavelength

The UV detection wavelength for nicorandil and the I.S. was chosen by using a solution of 100 μg/ml in methanol. The maximum absorption of nicorandil was at ca. 260 nm. In addition, a 254-nm wavelength offered a high absorption and a good selectivity for nicorandil plasma determination, so this wavelength was selected.

Pretreatment of plasma for drug determination

For the extraction of nicorandil and the I.S., a Bond Elut (1 ml volume, No. 608101, Analytichem International) solid-phase extraction (SPE) column

containing phenylsilica was used. The column was prewashed twice with 1 ml of methanol, followed by two washings with 1 ml of distilled water. Distilled water (0.75 ml), 20 μl of plasma containing nicorandil and 50 μl of 10 μg/ml I.S. solution were added to the column. Two 1-ml portions of distilled water were passed through the column, then nicorandil and the I.S. retained on the column were eluted with 250 μl of methanol. A 40-μl volume was used for injection into the HPLC system. This volume of methanol did not affect the shape of the chromatogram. The chromatographic peaks of nicorandil and the I.S. from plasma samples were coincident with those of authentic standards.

Calibration curve

Nicorandil solutions were prepared of 0.625, 1.25, 2.5, 5, 10 and 20 μg/ml by dilution of stock standard with 0.05 *M* $(NH_4)_2HPO_4$. These solutions were injected into the HPLC apparatus after the same pretreatment as for drug-free plasma. The calibration curve for nicorandil concentration was made from the peak-area ratio of nicorandil and the I.S.

Recovery

Blood samples of ca. 10 ml per animal were obtained from the interior vena cava of a non-drugged rat (sample I) and from that of a drugged rat (samples II, III and IV), 30–60 min after oral administration of nicorandil (10 mg/kg). The samples were centrifuged to obtain plasma. To 20 μl of this plasma, 20 μl of nicorandil solution at concentrations of 2.5, 5, 10 or 20 μg/ml (dissolved in 0.05 *M* $(NH_4)_2HPO_4$) and 50 μl of I.S. solution (10 μg/ml) were added. The recovery rate was calculated from data that were obtained using HPLC after pretreatment of 20 μl of the resulting solution.

Precision

To investigate the precision of plasma nicorandil measurements, plasma samples obtained from the interior vena cava were analysed ten times.

Correlation between plasma samples obtained from inferior vena cava and tail vein

Blood samples from the tail vein as well as the inferior vena cava obtained under laparotomy were carried out 0.5 h after nicorandil was administered orally at a dose of 10 or 15 mg/kg. Plasma was separated by centrifugation, and the correlation between the two sets of samples was determined as described above.

Concentration–time profile of plasma nicorandil

To determine the concentration–time profile of plasma nicorandil, blood samples were obtained from the tail vein 0.25, 0.5, 1, 2, 4 and 6 h after oral administration of nicorandil at doses of 5 and 10 mg/kg. The blood was sepa-

rated into plasma and erythrocytes, and the drug concentration in the plasma was measured. The pharmacokinetic parameters were obtained from the nicorandil concentration, using a personal computer program for non-linear least-squares regression [7]. The maximum plasma concentration (C_{max}) and its corresponding time (T_{max}), the absorption rate constant (K_a), the elimination half-life ($T_{1/2}$) and the area under the plasma concentration–time curve ($AUC_{0\to\infty}$) were estimated by the computer program.

RESULTS

Chromatography

As shown in Fig. 1, the retention times of nicorandil and the I.S. were 5.7 and 7.3 min, respectively. No other peaks corresponding to these retention times were noted in the chromatogram of drug-free plasma, indicating that interfering endogenous substances were not present. The concentration of nicorandil was 5.91 μg/ml, calculated from the peak-area ratio of nicorandil to I.S.

Calibration curve

The standard curve was prepared by analysing a nicorandil plasma solution at concentrations of 0.625, 1.25, 2.5, 5, 10 and 20 μg/ml. Between the concentration of nicorandil in blood and the peak-area ratio of nicorandil to I.S., an excellent linear correlation was noted in the range 0–20 μg/ml. The coefficient of correlation (r) was 0.999 and the regression equation was $y=17.84x+0.08$. The limit of detection at a signal-to-noise ratio of 3 was 0.1 μg/ml.

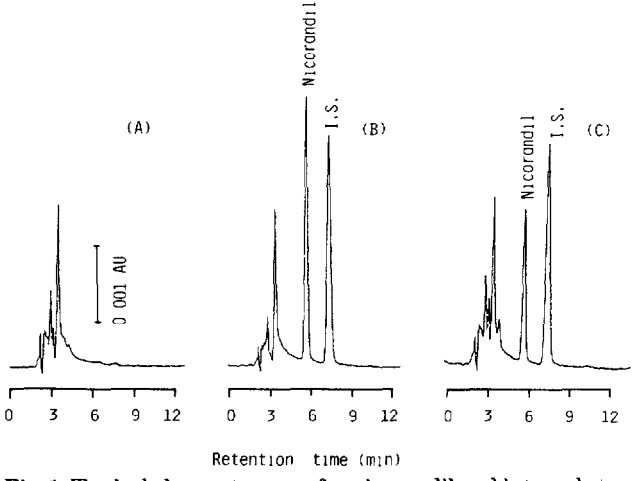

Fig. 1. Typical chromatograms for nicorandil and internal standard (I.S.). (A) Drug-free plasma; (B) spiked plasma (nicorandil 5 μg/ml, I.S. 10 μg/ml); (C) rat plasma after oral administration of nicorandil at a dose of 10 mg/kg (nicorandil 5.9 μg/ml).

Recovery rate and precision

The rates of recovery calculated by adding nicorandil of 2.5, 5, 10 and 20 μg/ml to plasma samples (sample I) of a non-drugged rat averaged 97.4% (92.3–100.8%) and by adding 10 and 20 μg/ml to plasma samples (samples II, III and IV) containing different concentrations of nicorandil averaged 103.9% (95.9–114.4%) (Table I). Coefficients of variation (C.V.) with reference to the simultaneous reproducibility in unknown samples I and II were 7.3% (5.5 ± 0.39 μg/ml, $n=10$) and 3.2% (7.0 ± 0.23 μg/ml, $n=10$), respectively (Table II), indicating a good reproducibility.

TABLE I

RECOVERY TEST IN METHOD PROPOSED FOR DETERMINATION OF NICORANDIL

Blood samples II, III and IV with nicorandil were obtained from inferior vena cava 30–60 min after nicorandil (10 mg/kg) was administered orally.

Plasma sample with nicorandil (*A*)	Nicorandil (μg/ml)			Recovery (*C*-*A*)/*B*×100 (%)
	Added (*B*)	Measured (*C*)	Recovered (*C*-*A*)	
Sample I (0 μg/ml)	2.5	2.47	2.47	98.8
	5	4.88	4.88	97.6
	10	9.23	9.23	92.3
	20	20.16	20.16	100.8
Sample II (9.56 μg/ml)	10	19.15	9.59	95.9
	20	29.17	19.61	98.1
Sample III (6.97 μg/ml)	10	18.12	11.15	111.5
	20	27.74	20.77	103.9
Sample IV (6.2 μg/ml)	10	17.64	11.44	114.4
	20	26.06	19.86	99.3

TABLE II

PRECISION OF NICORANDIL IN RAT PLASMA DETERMINED BY HPLC (WITHIN-DAY ASSAY)

Blood samples I and II with nicorandil were obtained from inferior vena cava after nicorandil (10 mg/kg) was administered orally.

Plasma sample with nicorandil	*n*	Concentration (mean ± S.D.) (μg/ml)	C.V. (%)
Sample I	10	5.5 ± 0.39	7.3
Sample II	10	7.0 ± 0.23	3.2

Correlation of plasma concentration between blood samples obtained from inferior vena cava and from tail vein

In the comparative analysis of the blood samples obtained from the interior vena cava and from the tail vein, the correlation coefficient, r, was 0.991 and the regression equation was $y=1.02x-0.22$, indicating a high correlation between the two.

Concentration–time profile of nicorandil

Concentration–time profiles for plasma nicorandil after doses of 5 and 10 mg/kg are shown in Fig. 2. The concentration of nicorandil in the plasma increased in a dose-dependent fashion. No nicorandil was found 24 h after the oral dose. For each administration group, the values of K_a, T_{max}, C_{max}, $T_{1/2}$ and AUC are shown in Table III. Thus, the pharmacokinetics of nicorandil in small animals can be studied by this method.

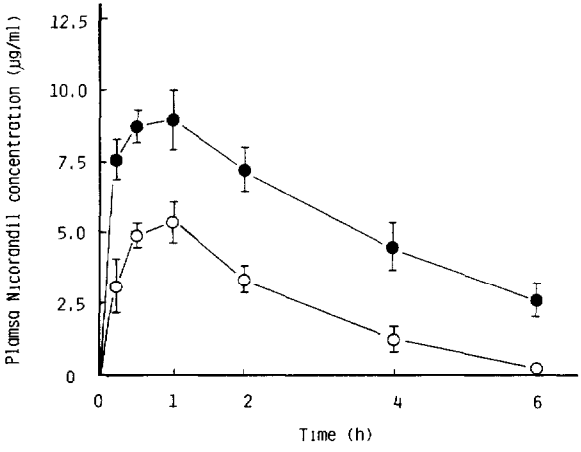

Fig. 2. Plasma nicorandil concentrations after oral administration in rats. Each point represents the mean value for three rats, and vertical lines represent the standard error of the mean. (○) 5 mg/kg; (●) 10 mg/kg.

TABLE III

PHARMACOKINETIC PARAMETERS OF NICORANDIL AFTER ORAL ADMINISTRATION

Nicorandil dose (mg/kg)	K_a (h^{-1})	T_{max} (h)	C_{max} (μg/ml)	$T_{1/2}$ (h)	$AUC_{0\to\infty}$ (μg·h/ml)
5	2.11	0.82	5.39	1.12	14.45
10	5.06	0.64	9.28	2.98	46.34

DISCUSSION

Kinoshita et al. [3] observed a significant correlation between the duration of heart movement and the blood level of nicorandil in patients with effort angina pectoris, and that the effect was dose-dependent. This indicates that the therapeutic effect of nicorandil may be influenced clinically by a significant change in the blood concentration, and hence that the monitoring of plasma nicorandil is necessary. Thus we developed a rapid and simple method for the determination of nicorandil in rat plasma. This method showed a favourable recovery (95.9–114.4%) after pretreatment with Bond Elut *PH*, as well as good reproducibility (C.V. 3.2–7.3%). This also enabled the determination of the plasma concentration using a small volume of plasma (20 μl), such as that obtained from a rat tail vein. In addition, the assay was rapid and easy to perform. Comparison of the plasma concentrations in the inferior vena cava and tail vein showed a good correlation ($r=0.991$). By sampling from rat tail veins the time-course of plasma nicorandil following oral administration of 5 mg/kg could be observed.

This method is useful in basic studies of plasma nicorandil in small animals. It is a rapid and simple way for microdetermination in plasma and obtaining the plasma nicorandil concentration–time data from a single animal, together with further pharmacokinetic parameters, such as K_a, T_{max}, C_{max} and $T_{1/2}$. The feasibility of observing plasma nicorandil dynamics in a chronological sequence represents an advantage. For instance, it will now be possible to study the relationship between the blood concentration and the pharmacological activity, and to take into account the effect of any other drugs used concurrently, or other influencing factors.

It has been reported that clinical doses of nicorandil produce plasma concentrations of 0.2–0.4 μg/ml as the C_{max} [3]. The present method may not be adequate to detect concentrations of 0.02 μg/ml (one tenth of the above C_{max}), because the detection limit in this method was 0.1 μg/ml plasma when 20 μl plasma samples were used. However, if the 250-μl methanol eluates are condensed or plasma sample volumes of more than 100 μl are used for detecting plasma nicorandil in patients receiving oral doses of the drug, it may be possible to monitor plasma concentrations of less than 0.02 μg/ml.

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