

# Fluorimetric determination of nicorandil in human plasma by a high-performance liquid chromatographic–postcolumn ultraviolet detection system equipped with on-line back-pressure tubing

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## Abstract

For the determination of nicorandil in plasma, a fluorometric technique using HPLC–postcolumn UV detection has been developed. The chromatographic system consisted of a single pump, photoreactor and on-line back-pressure tubing. The system was suitable for the separation of nicorandil under the present reaction conditions. The calibration graph was linear over the range 6.5–1170 ng ml<sup>-1</sup> using an injected volume of 100 μl. The pretreatment of the plasma samples consisted only of deproteinizing steps by adding perchloric acid. The mean recovery from plasma was 90.2%.

*Keywords:* Nicorandil

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## 1. Introduction

Nicorandil (NICR), a pyridinecarboxylic acid derivative, is a potent coronary vasodilator. The action mechanisms of NICR described are potassium channel activation and nitrate activity [1–3]. Consequently, the plasma NICR level in biological samples is expected to be indicative of the therapeutic efficacy. A number of procedures for the measurement of NICR in plasma have already been described including HPLC with spectrophotometric [4–7] and photoconductivity detectors [8]. Although HPLC is particularly useful in analyzing biological samples, its

spectrophotometric detector is liable to lack sensitivity and/or selectivity and requires tedious pretreatment. We have recently developed a photoirradiation system for pyridinecarboxylic acids [9–11]. A “black light” source was used for irradiation in order to obtain mild reaction conditions. However, the presence of a radical scavenger such as methanol interferes with the fluorescence reaction of the pyridinecarboxylic acids.

In the present study, NICR was found to fluoresce in the presence of H<sub>2</sub>O<sub>2</sub> and methanol with irradiation by germicidal light. For the HPLC–postcolumn UV detection system, a photoreactor equipped with an electric fan and a tube resistant to high pressure and on-line back pressure was developed. The UV detection system was suitable for the separation of

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NICR under the present reaction conditions. This assay allowed rapid and efficient measurement of NICR in plasma samples.

## 2. Experimental

### 2.1. Chemicals

Nicorandil was donated by Takeshima (Saitama, Japan). Nicorandil tablets (Sigmart) were purchased from Chugai (Tokyo, Japan) and contained 5.0 mg nicorandil. All other chemicals were obtained from Wako (Osaka, Japan).

### 2.2. Chromatographic system

The chromatographic system consisted of a high-pressure pump (Model ERC-8711, ERMA, Tokyo, Japan), a sample injector (Model 7125, Rheodyne, Berkeley, CA, USA) fitted with a 200- $\mu$ l loop, an analytical column, a photoreactor (Model S-3900, Soma Optics, Tokyo, Japan), a fluorescence monitor (Model RF-530 Shimadzu, Kyoto, Japan) with a 12- $\mu$ l flow cell and a Shimadzu Chromatopac C-R6A recorder-integrator. In the system, the analytical column (150 $\times$ 4.6 mm I.D.) packed with Capcell Pak C<sub>8</sub> (particle size 5  $\mu$ m, type AG-120, Shiseido, Tokyo, Japan) operated at room temperature. The S-3900 photoreactor consisted of a stainless-steel photoreactor, an electric fan and two GL-4 germicidal light tubes (4 W electric power, Nippon Denki, Tokyo, Japan). UV irradiation was carried out in ETFE [a copolymer of ethylene and tetrafluoroethylene (Tefzel), GL Sciences, Tokyo, Japan] tubing (3 m $\times$ 0.25 mm I.D. $\times$ 1.5 mm O.D.) which was wound around the two germicidal light tubes. Following the UV detection coil, a stainless-steel back-pressure tube (0.4 m $\times$ 0.1 mm I.D.) and PTFE tubing (2 m $\times$ 0.25 mm I.D.) were connected. The fluorescence of the effluent solution was measured with excitation and emission wavelengths of 323 and 386 nm, respectively.

### 2.3. Separation conditions

The mobile phase consisted of 60 ml 0.5 M acetic acid and 940 ml 0.5 M sodium acetate (pH 6.0)

containing 20% (v/v) methanol and 20 mM H<sub>2</sub>O<sub>2</sub>. The flow-rate was 0.8 ml min<sup>-1</sup> and chromatography was performed at ambient temperature.

### 2.4. Analytical procedure

To 200  $\mu$ l plasma was added 100  $\mu$ l 1.5 M HClO<sub>4</sub> in a polymer tube (1.5 ml) and the mixture was then vortex-mixed. It was centrifuged at 9600 g for 1 min. The whole mixture was added to 100  $\mu$ l 3.0 M K<sub>2</sub>CO<sub>3</sub>. After being vortex-mixed and centrifuged for 1 min, a 100- or 200- $\mu$ l portion of the supernatant was injected onto the chromatographic system.

### 2.5. Recovery study

A test was run to check the stability and absorption of NICR during the deproteinizing procedure. NICR at (13.2, 52.7 and 264 ng ml<sup>-1</sup>) was spiked in free human plasma, and run through the analytical procedure. The peak heights obtained for the samples were compared with those of the authentic NICR.

## 3. Results and discussion

### 3.1. Fluorescence reaction

Fig. 1 shows the excitation (a) and emission spectra (b) of the eluate for NICR obtained using the HPLC system. Excitation and emission maxima were 323 nm and 386 nm, respectively. With a low concentration of H<sub>2</sub>O<sub>2</sub>, nicotinic acid derivatives showed fluorescence with low-energy germicidal light and short reaction time, possibly due to bipyridine formation by nicotinic acid. Actually, the excitation and emission spectra of NICR resembled those of quinolinic acid [11] by UV detection with H<sub>2</sub>O<sub>2</sub>.

### 3.2. Optimization of chromatographic condition

NICR strongly interacted with the residual silanol groups of the C<sub>18</sub> column [8]. Even for a CN, C<sub>8</sub> and

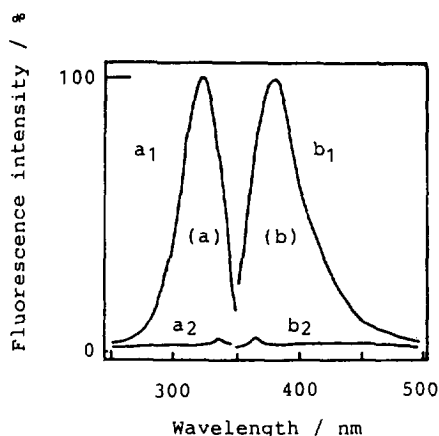


Fig. 1. Fluorescence excitation (a) and emission (b) spectra of the HPLC eluate of nicorandil.  $a_1$  and  $b_1$ ,  $13.2 \mu\text{g ml}^{-1}$  nicorandil;  $a_2$  and  $b_2$ , sample blank for  $a_1$  and  $b_1$ , respectively.

$C_1$  column, in the mobile phase without organic modifiers, the retention time was longer, being at least 40 min. A Capcell Pak  $C_8$  (endcapped) was then used to obtain a peak of good shape with the mobile phase containing 20% (v/v) methanol. The retention time of NICR was ca. 11 min.

### 3.3. Optimization of postcolumn UV detection

The conditions of the postcolumn UV detection were based on the chromatographic conditions. The reaction monitored in the present experiment was inhibited in the presence of a radical scavenger, such as methanol, mannitol and superoxide dismutase. Consequently, the selected light source was germicidal light that enabled the generation of a high amount of hydroxyl radicals from  $\text{H}_2\text{O}_2$ . However, it must be noted that  $\text{H}_2\text{O}_2$  production by germicidal light proceeds in a vigorous fashion (i.e., foaming) [12]. This was solved by connecting a stainless-steel tube of 0.1 mm I.D. and PTFE tube of 0.25 mm I.D.; then the back pressure changed to ca.  $40 \text{ kg cm}^{-2}$  and the foam completely disappeared. The PTFE tube was connected to prevent sudden variations in the flow cell due to back pressure. In the photoreactor, the use of PTFE tubing was not suitable for the reaction coil when irradiating with germicidal light. The PTFE tube broke several times because of the back pressure and irradiation energy.

Therefore, an ETFE tube was adopted that had high-pressure resistance ( $<150 \text{ kg cm}^{-2}$ ) and a transmittance similar to the PTFE tube. The photoreactor was cooled by an electric fan, with the temperature maintained usually at ca.  $40^\circ\text{C}$ .

Fig. 2a and Fig. 2b show the effect of  $\text{H}_2\text{O}_2$  concentration in the mobile phase and coil length on the UV detection. The optimum conditions were found to be 10–30 mM for the concentration of  $\text{H}_2\text{O}_2$  and a coil length of 2.5–3.5 m; 20 mM and 3.0 m were adopted for further experiments. The optimum pH of the mobile phase for the fluorescence reaction was 5.2–7.0; pH 6.0 was used. The retention time of NICR was not affected by the pH of the acetate buffer in the mobile phase.

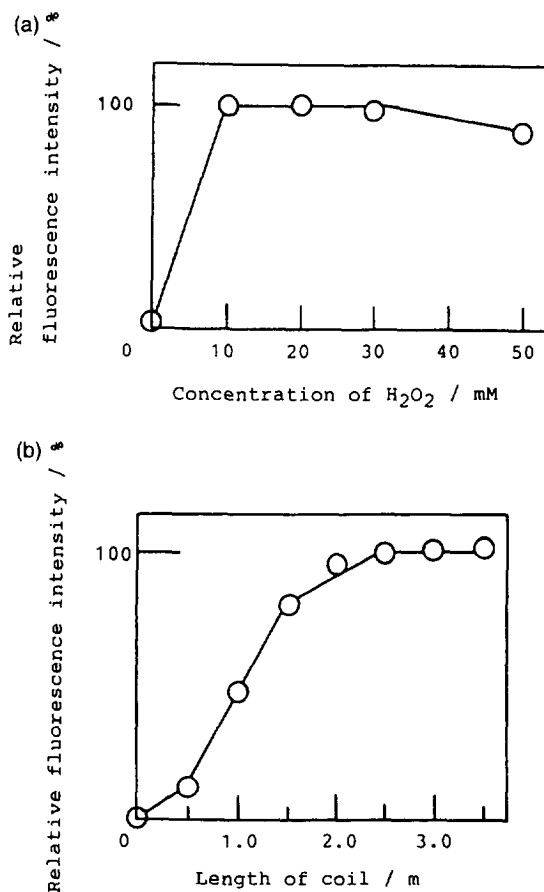


Fig. 2. (a) Effect of  $\text{H}_2\text{O}_2$  on the fluorescence intensity. (b) Effect of coil length on the fluorescence intensity. UV detection was carried out as described in Section 2. The amount of NICR was 26.4 ng.

### 3.4. Calibration and precision

The calibration graph was linear over the range of 6.5–1170 ng ml<sup>-1</sup> (seven data points) by injecting a volume of 100 μl NICR solution. The correlation coefficient was 0.9996, the slope was 3.76 and the intercept was -0.2, using a least-squares regression analysis. The relative standard deviation of the standard NICR was 3.9% at 10.5 ng ml<sup>-1</sup> (n=8) and 1.8% at 210 ng ml<sup>-1</sup> (n=11) when 100 μl was injected. The detection limit (S/N=3) was determined to be 7.0 ng ml<sup>-1</sup> using 200 μl deproteinized plasma.

### 3.5. Selectivity and recovery

Fig. 3 shows chromatograms of the authentic NICR and plasma sample. The NICR peak was observed in chromatograms (a) and (b) at the corresponding retention times, whereas chromatogram (c)

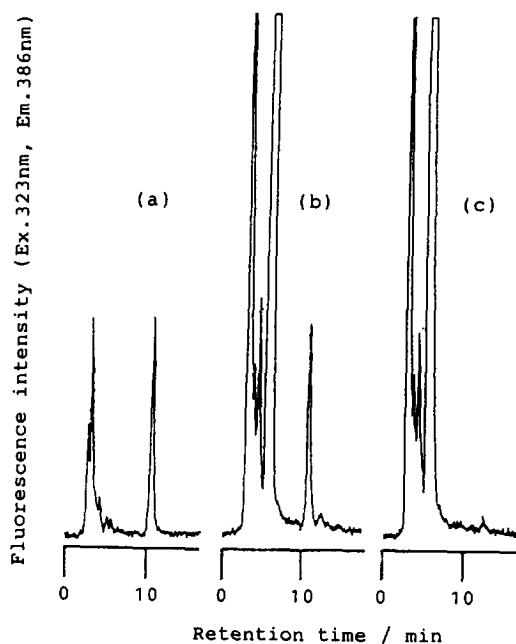


Fig. 3. Chromatograms obtained with standard and plasma samples. (a) Nicorandil standard; (b) plasma sample spiked with nicorandil; (c) plasma sample. Analytical procedure and conditions were as described in Section 2. The injection volume was 100 μl. The amount of nicorandil added was 264 ng (ml plasma)<sup>-1</sup>.

Table 1

Recovery of nicorandil from human plasma

Concentration added (ng/ml)	Recovery (mean ± S.D.) (%)	n
13.2 <sup>a</sup>	91.1 ± 5.0	6
52.7 <sup>a</sup>	92.0 ± 4.6	7
264 <sup>b</sup>	91.8 ± 2.5	6
Mean recovery	91.6	

<sup>a</sup> Injection volume 200 μl.

<sup>b</sup> Injection volume 100 μl.

of the free plasma sample shows no peak at that position. A comparison of the chromatograms demonstrates the specificity of the present method. In addition, several compounds such as kynurenic acid, nicotinic acid and its metabolites, tyrosine, phenylalanine, histidine, pyridoxine and thiamine, did not interfere with the analysis because they elute from the column before NICR. In Table 1 the recovery results from plasma are summarized. The mean recovery for all points on the spiked plasma was 91.6%.

### 3.6. Plasma concentration study

Fig. 4 shows a chromatogram obtained with a plasma sample at 1 h after oral administration (two 5-mg tablets) of NICR. The separation of NICR in the plasma sample by injecting a 200-μl aliquot was satisfactory. The plasma concentration curves of NICR are graphically shown in Fig. 5. The peak plasma NICR concentration was reached 30 min after oral administration, in accordance with Frydman et al. ( $C_{max}$ , 107 ± 30 ng ml<sup>-1</sup>;  $T_{max}$ , 0.42 ± 0.18 h) [2].

## 4. Conclusion

The present system, using a photoreactor and on-line back-pressure tubing is simple and easily assembled. This method is sensitive and specific enough to estimate NICR in human plasma, and is expected to be useful for therapeutic drug monitoring.

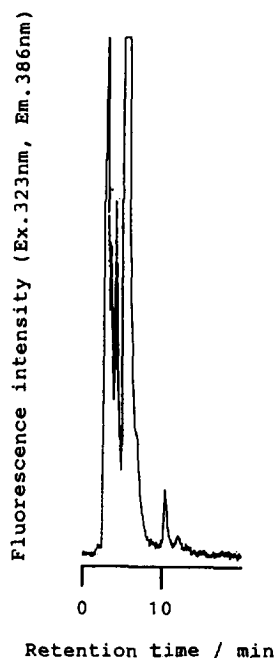


Fig. 4. Chromatogram from a plasma sample drawn 1 h after oral administration of nicorandil (two 5-mg tablets) to a normal man (b); 200  $\mu$ l deproteinized plasma was injected onto the chromatographic system. Concentration of nicorandil was 39.4 ng ml<sup>-1</sup>.

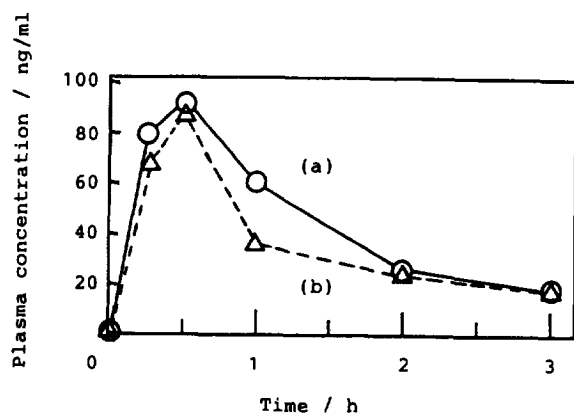


Fig. 5. Plasma concentration–time curve following oral administration of two 5-mg tablets of nicorandil, determined as described in Section 2.

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