Journal of Chromatography, 619 (1993) 336–341
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 7040

Short Communication

High-performance liquid chromatographic method for stability and pharmacokinetic studies of nicorandil

Elizabeth L. Bachert and Ho-Leung Fung*

Department of Pharmaceutics, School of Pharmacy, State University of New York at Buffalo, Buffalo, NY 14260 (USA)

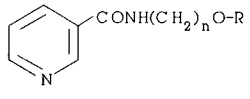
(First received April 27th, 1993; revised manuscript received June 22nd, 1993)

ABSTRACT

Nicorandil is a unique vasodilator that combines the actions of a potassium channel activator and a nitrovasodilator. Little literature is available on its chemical stability and pharmacokinetics in animals. We developed a simple, specific, sensitive, and precise high-performance liquid chromatographic method for the determination of nicorandil in aqueous solution and rat plasma, achieving a detection limit of 0.3 μg/ml with 180-μl samples. Nicorandil was found to be relatively stable between pH 2 and 10 at 37°C (half-life = 461–84 h) and it is subjected to specific base catalysis above pH 10. Both chemical degradation and *in vivo* metabolism produced N-(2-hydroxyethyl)nicotinamide, the denitrated product. Preliminary pharmacokinetic investigations showed that the assay is capable of quantitating nicorandil in rat plasma over a range of 0.3–100 μg/ml. These studies also suggested that the pharmacokinetics of nicorandil are dose-dependent.

INTRODUCTION

Nicorandil, N-(2-hydroxyethyl)nicotinamide nitrate ester (Fig. 1A), has been employed for the treatment of cardiovascular disorders such as angina pectoris [1,2], coronary spasm [3] and congestive heart failure [4]. The pharmacology of nicorandil is complex because it possesses both nitrate and potassium channel activating properties. While the exact mechanism for the therapeutic effect of nicorandil is unclear, it has been proposed that the potassium channel activation is related to the nicotinamide ring, whereas the nitrate activity is associated with the nitroester group [5,6]. The proximity of the two structurally active areas on nicorandil are apparently of great importance, since addition of an alkyl group in the side-chain, *e.g.*, in N-(3-hydroxypropyl)nicotinamide nitrate ester (Fig. 1B), decreases the potency by approximately 33% [5]. In humans and animals, nicorandil is metabolized to several me-



A: n=2, R= NO_2; nicorandil
B: n=3, R= NO_2; N-(3-hydroxypropyl) nicotinamide nitrate ester
C: n=2, R= H ; N-(2-hydroxyethyl) nicotinamide

Fig. 1. Structures of (A) N-(2-hydroxyethyl)nicotinamide nitrate ester (nicorandil), (B) N-(3-hydroxypropyl)nicotinamide nitrate ester, and (C) N-(2-hydroxyethyl)nicotinamide.

* Corresponding author.

0378-4347/93/$06.00

tabolites, with the primary metabolite in rats being the denitrated nicorandil, N-(2-hydroxyethyl)-nicotinamide (Fig. 1C).

High-performance liquid chromatographic (HPLC) assays have been reported for analyzing nicorandil in dog and rat plasma [7–9]. However, these assays require blood sample volumes of approximately 1–8 ml and are therefore unsuitable for extensive serial sampling in examining nicorandil pharmacokinetics in small animals such as the rat. We examined the possibility of scaling-down the available assays to suit this purpose and applied this scaled-down assay for the determination of the chemical stability of nicorandil in aqueous solutions as well as for the preliminary examination of the pharmacokinetics of nicorandil in rats.

EXPERIMENTAL

Chemicals and reagents

Nicorandil [N-(2-hydroxyethyl)nicotinamide nitrate ester], N-(3-hydroxypropyl)nicotinamide nitrate ester (internal standard), and N-(2-hydroxyethyl)nicotinamide were gifts of Chugai Pharmaceutical (Tokyo, Japan). Methanol was of HPLC grade, obtained from J.T. Baker (Phillipsburg, NJ, USA). All other reagents were of analytical grade.

Aqueous standard curves

Stock solutions of nicorandil in double-distilled water (0.01–1.0 mg/ml) were prepared in polypropylene microcentrifuge tubes (National Scientific Company, San Rafael, CA, USA). Stock standards were made by serial dilutions of the stock solution. These stock solutions were stored at 4°C for less than one month. Aqueous calibration standards were prepared daily from the stock solutions by spiking 10 μl of each standard with 10 μl of a solution of N-(3-hydroxypropyl)-nicotinamide nitrate ester (the internal standard, at 210 μg/ml) into microcentrifuge tubes containing 180 μl of double distilled water to give final nicorandil concentrations of 50, 25, 12.5, 5.0, 1.0, and 0.5 μg/ml. Additional standards were prepared for lower quantitation of nicorandil when necessary. The ratio of the peak area of nicorandil to that of the internal standard was plotted *versus* the concentration of nicorandil to obtain the calibration curve, and the slope and intercept were obtained by least-squares linear regression.

Plasma standards

Rat plasma samples (180 μl) were placed in 100 mm × 13 mm culture tubes, and 10 μl of 210 μg/ml internal standard and 10 μl of each aqueous stock solution of nicorandil were added to obtain the same range of final concentrations reported for aqueous standards. The sample preparation and extraction procedure of Sakai *et al.* [7] and Xu *et al.* [8] were adapted. To the plasma sample containing internal standard, one drop of 0.1 *M* NaOH was added, followed by 1.0 ml of ethyl acetate (J.T. Baker). The mixture was vortex-mixed for 30 s, capped with a PTFE-lined cap and centrifuged for 10 min at 2000 *g*. The upper ethyl acetate layer was removed by pasteur pipet and transferred to a 75 mm × 12 mm culture tube (Corning, NY, USA). The extraction procedure was repeated, and the ethyl acetate layers were combined and evaporated under nitrogen. Immediately when dry, 200 μl of double distilled water were added to the residue and vortex-mixed for 10 s to facilitate solubilization.

Apparatus and assay conditions

The assay conditions were modified from previous work [7–9]. The processed samples were injected on to the HPLC column (30–50 μl) either manually or via a Waters (Milford, MA, USA) WISP 710B automatic injector. The mobile phase consisted of filtered and degassed 45% methanol–double-distilled water which was pumped with a Waters Model 510 pump at a constant flow-rate of 1.0 ml/min. The analysis was carried out with a Waters μBondapak C_{18} HPLC column, 30 cm × 3.9 mm I.D. Detection was at 254 nm with a Hewlett Packard (Avondale, PA, USA) Model 1040M photodiode-array detector. The peak areas were integrated with Hewlett Packard 1040M software. The retention times for N-(2-hydroxyethyl)nicotinamide, nicorandil, and internal standard were 3.5, 5.5, and 6.7 min, respectively.

Aqueous degradation of nicorandil

Buffers in the pH range 2–12 with an ionic strength of 0.01 were prepared as described by Perrin [10]. Eight buffers were prepared: at pH 2 (0.01 *M* HCl), pH 3 (chloroacetic acid–potassium hydroxide), pH 4 (formic acid–potassium hydroxide), pH 6 (citric acid–sodium citrate), pH 8 (trishydroxymethylaminomethane–HCl), pH 10 (sodium bicarbonate–sodium carbonate), pH 11 (butylamine–HCl), and pH 12 (0.01 *M* NaOH); they were warmed to 37°C in an oscillating water bath. To each reaction vial, nicorandil and warm buffer were added to obtain a final concentration of 15 μg/ml. After mixing (approximately 1 min), a 50-μl aliquot was removed for assay. The solutions were maintained at 37°C for the study duration, and samples of 50 μl were removed periodically for up to one month. Aqueous nicorandil calibration curves were constructed each day when these samples were assayed. The degradation experiments were conducted in duplicate.

Statistics

Data are expressed as mean ± S.D. The pH–rate profile for nicorandil and degradation products were modelled using PCNONLIN (Statistical Consultants, Lexington, KY, USA).

RESULTS

Based on spectral information, 254 nm was chosen as the wavelength for nicorandil detection. Additional spectra of the internal standard and N-(2-hydroxyethyl)nicotinamide (not shown) suggested that a small shoulder at 262 nm is characteristic of the nicotinamide ring. Nicorandil and internal standard eluted at 5.5 and 6.7 min, respectively. The blank plasma chromatogram contained no interfering peaks for N-(2-hydroxyethyl)nicotinamide, nicorandil, and internal standard (data not shown).

Standard curves for both aqueous and extracted plasma standards were linear (0.5–50 μg/ml). The least-squares correlation coefficients for the aqueous and plasma curves were generally >0.998. Aqueous solutions of nicorandil degraded less than 5% over one month. Comparison of the standard curves for plasma and aqueous standards (0.5–50 μg/ml) showed the absolute recovery for nicorandil in plasma to be 95.0 ± 0.03% (n = 3). Using the stated conditions, the lowest detectable limit for aqueous standards was about 0.3 μg/ml or approximately 8.7 ng on column. The signal-to-noise ratio for the lowest detectable concentration was 3.6. The inter- and intra-day coefficients of variation for the assay, at nicorandil concentrations between 5 and 50 μg/ml, were less than 5%. The absolute recovery of the internal standard at 210 μg/ml from plasma samples was 98.7 ± 4% (n = 3). Spiked plasma samples were found to be stable at −20°C for 26 days with negligible degradation. Because of the similarities in recovery and detector responses between the aqueous and plasma standards, aqueous standards were subsequently used for all assays.

Nicorandil degraded via first-order kinetics over the pH range studied. A pH–rate profile was constructed using the observed first-order rate constant (k_{obs}). Between pH 3 and 10, the relationship between k_{obs} and pH can be adequately described by eqn. 1 (Fig. 2):

(Model 2)

$$k_{obs} = \frac{k_1 K_a}{[H^+] + K_a} + k_2 [OH^-] \quad (1)$$

where k_1 is the apparent rate constant for the reaction between water and the conjugate base of

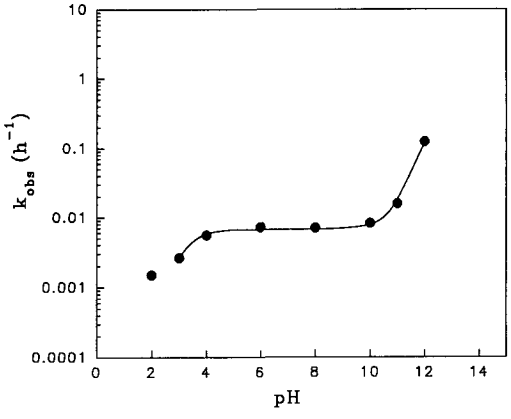


Fig. 2. pH–rate profile of nicorandil degradation in aqueous buffers. The points are the observed values and the line represents the fitted curve using eqn. 1.

nicorandil, k_2 is the apparent rate constant for the specific base-catalyzed reaction, $[H^+]$ and $[OH^-]$ are the hydrogen and hydroxide concentrations, respectively,and K_a is the dissociation constant of nicorandil (pK_a = 3.18). Estimates for k_1 and k_2 were 0.0062 and 11.8 h^{-1}, respectively.

From pH 6 through pH 12, two degradation products could be clearly detected at 254 nm. For example, at pH 12, the first degradation product (B) was detectable within 1 h of nicorandil incubation and reached a maximum at 24 h, after which degradation began to occur. At 24 h, a second degradation product (C) became detectable, and its concentration steadily increased throughout the study (Fig. 3). Product C was identified by photodiode-array spectral comparisons to be N-(2-hydroxyethyl)nicotinamide. The identity of B could not be readily identified. If the molar absorptivities of nicorandil, B, and C are assumed to be identical, the concentrations for the first degradation product can be calculated. This allowed computer curve fitting of the degradation cascade, either in terms of model 1 or model 2:

$$A \xrightarrow[k_A]{} B \xrightarrow[k_B]{} C \xrightarrow[k_C]{} \qquad \text{(Model 1)}$$

where A, B, C, D are, respectively, the concentrations of nicorandil, the first degradation product,

$$\begin{array}{ccccc} A & \xrightarrow[k_A]{} & B & \xrightarrow[k_{BC}]{} & C \\ & & \downarrow{\scriptstyle k_{B2}} & & \\ & & D & & \end{array} \qquad \text{(Model 2)}$$

N-(2-hydroxyethyl)nicotinamide, and a second unknown product (which has little absorptivity at 254 nm), and k_A, k_B, k_{BC}, k_{B2} and k_C are the rate constants designated for the respective reactions. Both models 1 and 2 can describe the degradation profiles reasonably well for pH 6–12. Additional computer curve fitting assuming B to have a molar absorptivity ten times higher than A was also conducted, with little changes in the quality of the fits, as determined by sums of squared deviations, values of the parameter estimates and observed *versus* predicted values. These results suggested that the kinetic fits were not too sensitive toward the assumed molar absorptivity value of the unknown intermediate.

Fig. 4 shows the pharmacokinetics of nicorandil in the rat after bolus intravenous doses of 0.75 and 12 mg. It is evident that the assay described is capable of quantitating nicorandil concentrations over a wide range (0.3–100 μg/ml). These preliminary studies also show that the elimination half-life ($t_{1/2}$) of nicorandil was dependent on the dose administered; increasing the intravenous dose 16-fold brought about a 3.6-fold increase in the $t_{1/2}$ (0.7 to 2.6 h).

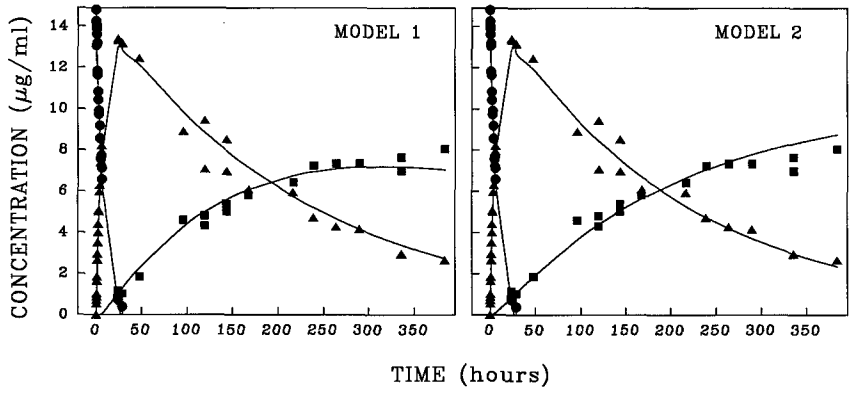


Fig. 3. Disappearance of nicorandil and appearance of two degradation products at pH 12. The points are the observed values (n = 2), and the lines represent the computer-fitted curves from kinetic models 1 and 2.

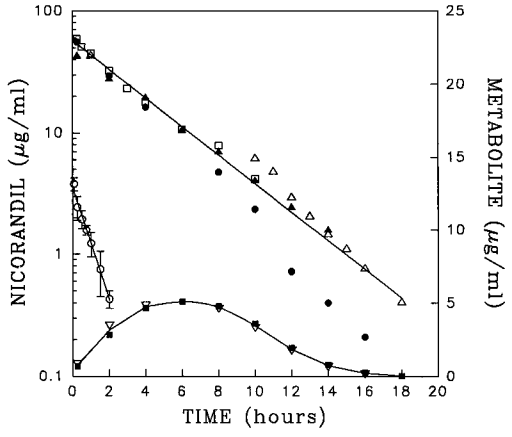


Fig. 4. Plasma nicorandil and N-(2-hydroxyethyl)nicotinamide concentration *versus* time curves after intravenous bolus doses of 0.75 mg (○; n = 5), 12 mg (data from different animals are represented by ●, △, ▲, and □; n = 4), and N-(2-hydroxyethyl)nicotinamide formation after a 12-mg nicorandil bolus dose (■ and ▽; n = 2).

DISCUSSION

The limit of detection of the present assay (8.7 ng on column under the stated conditions) is adequate for pharmacokinetic studies in small animals, from whom limited volumes (100–200 μl) of plasma are available. Previously reported assays for nicorandil [7–9] require 0.5–8.0 ml of blood for analysis. The absolute recovery for plasma was excellent (greater than 95%), which will allow use of aqueous standards rather than plasma standards for pharmacokinetic studies. Sample preparation is rapid, requiring less than 30 min per sample. Both nicorandil and the internal standard eluted from the column before 7 min post injection, with no detectable late-eluting components, thus the run time of each chromatogram is also quite short.

We have identified N-(2-hydroxyethyl)nicotinamide as a major degradation and metabolic product of nicorandil, which can be quantified through the same assay. An intermediate product (B) can be detected in the chromatogram, but its identity is currently unknown. A previous report suggests that this intermediate could be 2-(3-pyridyl)-2-oxazoline [11].

From the pH–rate profile of nicorandil, it was observed that the degradation $t_{1/2}$ (96 h) for nicorandil at the biological pH of 7.4 at 37°C was quite long. At approximately the same initial drug concentration, the *in vivo* $t_{1/2}$ is approximately 1.2 h. These results suggest that the *in vivo* disposition of nicorandil is primarily mediated by enzymatic processes.

Our preliminary pharmacokinetic studies showed the *in vivo* elimination of nicorandil to be dose-dependent. Interestingly, the two doses produced elimination curves that appeared linear over the entire sampling period, and the two different doses yielded different decay rates over the same concentration range. It is therefore apparent that simple Michaelis–Menten kinetics could not be used to describe the observed behavior. A more detailed study of the dose-dependent kinetics of nicorandil has been carried out and will be presented elsewhere.

In summary, we have demonstrated a simple and rapid HPLC assay for the analysis of nicorandil in small plasma samples as well as in aqueous solutions. This assay is suitable for carrying out stability studies of nicorandil and also pharmacokinetic studies in small animals.

ACKNOWLEDGEMENTS

This work was supported, in part, by NIH Grant GM42850. E. L. B. is a Merck Predoctoral Fellow. The authors would like to acknowledge the excellent technical assistance of Teresa Lopes and Lin Zhao.

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