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Short communication

Determination of nicorandil concentrations in human plasma using liquid chromatography

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1. Introduction

Nicorandil (*N*-(2-hydroxyethyl)nicotinamide nitrate) represents a novel type of compound in the treatment of angina pectoris, as it causes vasodilation of both coronary and systemic arteries, due to its dual mechanism of action as a potassium channel activator and as a nitrate compound [1]. Plasma concentrations of nicorandil in human pharmacokinetic studies have been measured either by GC-MS [2] or fluorimetric techniques with post column UV irradiation [3] or by utilising solid phase extraction coupled with liquid–liquid extraction, under relatively harsh chromatography conditions [4]. The purpose of this study therefore was to validate an alternate HPLC method with UV detection, for the assay of routine plasma samples in human pharmacokinetic studies, through modification of a previously reported method [4]. This method was applied to a study of nicorandil disposition in human subjects following oral administration of a 10-mg tablet.

2. Materials and methods

2.1. Chemicals

Nicorandil was gifted by Unichem Laboratories, Mumbai. The internal standard, (*N*-[2-(nitroxy) propyl]-3-pyridine carboxamide) chlorohydrate and metabolite (*N*-(2-hydroxyethyl)nicotinamide) were received as a gift from Rhone-Poulenc Rorer (Cedex, France). Concentrated hydrochloric acid, sodium hydroxide and methanol were purchased from Qualigens Fine Chemicals, Mumbai; sodium carbonate and dichloromethane (HPLC grade) from S.D. Fine Chemicals Ltd., Boisar and ethyl acetate and methanol (HPLC grade) from E. Merck (India) Ltd., Mumbai. All chemicals and solvents used

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were of analytical grade, unless otherwise indicated.

2.2. Standard solutions

Primary standard solutions (100 μg ml^{-1}) of nicorandil and internal standard (abbreviated as IS) were prepared in methanol and refrigerated. Suitable aliquots were further diluted to prepare working standard solutions of 500 and 100 ng ml^{-1} for nicorandil and 2 μg ml^{-1} for the internal standard. All standard solutions were stored at 4°C. Methanolic stock solutions of nicorandil degraded $< 3\%$ over a period of 2 months at 4°C.

2.3. Extraction procedure

Plasma was acquired from the Green Cross Blood Bank, Ahmedabad. Internal standard (200 ng) was added to 1 ml of plasma and the sample was vortexed for 30 s. Then one drop of 1.0 M sodium hydroxide and 7 ml of dichloromethane were added and the samples were extracted on a rotary mixer for 30 min. The mixture was then centrifuged at 2000 rev./min for 10 min. The aqueous layer was aspirated and the organic dichloromethane layer was transferred to another clean 10-ml extraction tube and evaporated to dryness, under a stream of nitrogen, in a water bath. The residue was redissolved in 4 ml of ethyl acetate; 200 μl of 2.0 M hydrochloric acid and 300 μl of water were added, and nicorandil was back-extracted (15 min) into the acid layer. The organic layer was separated and discarded after centrifugation (5 min) as previously stated; 600 μl of 1.0 M sodium carbonate was added to the aqueous acid layer, followed by addition of 4 ml dichloromethane. This mixture was once again extracted (15 min) and then centrifuged (5 min). The aqueous layer was aspirated and the organic solvent was transferred to conical centrifuge tubes and evaporated to dryness, under a stream of nitrogen, in a waterbath. The inner walls of the tubes were rinsed with 1 ml dichloromethane and the solvent was evaporated. The dried tubes were stoppered, sealed and stored at -4°C overnight. The samples were reconstituted in 150 μl of mobile phase and 100 μl was injected onto the chromatograph.

2.4. Chromatography

The HPLC system consisted of a Shimadzu LC-10AD pump, Shimadzu SPD-10A detector, Shimadzu SIL-10A autosampler, Waters temperature control module and column oven (Millipore, Milford, MA) and a Jasco 807-IT integrator with version 4.03 software (Jasco, Japan). Separation was achieved using a Finepak SIL C-8, 4.6×250 mm, 5 μm reverse phase column (Jasco, Japan) attached to a C-18/Corasil guard column (Millipore, Milford, MA) heated to 30°C, and mobile phase which consisted of methanol/water (15:85, v/v), pumped at a flow rate of 1.2 ml min^{-1}. The detector was set at a wavelength of 230 nm, 0.005 AUFS and 10 mVFS recorder output for maximum signal response. The retention times for nicorandil and IS were 35.3 and 59.7 min, respectively (Fig. 1). The retention time of the metabolite was 11.1 min and it did not interfere with the assay.

3. Results

3.1. Assay validation

Calibration curves generated by spiking blank plasma with nicorandil, yielding concentrations of 10–200 ng ml^{-1} were linear, with a mean $\pm$ S.E. of slope and intercept of 0.0059 ± 0.00005 and 0.0066 ± 0.0019, respectively, for 16 calibration curves, and an average linear regression correlation coefficient > 0.999.

Replicate analysis of plasma quality control samples spiked with nicorandil to yield concentrations of 120, 60, and 30 ng ml^{-1}, demonstrated an intra-day coefficient of variation of 2.77% ($n = 6$), 3.42% ($n = 6$) and 15.05% ($n = 5$), respectively, with corresponding mean assayed values of 117.74, 59.00 and 29.44 ng ml^{-1}. The inter-day coefficient of variation measured over a period of 2 months, was 4.16% ($n = 32$), 5.03% ($n = 32$), and 5.78% ($n = 29$), with corresponding mean assayed values of 116.65, 59.10 and 29.29 ng ml^{-1}, respectively.

The mean absolute extraction recovery of both compounds was in the range of 40–50%. The limit of quantitation of the assay was 10 ng ml^{-1}.

4. Discussion

Although a few methods have been reported for HPLC analysis of nicorandil in human [3,4], rat [5] and dog [6] plasma, these methods were found unsuitable for assay of plasma concentrations in human pharmacokinetic studies either due to inadequate UV detection sensitivity at the low concentrations occurring in human plasma, or due to the presence of unresolved plasma impurity peaks, or difficulty in replicating chromatography and extraction conditions.

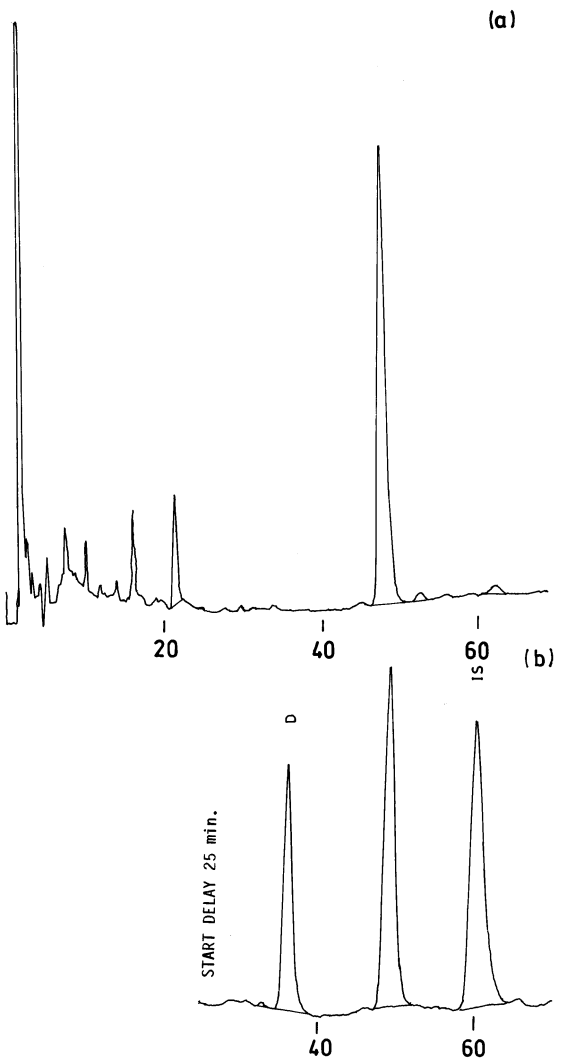

Fig. 1. Sample chromatograms of an extract of blank plasma (a) and plasma containing 70 ng ml^{-1} nicorandil [D] and 100 ng ml^{-1} IS (b).

Consequently, several sample cleaning extraction procedures were experimented with, including (1) direct extraction with dichloromethane under alkaline conditions; (2) sample pre-cleaning with ethyl acetate under acidic conditions, and re-extraction of the drug from the acidic aqueous layer with dichloromethane under alkaline conditions; (3) solid phase extraction followed by method (2); and (4) substituting solid phase extraction with dichloromethane extraction in method (3).

Although none of these methods completely eliminated plasma impurities, acid back-extraction from ethyl acetate was found to be the critical step for minimising plasma impurities and method (4) was adopted as the most efficient sample clean-up method, albeit a decrease in extraction recovery, due to the multiple steps involved.

Resolution of drug and IS from the residual plasma impurities was achieved by modifying the chromatography conditions as detailed in Table 1. Although pH had an appreciable effect on retention times, changing the pH of the mobile phase could not efficiently resolve the impurities on a C-18 column. However, a change in column packing from C-18 to C-8, with a concomitant increase in polarity of the mobile phase successfully achieved resolution, although, as expected, this resulted in a corresponding increase in retention times.

In summary, a sensitive and specific HPLC assay with UV detection has been validated for nicorandil and was used to quantitate drug concentrations in plasma after oral administration of a 10-mg tablet (Fig. 2).

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Table 1
Summary of the effect of chromatographic conditions on the resolution of nicorandil and IS. (A) Effect of pH of buffer (column: C-18 μBondapak, 3.9 × 300 mm; mobile phase-acetonitrile/phosphate buffer (0.05 M) (85:15, v/v)). (B) Effect of column and mobile phase composition

A	Exp. no.	pH	Retention times (min)		
			Drug	IS	Impurities
	1	7.0	13.6	20.8	8.4, 14.5, 15.4
	2	4.0	11.6	16.9	12.4, 13.4
	3	3.5	10.6	15.3	9.3, 12.5
	4	3.0	9.1	12.7	9.3, 12.4

B	Exp. no.	Column	Mobile phase[a]	Retention times (min)		
				Drug	IS	Impurities
	1	C-18, μBondapak (10 μm, 3.9 × 300 mm)	#1	10.2	14.5	7.8, 9.4, 11.3
	2	Finepak C-18 (5 μm, 4.6 × 250 mm)	#1	13.6	19.6	12.6, 14.6, 19.7
	3	Finepak C-8 (5 μm, 4.6 × 250 mm)	#2	24.4	38.2	15.1, 24.6, 33.4
	4	Finepak C-8 (5 μm, 4.6 × 250 mm)	#3	26.9	43.0	16.3, 27.4, 42.0, 44.5
	5	Finepak C-8 (5 μm, 4.6 × 250 mm)	#4	35.3	59.7	43.8, 46.2, 62.2, 115.0

[a] Mobile phase #1: phosphate buffer (pH 3.25; 0.05 M)/acetonitrile (85:15, v/v); #2: phosphate buffer (pH 5.0; 0.05 M)/acetonitrile (85:15, v/v); #3: acetonitrile/water (14:86, v/v); #4: methanol/water (15:85, v/v).

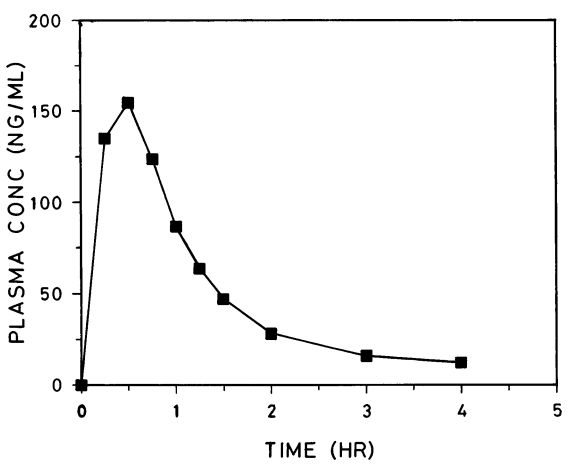

Fig. 2. Plasma concentration–time profile of nicorandil in a human subject, after oral administration of a 10-mg tablet.

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