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Routine and sensitive method for determination of nicorandil in human plasma developed for liquid chromatography with ultraviolet and m0ass spectrometric detection

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Abstract

A rapid and sensitive method using HPLC has been developed for the quantification of nicorandil (SG-75) in human plasma samples for routine bioequivalence studies. The sample preparation needs two liquid–liquid extractions, first with CH_3Cl and $HClO_4$ as denaturation reagent and second with addition of ethyl acetate and $Na_2CO_{3(aq)}$. Detection wavelength was 256 nm. The obtained correlation coefficient for weighted linear curve in the range from 5.0 to 300 ng/ml was higher than 0.9950. The limit of quantitation (LOQ) was established at 5.0 ng/ml. The HPLC separation was accomplished on Nucleosil Phenyl (5 μm) stainless steel column within 7 min. The mixture of 0.01 *M* ammonium acetate buffer (pH 6.2) and acetonitrile 10:3 (v/v) was used as the mobile phase. The same separation method was examined on HPLC–MS system. Using this system, the LOQ was established at 1.0 ng/ml and the linearity was obtained in the range from 1.0 to 150 ng/ml.

Keywords: Nicorandil

1. Introduction

Nicorandil, *N*-[2-(nitroxy)ethyl]-3-pyridine-carboxamide, Fig. 1, is a coronary vasodilator. It is clinically used for the treatment of angina pectoris [1–4].

In general, the determination of nicorandil has previously been performed by reversed-phase high-performance liquid chromatography after cartridge extraction [5–7], or liquid–liquid extraction [8]. These methods gave the detection limit from 10 to 7.0 ng/ml for nicorandil in human plasma. However, a more sensitive method was required in clinical studies. For this purpose, methods using photoconductivity detectors [9] or gas chromatography–mass spectrometry [10] have been used. Though these

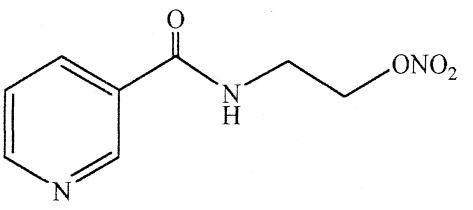

Fig. 1. The structural formula of nicorandil, *N*-[2-(nitroxy)ethyl]-3-pyridine-carboxamide.

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methods have high sensitivity (2 ng/ml, respectively), the first requires a special system and latter needs a lot of time for sample preparation.

Tanikawa et al. [11] developed the extraction method, which gave excellent recoveries and sample clean-up. This improvement enabled the measurement of nicorandil levels at a wavelength of 220 nm, which gave better limit of detection of nicorandil in human plasma samples (3 ng/ml). The authors concluded that the quantification (LOQ) of nicorandil at 5.0 ng/ml with UV detector at 256 nm is impossible because of a low nicorandil molar absorption coefficient. In spite of this, we developed a new liquid–liquid extraction and pre-concentration method for the quantification of nicorandil at 5.0 ng/ml with a HPLC–UV system (λ=256 nm). The same separation procedure was applied using MS detector, where we reach the limit of quantitation of 1.0 ng/ml of nicorandil in human plasma.

2. Experimental

2.1. Materials

Nicorandil (Batch No. CB 3652 Chemo S.A., purity >98%) has been made by CIBA, Basel, Switzerland. Water was distilled twice in an all-glass apparatus and deionized using Milli-Q system (Millipore, Bedford, MA, USA). All reagents were reagent grade and all solvents were HPLC grade.

2.2. Equipment

The HPLC system was consisted of a pump LDC Constametric 4100 (TSP, Thermo Separation Product, Riviera Beach, CA, USA), an autosampler TSP (AS1000) with fixed loop of 50 μl, a LDC Spectromonitor 3200 (TSP), a computer PC (IBM compatible 486) TSP-PC 1000 with OS2-TSP 1000 software and LCQ ion trap mass spectrometer (Finnigan, MAT, San Jose, CA, USA). A stainless-steel column (150×4.6 mm I.D.) packed with Nucleosil-Phenyl stationary phase, particle size 5 μm was used.

2.3. HPLC conditions

A degassed mixture of 0.01 *M* ammonium acetate aqueous buffer and acetonitrile (10:3, v/v) was used as mobile phase. The pH of the buffer solution was adjusted at 6.2 with 2 *M* aqueous solution of acetic acid. The detection wavelength was set at 256 nm. The flow-rate was 1.0 ml/min and room temperature at 25°C was maintained through analysis. The injection volume was 50 μl.

Mass detection conditions: capillary temperature was set at 150°C, capillary voltage at 4.0 V, vaporiser temperature at 450°C, sheath gas flow (N_2) at 0.65 MPa, auxiliary gas flow (N_2) at 0.05 MPa, tube lens offset at 30 V, discharge voltage at 4.0 kV, discharge current was 10 μA and multiplier voltage was −950 V. An atmospheric pressure chemical ionisation (APCI) interface was used for direct on-line sample introduction into mass detector.

2.4. Sample preparation

The 1.0 ml of human plasma sample containing nicorandil was deproteinised by 20 μl of $HClO_4$ (60%). The extraction was performed with 1.0 ml of dichloromethane. The suspension was vigorously shaken for 20 s on Vortex and phases were separated by centrifugation at 2500 *g* for 10 min. The water phase was decanted and 90 μl of 1.0 *M* $Na_2CO_{3(aq)}$ was added. Nicorandil was extracted with 1.2 ml of ethyl acetate by vigorously shaking on Vortex and the phases were separated by centrifugation at 2500 *g* for 5 min. The 850 (±2 vol.%) μl of upper organic layer was removed with a validated pipette and dried under nitrogen at 35°C in water bath. The residue was dissolved in 150 μl of mobile phase and the 50 μl of the solution was injected into the HPLC system.

2.5. Calibration curve

Standard aqueous solutions containing 50, 100, 200, 300, 500, 1000, 2000 and 3000 ng/ml of nicorandil were prepared by a serial dilution technique of an initial stock solution of 100 μg/ml. Aliquots (1.0 ml) of these standard solutions were spiked into control human plasma (9.0 ml) in 10.0 ml glass flasks to give calibration curve concentrations of 5.0, 10, 20, 30, 50, 100, 200 and 300 ng/ml. Aliquots of spiked human plasma samples (1.0 ml) were treated as described in Section 2.4, (Sample preparation). The peak area values of

nicorandil were used to calculate the calibration curve of nicorandil in human plasma.

2.6. Validation

The intra-day ($n=10$) and inter-day ($n=10$) reproducibility were examined using quality control (QC) samples. The 10.0 ml volume of the standard solutions containing 150, 600 and 1200 ng/ml of nicorandil was added into 90.0 ml of control human plasma to produce the nominal concentrations of 15, 60 and 120 ng/ml of QC samples and apportioned into 1.0 ml aliquots for storage and subsequent analysis. These working samples were stored at −20°C until analysis. The inter-day variability was assessed with ten replicates at each concentration over five days. Similarly, the intra-day variability was assessed with ten replicates for each of three working sample concentrations in a single day. The nicorandil concentrations were calculated from the calibration curve obtained on the same day as working samples.

The limit of quantitation and detection of this method was examined [12] using QC samples of 0.5, 1.0, 2.5 ng/ml for MS detection and 2.5, 5.0 and 10 ng/ml for UV detection. This QC samples were prepared in human plasma as it has been already described above.

The percentage recovery was determined by comparing the average peak area values of the plasma working samples of nominal concentrations 15, 60, and 120 ng/ml and the average peak area values of the water standard samples. The short time stability (5 h at room temperature) of spiked plasma samples and the 24 h stability of extracted (as describe above) plasma samples were also examined.

3. Results and discussion

3.1. Preparation

In previously reported methods for nicorandil determination in plasma samples [5–8,11], a rapid

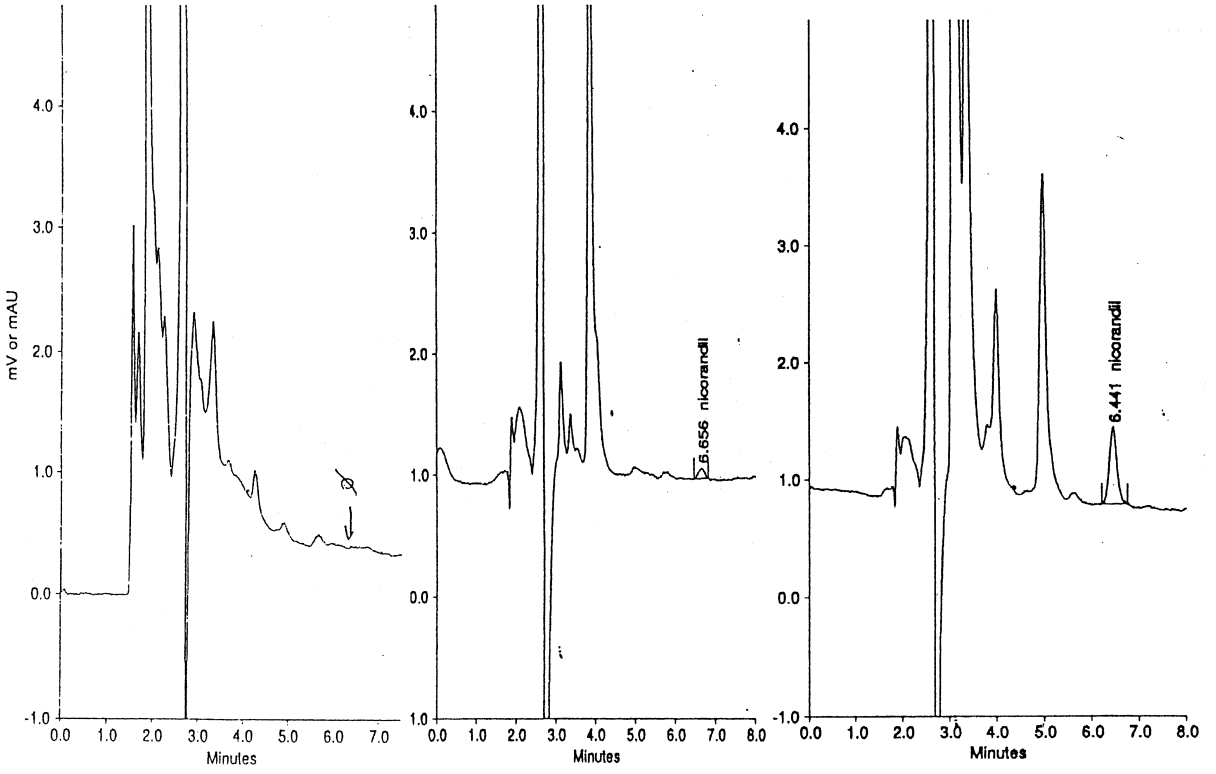

Fig. 2. Representative chromatograms with UV detection at 5.0 and 15.0 ng of nicorandil in 1.0 ml of human plasma.

and highly sensitive quantification with HPLC–UV at 254 nm was impossible because of low nicorandil molar absorption coefficient (ϵ=3500), although UV detection at 220 nm was impossible because of matrix interferences. The problem has been resolved by the group of Tanikawa [11] with quite exhaustive and excessive solvent consumption sample preparation steps. However, our method provides a sim-

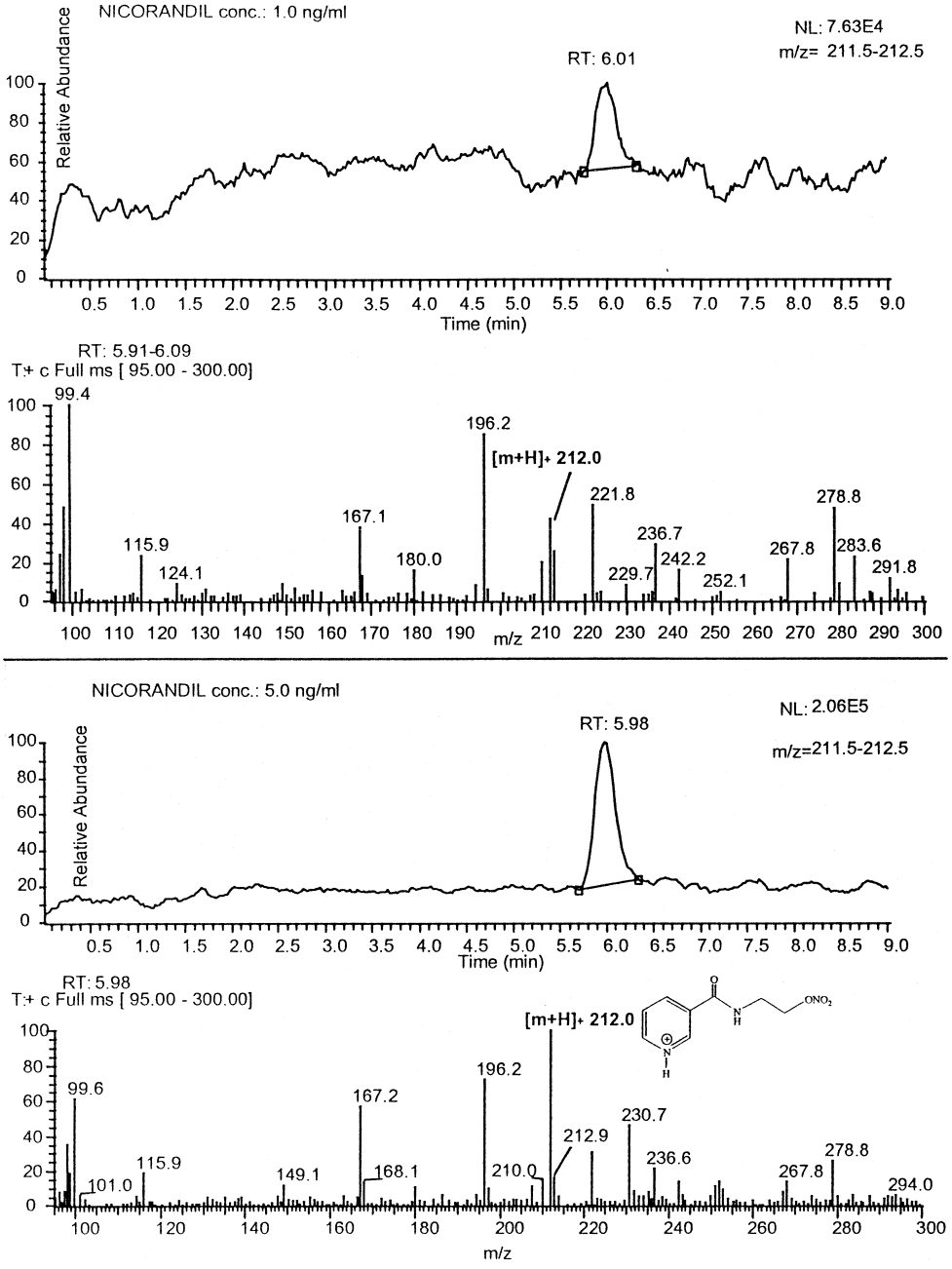

Fig. 3. Representative chromatograms and spectra information with MS detection at 1.0 and 5.0 ng of nicorandil in 1.0 ml of human plasma.

plified extraction procedure and requires minor usage of solvents. Typical chromatograms are presented in Fig. 2.

The HPLC separation conditions were proper for LCQ detector use, Fig. 3. Because of the improved sensitivity and selectivity of this system, the quantitation of nicorandil may be improved to a concentration of 1.0 ng/ml of nicorandil in plasma, even if some coeluting compounds are present.

3.2. Validation and application

The relative standard deviation levels for nominal concentrations of 2.5, 5.0 and 10.0 ng/ml were 26.3%, 4.3% and 3.2%, respectively. The limit of quantitation of nicorandil was established [12] at 5.0 ng/ml and the limit of detection was below 3 ng/ml.

Relative standard deviations (RSD%) for linear curve points at concentrations of 5, 10, 20, 30, 50, 100, 200 and 300 ng/ml of nicorandil were 11.0, 2.8, 4.8, 3.4, 7.8, 4.3, 7.7 and 9.5%, respectively. The deviations of back calculated values for calibration curve points were within 5% of the respective nominal values.

The weighted calibration curves over five days were linear in the range from 5.0 to 300 ng/ml with correlation coefficients (r) of at least 0.9950.

The plasma extraction recoveries for nominal nicorandil concentrations at 15.0, 60.0 and 120 ng/ml were 54.8%, 54.6%, and 46.4%, respectively.

The relative standard deviations (RSD) evaluated at concentrations of 15, 60, 120 ng/ml for intra-day variations were 6.22, 7.56 and 9.06%, respectively, and for inter-day variations were 2.21, 6.31 and 12.19%, respectively (Table 1).

The average measured concentration for short-term stability of nicorandil plasma samples were 16.5 and 123.8 ng/ml for nominal concentrations of 15 and 120 ng/ml. The RSD values for these QC samples were 5.58% and 9.76%, respectively. The RSD values of 24 h stability extracted plasma samples of nicorandil at the same nominal concentrations were 5.68% and 9.04% and the measured values were 13.8 and 124.7 ng/ml, respectively (Table 2). Comparison plasma samples of the same concentrations were stored at −20°C and than were freshly prepared by the extraction procedure as described above and immediately injected. From the data in Table 2, we can conclude that there is no statistical significant difference between comparison and stability samples.

Table 1
Intra-day and inter-day variations in the determination of nicorandil in human plasma[a]

n	Nicorandil (ng/ml)			
	Added	Measured	SD	% RSD
Intra-day				
10	15.0	14.0	0.87	6.22
10	60.0	58.2	4.40	7.56
9	120.0	123.3	11.17	9.06
Inter-day				
10	15,0	13.1	0.29	2.21
10	60,0	58.6	3.70	6.31
9	120,0	121.5	14.81	12.19

[a] Column: Nucleosil-Phenyl, particle size: 5 μm, 150×4.6 mm I.D., Mobile phase: 0.01 *M* ammonium acetate aqueous buffer and acetonitrile (10:3, v/v), pH=6.2, Flow: 1.0 ml/min, Temperature: 25°C, Injection: 50 μl, Detection wavelength: 256 nm.

3.3. MS determination

The same extraction procedure and chromatographic system as already have been described, were used for system equipped with ion trap MS detector. The atmospheric pressure chemical ionisation (APCI) interface was used for in-line introduction of the plasma sample into MS after the chromatographic separation.

The established limit of quantitation, according to the measured peak area, was 1.0 ng/ml [12]. Detection of nicorandil is possible below 0.5 ng of nicorandil per milliliter of plasma. Table 3 represents LOD and LOQ data.

The calibration curves were linear in the range from 0.5 to 150 ng/ml with correlation coefficients (r) of at least 0.9950.

The calibration curve obtained using the HPLC–MS system was compared to the calibration curve obtained on the HPLC–UV system (Fig. 4).

Blood samples were collected 0.5, 1.0, 1.5, 2.0, 4.0, 6.0, 8.0, 12.0, 18.0, 30.0, 42.0 h after oral administration of 20 mg dose of nicorandil. The plasma concentration–time profile calculated by this method represent the Fig. 5. The maximum plasma

Table 2
Stability of spiked plasma and extracted plasma samples[a]

No. of samples	Nicorandil (ng/ml)*				
	Added	Measured	% Nominal.	SD	% RSD
Short term spiked plasma stability (5 h at room temperature 25°C)					
Comparison samples					
8	15.0	15.9	105.8	1.41	8.89
9	120.0	123.0	102.5	9.95	8.09
Stability samples					
9	15.0	16.5	109.9	0.92	5.58
9	120.0	123.8	103.2	12.1	9.76
24 h stability of extracted plasma samples in autosampler at room temperature 25°C					
Comparison samples					
8	15.0	13.4	89.3	0.77	5.74
9	120.0	111.6	93.0	9.38	8.40
Stability samples					
9	15.0	13.8	92.0	0.78	5.68
10	120.0	124.7	104.0	11.3	9.04

[a] Column: Nucleosil-Phenyl, particle size: 5 μm, 150×4.6 mm I.D. Mobile phase: 0.01 *M* ammonium acetate buffer$_{(aq)}$ and acetonitrile (10:3, v/v), pH=6.2 FLOW: 1.0 ml/min, Temperature: 25°C, Injection: 50 μl, Detection wavelength 256 nm.

Table 3
Established limit of quantitation [12] and limit of detection[a]

	Mean peak area	SD	RSD (%)
(*n*=6) 0.5 ng/ml	20405	6305	30.9
(*n*=6) 1.0 ng/ml	58945	24497	4.9
(*n*=6) 2.0 ng/ml	9819470	490974	5.0

[a] Column: Nucleosil-Phenyl, particle size: 5 μm, 150×4.6 mm I.D. Mobile phase: 0.01 *M* ammonium acetate aqueous buffer and acetonitrile (10:3, v/v), pH=6.2, Flow: 1.0 ml/min, Temperature: 25°C, Injection: 50 μl, Capillary temperature: 150°C, Capillary voltage: 4.0 V, Vaporiser temperature: 450°C, Sheath Gas flow (N_2): 80 units (0.65 Mpa), Auxillary gas flow (N_2): 5 units (0.05 Mpa), Tube lens offset: 30 V, Discharge voltage: 4.0 kV, Discharge current: 10 μA, Multiplier voltage: −950 V, Atmospheric pressure chemical ionisation (direct on-line sample introduction into MS).

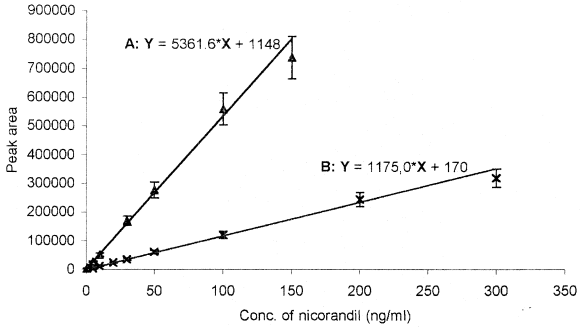

Fig. 4. The comparison of weighted linear curves made by using (A)-MS detector and (B)-UV detector

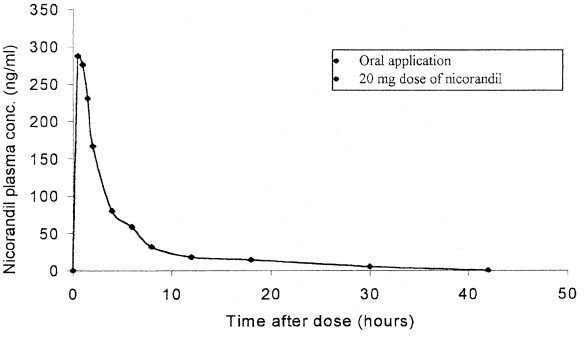

Fig. 5. Nicorandil concentrations in human plasma after oral application.

concentration of nicorandil was reached soon after administration, and it was 288 ng/ml. Until 8 h time interval, the plasma concentration curve declined very rapidly, and after that it shows a gradual decline with a terminal elimination half by value of 2.5 h.

4. Conclusion

However, this new, fully validated, analytical method is one of the most sensitive and the most rapid method for the determination of nicorandil blood plasma levels in clinical studies, using reversed-phase HPLC with UV or MS detection. According to the method validation results, the extraction procedure is highly reproducible and numerous samples can be analysed over night with an automated recording and calculating system. Therefore this assay method may represent the state of the art of present methods for bioequivalence studies.

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