

A validated, stability-indicating HPLC method for the determination of Nicorandil related substances from Nicorandil tablet dosage form

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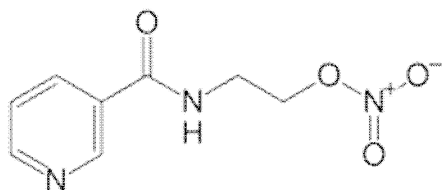
Abstract: Nicorandil is a potent drug with its dual mechanism of action results in its wide application in treating angina patients. Since the literature review showed no reported method for evaluation for the impurities generated in process and by force degradation. Thus the paper describes a method for estimation of impurities of Nicorandil in tablet dosage form using HPLC. The best separation was achieved using an Inertsil C₈ 250 x 4.6mm; 5 μ HPLC column at 30°C employing a gradient elution. Mobile phase consisting of solvent A (1.42 gm of Disodium hydrogen phosphate anhydrous and adjust the pH 6.4 with ortho phosphoric acid. Solvent B (Acetonitrile) and solvent C (methanol) was used at a flow rate of 1.0 ml min⁻¹. UV detection was performed at 262 nm. This method can separate all impurities of Nicorandil in a run time of 35minutes. Specificity of the method was established from the peak purity indices obtained with the aid of PDA detection and satisfactory resolution between related impurities. The formulation was subjected to oxidation, hydrolysis, photolysis, and heat as stress conditions. Relevant degradation was found to take place under photolytic degradation condition. The method was linear over the concentration range 0.003-0.4 mg mL⁻¹ for Nicorandil respectively. Robustness against small modification in pH, column oven temperature, flow rate and percentage of the mobile phase composition was ascertained. Validation of the method was done as per ICH guidelines, demonstrating to be accurate and precise (repeatability and intermediate precision level) within the corresponding linear range of known impurities of Nicorandil in tablet dosage form.

Keywords: Nicorandil; Related substances; RP-HPLC; Stability Indicating; Validation.

1. Introduction

Nicorandil (2-[(pyridin-3-ylcarbonyl) amino]ethyl nitrate) a nicotinamide derivative used as vasodilatory drug used in treatment of angina. The drug act through two methods, firstly, by activating potassium channels, and secondly by donating nitric oxide to activate the enzyme guanylate cyclase. This enzyme causes activation of cGMP leading to both arterial and venous vasodilatation by de-phosphorylation of the myosin

light chain. As it is selective for vascular potassium channels, it has no significant action on cardiac contractility and conduction. Thus is a novel drug for treatment of angina pectoris.¹ Further many studies have suggested that the drug possess similar safety and efficacy as the other drugs used for angina but efficacy increase after a year on continued treatment.^{2,3} The drug has also now been evaluated in combination with other drugs like Lamotrigine⁴.

Figure 1: Nicorandil

The literature revealed that the assay of the drug in pure and dosage forms is not official in any pharmacopeia and, therefore, requires much more investigation. The estimation of Nicorandil from biological fluids and/or pharmaceutical formulations has been conducted using several analytical methods include high-performance thin layer chromatography^{5, 6,7} high-performance liquid chromatography^{8,9,10,11,12,13, 14} and gas chromatography coupled with mass spectrometry¹⁵. A review of literature revealed no stability indicating HPLC method for the related substances of Nicorandil in pharmaceutical formulations.

The Regulatory agencies recommend the use of stability indicating methods (SIMs) FDA Analytical Procedures and Method Validation^{16, 17} for the analysis of stability samples¹⁸. This requires stress studies in order to generate the potential related impurities under stressed conditions; method development and validation¹⁹. With the evident of the International Conference on Harmonization (ICH) guidelines (ICH)^{20, 21}, requirements for the establishment of SIMs have become more clearly mandated. The productions of the potential impurities in a drug product generally take place under various environmental conditions like exposure to light, heat, hydrolysis or oxidation. Hence Stress testing can help identifying degradation products and provide important information about intrinsic stability of the drug product.

Therefore, present article reports the results of stability study of related substance of Nicorandil form Nicorandil tablet with the aim of determining the extent of the influence of different stress conditions on the stability of drug product.

2. Experimental

2.1 Reagents and Materials

Nicorandil active pharmaceutical ingredient (API) and test sample (Each tablet containing 20mg Nicorandil tablet) were kindly supplied by Getz Pharma Research, Ambarnath, India. Individual reference standards for Nicorandil impurities (Fig 1) were not available. The EP CRS for system suitability, consisting of a mixture of all the impurities was procured from (LGC Promochem, India). The

chemical names for all components are listed in Table 1.

Disodium hydrogen phosphate was obtained from Merck Limited, Mumbai, India; Methanol was procured from Merck Mumbai, India; Acetonitrile was obtained from Rankem Mumbai, India; Ortho-Phosphoric acid, Sodium Hydroxide, Hydrochloric acid, 50% Hydrogen peroxide were also obtained from Merck Limited, Mumbai, India. High purity deionised water was obtained from [Millipore, Milli-Q (Bedford, MA, USA)] purification system.

2.2 Instrumentation

HPLC system (Waters 2695 Alliance Separation Module) (eg. Waters Milford, USA) equipped with inbuilt autosampler and quaternary gradient pump with an on-line degasser was used. The column compartment having temperature control and Photodiode Array/ Ultraviolet (PDA/UV) Detector (2996/2487) was employed throughout the analysis. Chromatographic data was acquired using Empower software. The Analytical Balance used for weighing was of the make –Mettler Toledo, Model- XS205DU. The pH meter used was of the make -Thermo Electron Corp., Model-Orion-4star 1117000

2.3 Chromatographic conditions

Inertsil C8, 250 x 4.6 mm, 5µm or equivalent column was used as stationary phase maintained at 30°C. The mobile phase involved a variable composition of solvent A (1.42 gm of Disodium hydrogen phosphate anhydrous transfer into 1000 ml of water. Adjust the pH 6.4 with ortho phosphoric acid. Filter through 0.45µ Nylon membrane filter and sonicate to degas.), solvent B (Acetonitrile) and Solvent C (Methanol). The mobile phase was pumped through the column with at a flow rate of 1ml min⁻¹. The gradient program is shown in Table 1.

The optimum wavelength selected was 262 nm which represents the wavelength where all impurities have suitable responses in order to permit simultaneous determination of related impurities of Nicorandil tablets. The stressed samples were analyzed using a PDA detector covering the range of 200–400 nm.

2.4 Solution Preparation

2.4.1 Diluents

The following was used as diluent:

A mixture of Methanol: Water in the ratio 50:50 v/v.

2.4.2 System Suitability Solution

The standard solution prepared was used for system suitability evaluation.

2.4.3 Standard Solution

2.4.3.1 Preparation of Standard Solution

For standard preparation Nicorandil standard 42mg was accurately weighed and transferred to 250 ml amber coloured volumetric flask. 100 ml of diluents was added and sonicated to dissolve, followed by dilution till volume with diluent. 3 ml of this diluted solution was transferred to 250 ml volumetric flask and volume is made up with diluents.

2.4.4 Sample Solution

5 tablets weighed and transferred to 100ml amber coloured volumetric flask, 70ml of diluents was added and sonicated for 15minutes. The flask was allowed to cool at room temperature. The volume was made up to the mark with diluent. The sample solution was filtered through 0.45 μ Nylon membrane filter.

2.4.5 Forced degradation sample solution for specificity study

Multiple stressed samples were prepared as indicated below. They were carried out on the tablets and chromatographed along with a non-stressed sample (control).

2.4.5.1 Hydrolytic conditions: acid-induced degradation.

5 tablets of Nicorandil were weighed into 100 ml amber colored volumetric flask, to this 10 ml of diluent was added and sonicated for 15minutes. 2 ml of 5N Hydrochloric acid was further added. The solution was heated on the water bath at 70°C for 3 hours. The solution was cooled and neutralized with same volume and same strength alkali. The solution was made up to the volume with diluent. Placebo was weighed equivalent to tablet and treated as sample.

2.4.5.2 Hydrolytic conditions: base-induced degradation.

5 tablets of Nicorandil were weighed into 100 ml amber colored volumetric flask, to this 10 ml of diluent was added and sonicated for 15minutes. 2 ml of 0.5N sodium hydroxide was further added. The solution was heated on the water bath at 70°C for 1 hour. The solution was cooled and neutralized with same volume and same strength alkali. The solution was made up to the volume with diluent. Placebo was weighed equivalent to tablet and treated as sample.

2.4.5.2 Oxidative condition: hydrogen peroxide-induced degradation.

5 tablets of Nicorandil were weighed into 100 ml amber colored volumetric flask, to this 10 ml of diluent was added and sonicated for 15minutes. 1 ml of 50 % Hydrogen peroxide was further added. The solution was heated on the water bath at 70°C for 45minutes. The solution was cooled and made up to

the volume with diluent. Placebo was weighed equivalent to tablet and treated as sample.

2.4.5.3 Thermal degradation study.

10 tablets of Nicorandil were weighed and transferred into 100 ml volumetric flask. 10 ml of diluent-B was added and sonicated for 5 to 10 minutes with intermittent shaking till the tablets disintegrate. The solution was cooled at room temperature and 10 ml of diluent C was added. The solution was heated in the oven at 70°C for 24 hours, cooled and volume was made up the with diluent-D. Further 3 ml of this solution was diluted to 25 ml with diluent-D.

2.4.5.4 Photolytic degradation study.

As per guidelines for photostability testing of new drug substances and products, samples should be exposed to light providing an overall illumination of not less than 1.2 million lx hours and an integrated near ultraviolet energy of not less than 200Wh/m² to allow direct comparisons to be made between the drug substance and drug product.

For photo stability testing 5 tablet of Nicorandil was transferred to 100 ml flask covered with aluminum foil, to it 10 ml of diluent is added and sonicated for 15minutes. The flask was kept under UV and white light for 1.2 million lux hours in photo stability chamber. After study the sample was cooled and diluted up to the mark with diluent. Placebo equivalent to tablet was weighed, transferred and treated as tablet sample.

2.4.6 Preparation of Placebo solution

Placebo equivalent to 5 tablets (450 mg) were accurately weighed and transfer into 100 ml amber coloured volumetric flask, to it 70ml of diluent was added and sonicated for 15minutes. The flask was allowed it to cool at room temperature. The volume was made up to the mark with diluent. The sample solution was filtered through 0.45 μ Nylon membrane filter.

3. Results and Discussion

3.1 Optimization of chromatographic conditions

3.1.1 Selection of stationary phase:

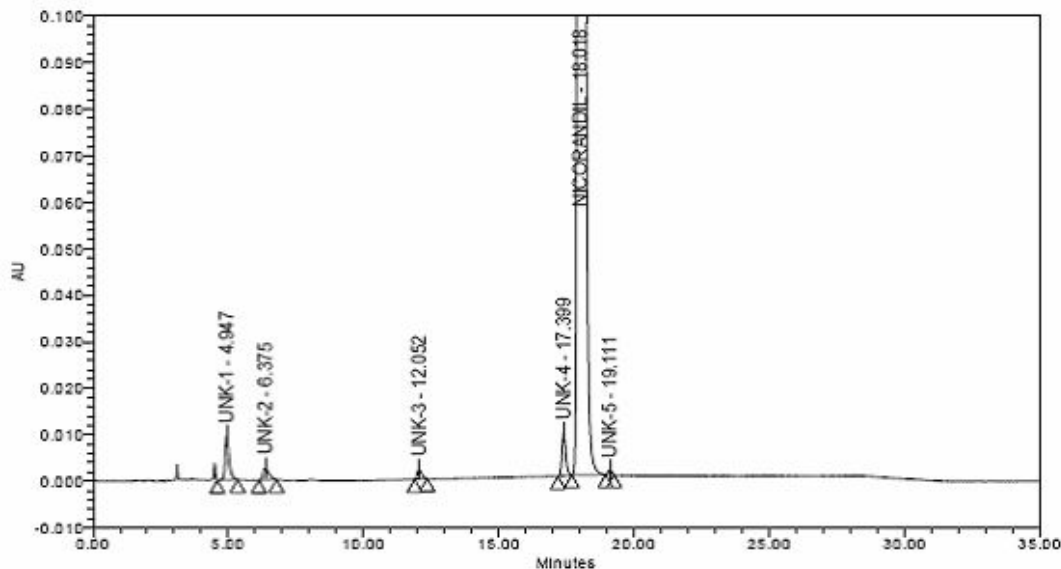
Different reversed phase column were used as stationary phase selection during column selection. The column differed in length and bonding (C18) but the desired separation was achieved using Inertsil C₈ 250 x 4.6 mm, 5 μ m and thus it was proven robust in nature.

Table 1: Gradient program

Time in minutes	Mobile phase A %	Mobile phase B %	Mobile phase C %
0	90	6	4
5	90	6	4
15	70	20	10
25	70	20	10
26	90	6	4
35	90	6	4

Table 2: Validation summary

Parameters	Result obtained	
System suitability % RSD for Standard solution.	1.28%	
USP Tailing Factor	1.14	
USP Plates	84820	
Forced degradation	The peak due to known impurities was pure as shown in PDA	
Limit of Detection	% RSD	Concentration (%)
	11.70	0.005
Limit of Quantitation	1.71	0.015
Linearity	Response is linear between 0.003mg/ml to 0.4 mg/ml	
	Correlation coefficient: 1.0000	
	Y- Intercept is within $\pm 10.0\%$ of corresponding Y-co-ordinate.	
Accuracy (Recovery)	% Mean Recovery	
	At LOQ: 97.8%	
	At 80-120%-100.5%	
Method Precision	RRT-0.27	RRT-0.97
	1.66	5.11
Intermediate Precision (Ruggedness) RSD for % Single max Impurity content	Complies	
	RRT-0.27	RRT-0.97
	1.58	8.21
RSD for pooled result (Analyst-I and II)	2.86	8.46
Stability in analytical solution	Sample is stable for 6 hours at 15°C	
Robustness Change in Flow rate (± 0.1 ml/min) Change in wavelength (± 5 nm) Change in Buffer pH (± 0.2) Column oven temperature ($\pm 5^\circ\text{C}$)	No significant change in System suitability	
Filter compatibility	% Difference for impurity content of Centrifuge and filtered was ± 0.05 .	

Figure 2: Typical Chromatogram for RS of Nicorandil

3.1.2 Influence of pH of mobile phase buffer

A pH change of ± 0.2 units did not have any adverse effect on the separation.

After optimizing various parameters, the method was finalized on Inertsil C₈ 250 x 6mm; 5 μ HPLC column using variable composition of solvent A: anhydrous Na₂HPO₄ (1.42 g L⁻¹) pH 6.4 with orthophosphoric acid, solvent B: Acetonitrile and solvent C: Methanol. The separation was achieved using gradient programming as shown in table 1. The mobile phase pumped through the column at a flow rate of 1.0 ml min⁻¹ and column compartment temperature kept at 30° C. The detector response for all the components found suitable at 262 nm; with sample volume of 20 μ l; hence the typical chromatogram was recorded at this wavelength. The typical HPLC chromatograms (Fig. 2) represent the satisfactory separation of all components among each other.

3.2. Method validation

The optimized RP-HPLC method was validated according to ICH guidelines. The various validation parameters that were performed are as follows: Specificity, Accuracy, and Precision (Repeatability and Intermediate Precision), Linearity, Range and Robustness. System suitability features were also assessed. Solution stability and filter compatibility were also studied.

3.2.1. System suitability test

The system suitability test performed according to USP 30. The Standard solution was injected six times into the chromatograph and the chromatograms were

recorded. The relative standard deviation of the area for individual peaks, for six replicate injections of standard solution should not be more than 5.0 %. The USP theoretical plates for Nicorandil should not be less than 50000.

The relative standard deviation for six replicate injections of standard solution was found to be less than 5.0 % which is tabulated in Table 2. The results obtained for Theoretical plates, USP tailing factor (T_f) were also all within acceptable limits.

3.2.2. Specificity

The peak purity indices for the analytes in stressed solutions determined with PDA detector under optimized chromatographic conditions were found to be better (purity angle < purity threshold) indicating that no additional peaks were co-eluting with the analytes and evidencing the ability of the method to assess unequivocally the analyte of interest in the presence of potential interference. Baseline resolution was achieved for all investigated compounds. The FDA guidelines indicated that well separated peaks, with resolution, R_s > 2 between the peak of interest and the closest eluting peak, are reliable for the quantification. All the peaks meet this specification, visibly confirmed in Fig.2. Table 2 summarizes the tabulated results for specificity.

3.2.3 Forced degradation results:

The acid degradation carried out using 5N HCL resulted in adequate degradation of the sample with percentage purity of 89.51%. 92.84% degradation was observed after degradation with 0.5N NaOH base. The

forced degradation of sample with 50% hydrogen peroxide degraded the drug Nicorandil till purity of 85.15%.

The drug Nicorandil was maximum degraded with heat degradation to give sample purity of 78.67%. About 80% purity of Nicorandil was after photolytic degradation of sample under different condition. The values of % purity have been summarized in table 2. The table also shows that the purity angle is less than purity threshold, for Nicorandil peak for all conditions thus the peak is pure and the method is stability indicating with respect to forced degradation studies.

3.2.4. Linearity and Range

The nominal concentration of test solution for Nicorandil was 0.003 mgml^{-1} and 0.4008 mgml^{-1} , respectively.

The limit of any impurity related to Nicorandil was kept at NMT 0.1% for unknown. The plots of area under the curve (AUC) of the peak responses of the analytes against their corresponding concentrations fitted straight lines responding to equations.

The y-intercepts were close to zero with their confidence intervals containing the origin. The correlation co-efficient (r) of Nicorandil was found to be 1.0000.

The results are tabulated in Table 2. The linear regression plot is shown in figure 3.

3.2.4.1. Determination of limit of quantification and detection (LOQ and LOD).

The linearity was performed with series of dilutions of decreasing concentrations were injected into the system and the areas were determined, Graph of concentration vs. area were plotted and SLOPE of the line was calculated. This was then used for the determination of limit of quantification and detection. Residual standard deviation (σ) method was applied and the LOQ and LOD values were predicted using following formulas (a) and (b) and established the

precision at these predicted levels.

$$\text{LOQ} = 10 \frac{\sigma}{S} \quad (\text{a})$$

$$\text{LOD} = 3.3 \frac{\sigma}{S} \quad (\text{b})$$

Where

σ = residual standard deviation of response and

S = slope of the calibration curve.

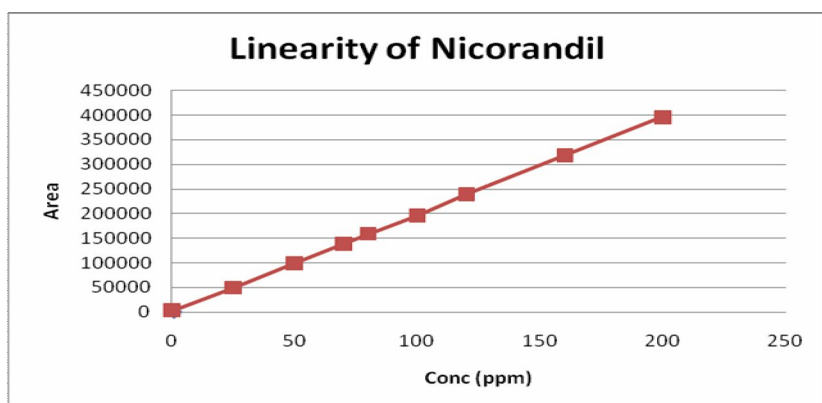
The linearity results are tabulated in Table 6. The predicted concentration from the formula was further subjected to reproducibility to ensure the validation of the method. The results of reproducibility are tabulated in table 2, which show that % relative standard deviation of 11.70% at LOD and 1.71% at LOQ. Thus from the results an inference can be drawn that the method can detect and quantify the lowest concentrations with minimum error in a reproducible manner.

3.2.5 Accuracy

Accuracy was evaluated by the determination spiking placebo with Nicorandil in solution by standard addition method. The experiment was carried out by adding known amount of analyte corresponding to four concentration levels of LOQ, 50%, 100% and 200% of the specification level in sample solution. The samples were prepared in triplicate at each level. The quantification of added analyte (%weight/weight) was carried out by using an external standard of corresponding main drug prepared at the analytical concentration.

The accuracy limits were kept at 75 to 125% at LOQ levels and 80 to 120% for other levels. The experimental results revealed that approximately 93.3–101.7% recoveries were obtained at different levels. Therefore, based on the recovery data (Table 2) the estimation of related compounds that are prescribed in this report has been demonstrated to be accurate for intended purpose and is adequate for routine analysis.

Figure 3: Linearity of Nicorandil



3.2.6 Method precision and ruggedness

ICH (International Conference on Harmonization of technical Requirements for Registration of Pharmaceuticals for Human Use) considers ruggedness as the method reproducibility and intermediate precision.

During Method precision six independent sample preparations were injected. During intermediate precision the same exercise was repeated using a fresh set of samples on a separate day, on a separate instrument, using a different HPLC column serial number by a different analyst. The results of the precision for the tablet strength of 80_12.5 are revealed in the data given in Table 2.

3.2.7. Robustness

In order to demonstrate the robustness of the method, system suitability parameters were verified by making deliberate change in chromatographic conditions, i.e. change in flow rate by $\pm 0.1 \text{ ml min}^{-1}$, change in wavelength at 257 and 267nm change in pH of the buffer 6.2 and 6.6, change in column oven temperature by $\pm 5^\circ\text{C}$. The standard and sample was injected and the system suitability conditions and final result was monitored. The results of the method demonstrated to be robust over an acceptable working range of its HPLC operational conditions. The results for the robustness are summarized in Table 2. Hence it was concluded that method is Robust.

3.2.8 Solution Stability

The standard and sample solution was kept at sample temperature for 24 hours were injected on to the HPLC. The data could be summarized as the RSD of Standard solution up to 24 hours is less than 10% and % difference up to 24 hours is ± 0.05 . Thus the

standard solution found to be stable for at least upto 24 hours at 20°C .

3.2.9 Filter Compatibility

The Filter compatibility was evaluated by spiking sample solution filtered through different types of membrane syringe filters (Centrifuged, Glass, Nylon, PVDF and Teflon) were injected on HPLC. The % difference was calculated against centrifuged sample solution. The data shows that % Difference against centrifuged is within the limit ± 0.05 except PVDF filter where it was observed to be 0.13% difference in total impurity. The Single most impurity did not exceed 0.5% and the total impurity is not more than 2% in all the filter paper used.

4. Conclusion

A stability study was carried out and an efficient HPLC method for the quantification of related substances of Nicorandil in drug product was developed and validated. The results of the stress testing of the drug, undertaken according to the ICH guidelines, revealed that the photo-degradation generated two new unknown impurity degradation products were formed.

The validation experiments provided proof that the HPLC analytical method is linear in the proposed working range as well as accurate, precise (repeatability and intermediate precision levels) and specific, being able to separate the main drug from its degradation products. The proposed method was also found to be robust with respect to flow rate, column oven temperature, pH of mobile phase. Due to these characteristics, the method has stability indicating properties being fit for its intended purpose; it may find application for the routine analysis of the related substances of Nicorandil in Nicorandil tablets.

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