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Determination of inorganic nitrate impurity from nicorandil and its tablet dosage form by simple reversed phase liquid chromatographic method

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Abstract

A simple, fast, precise, specific and economical reverse phase liquid chromatographic method was developed for determination of nitrate (NIT) impurity from nicorandil (NIC) and its tablet dosage form. NIT is process impurity as well as a degradation product of NIC. This article is based on application of amino propyl silane (APS) column for determination of NIT in presence of NIC and various excipients using potassium nitrate as a standard. The developed method was validated for its intended use by evaluating various important parameters like linearity, accuracy, recovery and specificity. The developed method was applied to evaluate the compatibility study of NIC with various excipients by monitoring the NIT content.
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Keywords: Inorganic nitrate; Nicorandil; APS column; Specific

1. Introduction

NIC is potent vasodilator and categorized as life saving drug, chemically it is [*N*-[(2-(nitroxy)ethyl]-3-pyridinecarboxamide (Fig. 1). NIC is a nitrate in chemical structure, however, at basic pharmacology it has dual mechanism of action: one is related to its chemical structure as a nitrate and other is unrelated. Release of NIT is inherent property of NIC in vivo as well as in vitro [1]. NIC as drug and dosage form, upon storage produces NIT as a major impurity. Quantification of NIT as an impurity is considered as an important parameter to evaluate the stability of the NIC as active pharmaceutical ingredient (API) and its dosage form.

Various analytical techniques were used to determine NIC, like difference spectroscopy [2], spectrophotometric [3], HPLC [4–6], and HPTLC [7,8]. Stability of NIC is biggest hindrance for the formulator as it is highly unstable. A report related to in vitro and in vivo formation of de-nitrated product, *N*-(2-hydroxyethyl)nicotinamide [HEN], and free inorganic nitrate ion from NIC is reported [9]. Formation of free NIT is reported but not tried to determine in any publication, but free nitrate is an impurity and should be monitored. Determination of NIT from pharmaceuticals [10], water [11,12] was reported using ion exchange chromatography.

Determination of NIT as an impurity from NIC and its pharmaceutical product is not attempted yet. Determination of NIT was reported using ion exchange column employed with various detection techniques like spectrophotometric [13,14], and fluorescence [15]. Use of ODS column along with ion interaction reagent [16] and column immobilized with bovine serum albumin as a stationary phase [17] were published for determination of NIT.

The ion exchange columns are very costly and use of it for the formulation is limited, so it was taken as a major aim to develop a simple, sensitive and precise LC method using a commonly used column for the determination of NIT from NIC and its tablet dosage form. The developed method is validated for its intended purpose and successfully applied for the determination of NIT as an impurity from NIC and tablet dosage form. The developed method is successfully applied to determine the increased level of NIT in the course of routine and accelerated stability study of dosage form. It was also applied to study the excipient compatibility of

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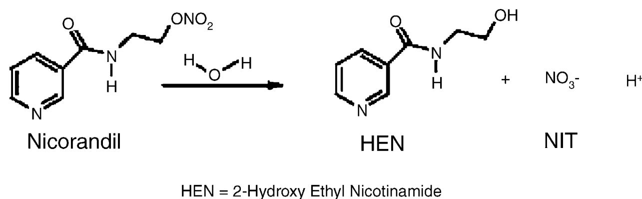

Fig. 1. Structure of nicorandil and formation of nitrate.

NIC using determination of NIT level at each specified intervals.

2. Experimental

2.1. Materials

(a) Potassium nitrate—references standard grade was purchased, with claimed purity of 100% from Sigma–Aldrich (Banglore, India).
(b) Nicorandil—working standard grade (99.75%) was supplied by Torrent Research Center (Gandhinagar, India).
(c) NIC tablets (20 mg), Placebo and various excipients were supplied by Torrent Research Center (Gandhinagar, India).

2.2. Reagents

(a) H_3PO_4—Merck, India.
(b) Boric Acid—Merck, India.
(c) KH_2PO_4—Merck, India.
(d) 0.2 M hydrochloric acid—aqueous solution (Merck, India).
(e) 1 M NaOH—aqueous solution (Merck, India).
(f) Methanol – HPLC grade, (Rankem, India).
(g) Milli-Q water—it was purified by Millipore Corporation's system (Millipore USA).
(h) KCl—GR grade, (Merck, India).
(i) KOH—GR grade, (Merck, India).

2.3. Instruments

(a) Walk-in stability chamber—(Newtronic, India).
(b) Hot air oven (Labline, India) were used.

2.4. Chromatographic system

A Shimadzu model, LC-2010C chromatograph with auto-injector, sample cooler attached with UV–vis and photo diode array (PDA) detector (SPD 10Ma vp) was used. Separation was achieved on an APS column (Hypersil–BDS, 150 mm × 4.6 mm i.d., 3 μm particle size) with a mobile phase consisting of a mixture of KH_2PO_4 buffer (10 mM) and methanol (70:30, v/v), pH was adjusted to 5.0 (if required) with 1.0% (w/v) aqueous solution of KOH or 1.0% (v/v) H_3PO_4. UV visible detector was set at 220 nm, while during the course of method development PDA detector was set from 190 to 400 nm range. The flow was adjusted to 1.0 ml/min, and methanol was used as a diluent for sample and standard preparation.

2.5. Standard and test solutions

2.5.1. Standard preparation (0.5%, of test concentration)

32.2 mg of potassium nitrate equivalent to 20 mg NIT was weighed in 50 ml volumetric flask and dissolved using 2 ml of Milli-Q water; volume was adjusted to the mark with methanol. The solution was dilute suitably to prepare the standard solution containing 0.2 μg/ml of NIT.

2.5.2. Test preparation

Five tablets (equivalent to 100 mg NIC) were weighed and transferred to 250 l volumetric flask, 200 ml of methanol was added to it and sonicated for 30 min. After cooling the solution volume was adjusted to the mark with methanol, and 10 ml of the solution was centrifuged at 3500 rpm for 10 min and the supernatant was suitably diluted to get 40 μg/ml concentration of NIC. Complete dispersion of tablet is difficult by employing only sonication; so vigorous shaking of flask during initial 15 min sonication was found essential.

The standard containing NIT was found stable for 24 h at 25 °C, while in the test solution containing NIC, the content of NIT was found increased after 4 h of sample preparation at 25 °C, while found stable for 6 h at 5 °C.

2.6. Method validation

The developed method was validated for linearity at various concentration range (0.001–0.028 μg/ml) of NIT. System precision was evaluated by analyzing the standard solution for five times ($n = 5$). Method precision (repeatability) was evaluated by performing six replicate determination of NIT for the same sample of NIC; here sample concentration was 0.04 mg/ml of NIC ($n = 6$), on same day. Reproducibility of the method was evaluated by analyzing the same sample on three different days in triplicate ($n = 3$). Recovery of the method was evaluated at four different concentration levels by spiking a known amount of potassium nitrate in pre-quantified sample preparation (with respect to NIT). Limit of detection (LOD) and limit of quantification (LOQ) of the method for determination of NIT was derived by calculating signal-to-noise ratio (S/N ratio) of diluted standard (0.001 μg/ml). Data related to method validation are represented in Tables 1 and 2. Specificity of the developed method was evaluated by studying the each excipients separately and also by degradation of NIC in various buffer solutions [prepared as per United State Pharmacopoeia (USP) (Table 3)]. All the samples were degraded by exposing to 60 °C for 24 h and analyzed at concentration of 0.04 mg/ml of NIC. Peak purity of NIT peak monitored on PDA detector and data obtained by this exercise are represented in Table 3.

3. Results and discussion

Various published literature suggested formation of NIT due to degradation of NIC, as explained in Fig. 1. The chemistry of NIC degradation is very complex and it generates various other impurities along with NIT [18], determination of other impurities is under scope of further research. During the development

Table 1
Data indicating various validation parameters of the developed method

Parameter	NIT at 220 nm
Limit of detection	0.0005 μg/ml
Limit of quantification	0.001 μg/ml
Linearity range	0.001 to 0.028 μg/ml
Linearity equation	$y = 1800645x + 511$
Correlation coefficient	0.9999
System precision (%, RSD) ($n = 5$)	0.4
Repeatability of measurement (% of nitrate) ($n = 6$)	Mean ± SD, %RSD, 5.56 ± 0.02, 0.4
Reproducibility of measurement, (% Of Nitrate) ($n = 3 \times 3$)	Mean ± SD, %RSD
Day 1	5.52 ± 0.05, 0.3
Day 2	5.50 ± 0.04, 0.4
Day 3	5.58 ± 0.06, 0.5
Overall %RSD	0.4

Table 2
Data indicating recovery study of NIT

Amount of NIT added (μg)	Amount of NIT found (μg)	Recovery (%)	Average recovery (mean) (%)
0.001	0.001	101.6	98.4
0.01	0.010	99.7	
0.02	0.019	95.8	
0.03	0.029	96.6	

trials it was thought of interest to develop a single method for determination of all the possible impurities using single liquid chromatography method. Earlier trials include the aim of resolution of NIT from other impurities, excipients and NIC using single method employed with routine C_8 and C_{18} column, but it was found problematic. The major draw back of this method was that the elution of NIT was found at ≅2.5 min, which is considered as a dead volume of the system. Determination of NIT in tablet dosage form was found very difficult due to co-elution of almost all the excipients with NIT peak, and it was found very difficult to resolve the NIT peak from excipients using common reverse phase columns like C_8 and C_{18}.

Various reverse phase columns were tried to resolve the NIT peak from the excipients peak and to retain the NIT peak longer than 4 min to quantify it comfortably. Unfortunately we did not achieve it all together while the determination of NIT is considered as a very important aspect for evaluation of the stability of the dosage from.

Table 3
Data indicating specificity of the developed method

Number	Degradation condition	Total peak purity (NIT)
1.	NIC in pH 1.5 solution[a]	0.9997
2.	NIC in pH 6.8 pH phosphate buffer[a]	0.9997
3.	NIC in pH 9.0 pH borate buffer[a]	0.9987
4.	NIC in 0.5% H_2O_2 in water, for oxidative degradation	0.9999

[a] Various pH solutions and buffers were prepared as per USP 28 and exposed at 60 °C for 24 h.

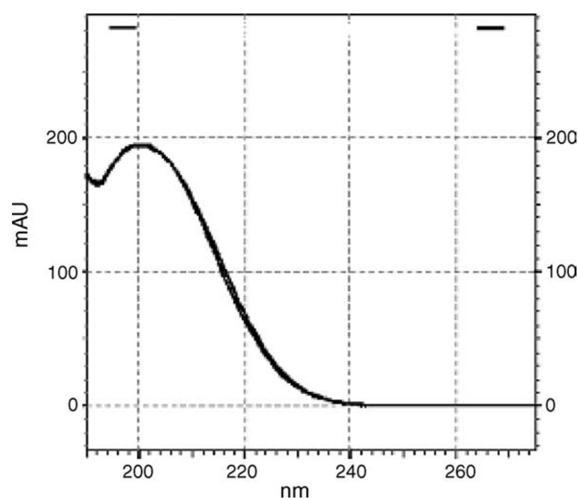

Fig. 2. Verlaid UV spectrum of NIT (2.0 μg/ml) standard (▬) and spectra of NIT in degraded sample of NIC (—).

The use of ion pairing agents as mentioned in [19] was also tried to retain the NIT but the reagent has altered the retention of other organic impurities and lead to longer run time with less effective resolution among the other impurities. Other methods like electro analytical method and discussed in [20] were not tried as our aim was to develop simple liquid chromatographic method, so at last after all facts and circumstances led us to develop a separate method for determination of NIT from drug substance and tablet formulation of NIC. Various published literatures were studied to do the same, but all the methodology published for determination of NIT from water and pharmaceuticals were mainly based on techniques, like ion exchange chromatography attached with conductometric and fluorescence detection etc. All this methods have limited application for the determination of NIT in presences of various excipients like mannitol, hydroxy propyl methyl cellulose (HPMC), eudragit, talc, fumaric acid, stearic acid, and maleic acid.

By considering the negative charge of the NIT ion, application of amino propyl silane column was tried to explore. The logic behind the use of this column was to retain NIT and other ionized (negatively) molecules for sufficient time on column. The free NIT present in the tablet dosage form is easily attached to the positively charged amino group present at the top of amino propyl chain attached with silica bed of the column, which ultimately led the retention of NIT on the column. While other non polarized or positively charged organic components would not retain for longer time on the column and ultimately leads the zero interference in the determination of NIT from the drug and excipients employed in the formulation.

Detection wavelength was selected on the basis of achieving maximum sensitivity of the method, for this purpose the detection wavelength was kept 220 nm and it was found close to the λ max of the NIT in methanol (Fig. 2) and avoided unnecessary base line noise found at lower λ (i.e. 205–210 nm). Selection of mobile phase pH was based on the stability of APS column. Stability of APS column is very less at highly acidic and basic

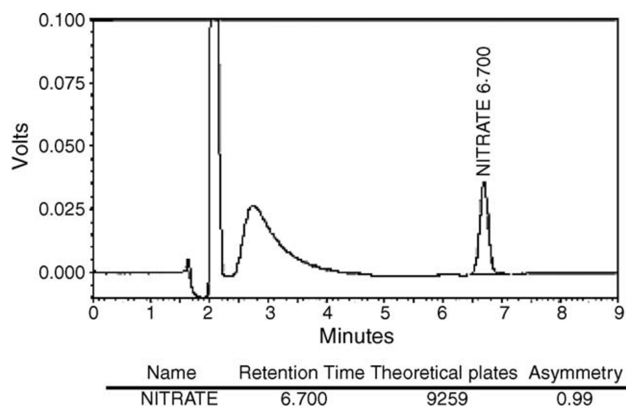

Fig. 3. Chromatogram indicating standard preparation of KNO_3 equivalent to 0.2 μg/ml of NIT.

Table 4
Data indicating compatibility of NIC with various excipients and percentage of NIT at 25 °C and 60% relative humidity

Number	Sample	%NIT @ 2–8 °C	%NIT @ 25 °C/60% RH
		After 3 months	
1	Nicorandil	0.12	0.20
2	Nicorandil + mannitol	0.19	0.19
3	Nicorandil + stearic acid	0.08	0.15
4	Nicorandil + cutina	0.16	0.18
5	Nicorandil + HPMC	0.14	0.36
6	Nicorandil + HPMC K4M	0.17	1.58
7	Nicorandil + aerosil 100	0.17	0.64
8	Nicorandil + talc	0.25	0.45
9	Nicorandil + eudragit L101	0.17	0.49
10	Nicorandil + eudragit RSPO	0.42	1.16
11	Nicorandil + fumaric acid	0.16	0.11

pH of the mobile phase. To achieve the maximum retention of NIT and longer column life, pH of mobile phase is kept around 5.0.

Ideally the presence of buffer part in the mobile phase composition has not play a significant role for resolution and retention of NIT, but as we have discussed that the pH of mobile phase was required to be adjusted around 5.0 which may or may not be achieved using simple mixture of water and methanol and/or acetonitrile. The use of 10 mM potassium dihydrogen phosphate buffer serves both the purpose like it helps to adjust the buffer pH around 4.5, upon addition of required quantity of methanol it shifts the pH around 5.0 (desired pH).

Various trials were taken by alteration of buffer (50, 60, 70, 75, 80 and 90) and methanol (50, 40, 30, 25, 20, 10) ratios to adjust the retention time of NIT and peak of NIC. Finally the ratio was finalized as buffer and methanol (70:30), pH was adjusted to 5.0 with 1.0% (w/v) KOH/1.0% (v/v) H_3PO_4 (if required). It has given the good peak shape of NIT and zero interference from NIC and other common excipients used in the formulation (Figs. 3 and 4), at a flow rate of 1 ml/min.

Response factor method is applicable for precise determination of any component but here it is not applicable as peak of NIC was found at dead volume of the system. In this scenario it was achieved using potassium nitrate as a standard. The retention time of NIT and its peak performance data is represented in Fig. 3.

The proposed method was validated for its intended purpose. The developed method was applied for the determination of NIT from drug substance, and tablet dosage form of NIC. Additionally it was applied to establish the compatibility of NIC with various common excipients and its relation with degradation of NIC and formation of NIT was studied. Data suggests that the NIC is unstable at 25 °C with relative humidity of 60%, while found comparatively stable at 2–8 °C with proposed excipients (Table 4). In all the cases the presence and increment of NIT level plays significant role to claim the stability of drug substance and it is found useful for the development of dosage form containing NIC.

The developed method is used for the optimization of the dosage form and in this context the method has been employed for the evaluation of compatibility study of NIC with various probable excipients. Data related to excipients compatibility study were summarized in Table 4. Data suggests that the increment in NIT level indicates incompatibility of NIC with the particular excipients. The developed method has been successfully employed for the determination of NIT content during the stability study of drug substance and its tablet formulation.

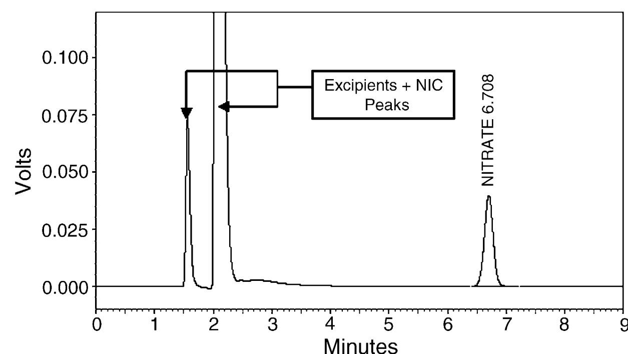

Fig. 4. Chromatogram indicating formation of NIT in sample preparation and its resolution with NIC.

4. Conclusions

The developed method is found economical, fast, precise and specific for the determination of NIT from NIC and its dosage form using simple APS column. The developed method is found suitable to detect NIT from NIC and its dosage form with high degree of precision and reproducibility. NIC is very unstable molecule and has got very complex degradation patterns. It generates other organic impurities along with NIT. Determination of other impurities along with NIC is under scope of future research work. At present proposed method is found suitable for quantification of NIT, as an impurity and validated for its intended use. The developed method is suitable to apply as a simple quality control tool for the determination of NIT as an impurity from NIC and its tablet dosage form.

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