International Journal of Applied Pharmaceutics

ISSN- 0975-7058 Vol 13, Issue 1, 2021

Original Article

A VALIDATED RP-HPLC METHOD FOR IMPURITY PROFILING OF SODIUM NITROPRUSSIDE IN INJECTION DOSAGE FORM

MURALI KRISHNAM RAJU P.[a,b], VENKATA NARAYANA B.[b], SHYAMALA P.[a*], SRINIVASU KONDRA[b], HSN RAJU DANTULURI[b]

[a]Department of Physical, Nuclear Chemistry and Chemical Oceanography, School of Chemistry, Andhra University, Visakhapatnam 530003, Andhra Pradesh, India, [b]Aurobindo Pharma Limited, Bachupally, R. R District, Hyderabad 500090, Telangana, India
Email: shyamalapulipaka06@gmail.com

Received: 25 Aug 2020, Revised and Accepted: 08 Oct 2020

ABSTRACT

Objective: The main objective of this research work is to develop and validate a single reverse-phase high-performance liquid chromatography (RP-HPLC) method. This method should be capable of quantifying all the known, as well as other possible degradation impurities of sodium nitroprusside (SNP) in its injection formulation.

Methods: Of all method development trails, we have observed better separations between known and degradation impurities in Inert sustain C18, (250 x 4.6) mm, 5 µm column at 30 °C temperature. Isocratic elution was carried out by using pH 8.6 phosphate buffer and acetonitrile in the ratio of 65:35 %v/v with a flow rate of 0.8 ml/min. The detection was carried out at 220 nm, with an injection volume of 10 µl.

Results: In the proposed method, SNP was eluted at 22.5 min. Nitrite, nitrate, and ferrocyanide were linear from 0.25 to 37 µg/ml, ferricyanide was linear from 1.0 to 37 µg/ml, and SNP was linear from 0.75 to 37 µg/ml. The % RSD for six spiked samples (precision) was found to be less than 0.5 %. Accuracy was performed for known impurities from LOQ to 150 % for a 0.5 % specification level. The results were found to be in the acceptance range of 90-110 %. The LOQ concentration of nitrite, nitrate, and ferrocyanide was 0.25 µg/ml each, LOQ of ferricyanide and SNP was found to be 1.0 µg/ml and 0.75 µg/ml, respectively. The SNP injection samples were exposed to different degradation conditions, and the results were found specific in the proposed methodology.

Conclusion: The proposed RP-HPLC method is specific, precise, accurate, linear, stable, and robust for quantification of known and other possible degradation impurities in SNP injection formulation.

Keywords: RP-HPLC, Impurity profiling, Injection formulation, Sodium nitroprusside, Degradation

DOI: http://dx.doi.org/10.22159/ijap.2021v13i1.39534. Journal homepage: https://innovareacademics.in/journals/index.php/ijap

INTRODUCTION

SNP is a sodium salt of di anionic metal complex dihydrate with molecular formula $Na_2[Fe(CN)_5NO].2H_2O$. It is chemically named as Ferrate (2-), pentakis(cyano-C)nitrosyl-, disodium, dihydrate [1, 2]. It is a potent rapid-acting hypotensive agent and when administered intravenously. SNP is used to lower blood pressure during surgical operations**.** Continuous injection of SNP might cause cyanide poisoning; therefore, intravenous administration rate (10 mcg/kg/min) is strictly controlled [3, 4]. However, there are several problems associated with the clinical use of sodium nitroprusside, including tolerance and the toxicity of its metabolites, cyanide, and thiocyanate [5, 6].

The literature survey of SNP revealed some of the reported pharmacopeia methods for the estimation of ferricyanide and ferrocyanide in SNP [7, 8]. The reported HPLC methods were found for estimation of nitrates and nitrites [9, 10]. A HPLC method was proposed for the estimation of ferrocyanide in food-grade salts [11]. A spectrophotometric method was published for the estimation of SNP and its photo degradants [12]. An assay by HPLC method was reported for SNP [13], and an *in vitro* stability of SNP for intravenous administration methodology was reported [14]. Based on the literature survey, it was observed that most of the reported works concentrated on the assay of SNP, the content of ferricyanide, and ferrocyanide in SNP. To date, no analytical method was published to quantify all known and possible degradation impurities of SNP simultaneously in the single liquid chromatography (LC) method for injection formulation.

Taking cues from literature, we have decided to develop and validate a short RP-HPLC method for detecting all possible impurities of SNP in injection formulation. During development, we have performed several stress conditions such as hydrolysis, oxidation, photolysis and thermal conditions in order to highlight the possible degradation impurities in the proposed methodology [15-17]. The developed method was checked for specificity, precision, linearity, accuracy, robustness and stability of solutions based on ICH Q2 (R1) [18]. Based on method development and validation results, the proposed LC method could quantify all known and other possible degradation impurities. The chemical structures of four known impurities and SNP are given in fig. 1.

MATERIALS AND METHODS

Chemicals

SNP standard and injection formulation (labelled 25 mg/ml) samples were provided by Aurobindo pharma research center-1, Hyderabad, Telangana, India. Impurities, sodium nitrite USP reference standard and sodium nitrate USP reference standards were procured from the United States pharmacopeial convention, USA. Potassium ferrocyanide trihydrate and potassium ferricyanide procured from Sigma Aldrich, USA. Chemicals, such as Di-sodium hydrogen phosphate (ACS), orthophosphoric acid (AR), sodium hydroxide (AR), hydrogen peroxide (LR), and acetonitrile (HPLC) were procured from Merck, India. HPLC grade water and Hydrochloric acid (AR) were procured from Rankem, India. Tetra-n-butylammonium hydroxide 40% solution in water was procured from Alfa aesar, UK.

Instrumentation

This research work was carried out on Shimadzu prominence HPLC (LC-20AD), consisting of a quaternary solvent manager and an online degasser unit (DGU-20A5R). This instrument is equipped with a photodiode array detector (SPD-M20A), it operates and data processes with waters empower 3 software. Waters Alliance e2695 separations module HPLC with a quaternary solvent delivery system and an online degasser unit. This instrument is equipped with a photodiode array detector (SPD-M20A), it operates and data

processes with waters empower 3 software. Other instruments used in this research include Sartorius make Microbalance (SE2), Sartorius make Analytical balance (CPA225D), Eutech make pH meter (pH 2700), Cintex make Water bath (CIC-2BM) and Borosil make filtration kit equipped with Millipore 230V, 50HZ vacuum pump.

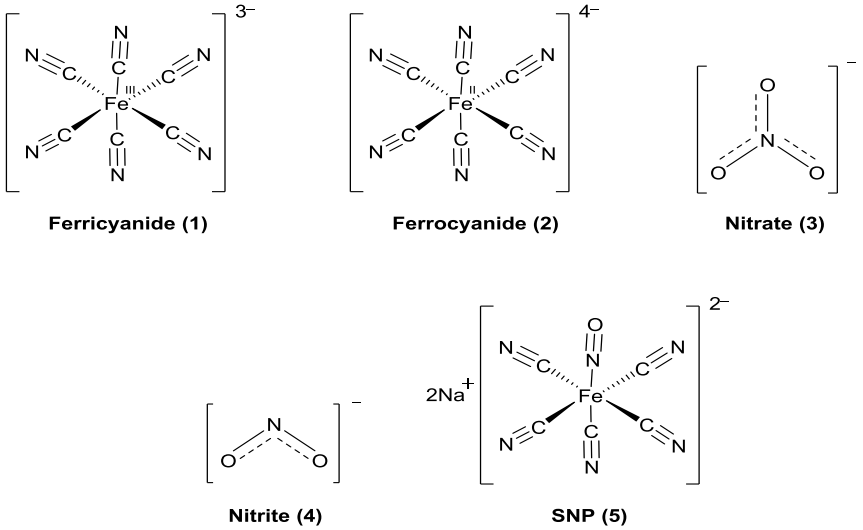

Fig. 1: Chemical structures of ferricyanide, ferrocyanide, nitrate, nitrite and SNP

Chromatographic conditions

Optimum results were obtained in isocratic mode using a mobile phase containing a degassed mixture of disodium hydrogen phosphate dihydrate (25 mmol), 13 ml/l of Tetra-n-butylammonium hydroxide 40% solution in water [19], pH adjusted to 8.6 with orthophosphoric acid and acetonitrile in the ratio of 65:35 %v/v [19]. Other chromatographic conditions include Column: Inertsustain C18 (250×4.6 mm, 5 µm) (make: GL Sciences) [19], Column temperature: 30 °C, Flow rate: 0.8 ml/min [20], Injection volume: 10 µl [20] at Detector wavelength: 220 nm. The mobile phase, standard, and sample solution were filtered through a 0.45 µm membrane filter before injecting it into the HPLC system.

Diluent preparation

Mobile phase was used as diluent.

Preparation of standard stock-1: (1000 µg/ml)

Accurately transferred 38 mg of sodium nitrite, 41 mg of potassium nitrate, 50 mg of potassium ferrocyanide trihydrate and 25 mg of SNP standard into a 25 ml volumetric flask containing 10 ml of diluent, dissolved and made up to the mark with diluent, mixed well.

Preparation of standard stock-2: (1000 µg/ml)

Accurately transferred 39 mg of potassium ferricyanide standard in to a 25 ml volumetric flask containing 10 ml of diluent, dissolved and made up to the mark with diluent, mixed well.

Preparation of standard solution-1: (10 µg/ml)

Accurately transferred 1 ml of standard stock-1 into a 100 ml volumetric flask, made up to the mark with diluent and mixed well.

Preparation of standard solution-2: (10 µg/ml)

Accurately transferred 1 ml of standard stock-2 into a 100 ml volumetric flask, made up to mark with diluent and mixed well.

Preparation of sample solution: (5000 µg/ml)

Pooled sample was prepared by mixing the contents of 3 vials of SNP injection formulation (fill volume: 2 ml each). Accurately transferred 2 ml of pooled sample into a 10 ml volumetric flask, made up to mark with diluent and mixed well.

RESULTS AND DISCUSSION

Method development and optimization

According to available pharmacopoeia monographs, titrimetric methods were used for the quantification of ferricyanide and ferrocyanide in SNP. These methods are only specific for quantification of ferricyanide and ferrocyanide, where other impurities cannot be quantified [21, 22]. In some articles, authors reported ferricyanide, ferrocyanide and other possible photodegradation impurities by using spectrophotometry in SNP [23, 24]. By some articles, authors reported A HPLC method for determining nitrate and nitrite levels in vegetables [25]. Till now, no reported article was published by addressing ferricyanide, ferrocyanide, nitrate, nitrite and other possible degradation impurities, likely to be formed during the product shelf life all together in a single chromatographic method.

Taking cues from the available literature, we have taken it as a challenge to develop a short, stable, sensitive, accurate and robust, RP-HPLC method for detecting all possible impurities of the SNP with sensitivity less than 1 µg/ml. The SNP and their impurities are charged species and are highly polar. Hence conventional HPLC mobile phases and columns could not hold the polar components in reverse phase chromatography, as it leads to poor resolutions. To get adequate resolutions between the negatively charged components such as ferricyanide, ferrocyanide and SNP in an octadecylsilane bonded column (C18), basic ion pain reagent like tetrabutylammonium hydroxide (TBAH) was introduced into the mobile phase [26].

Along with TBAH, other basic ion-pair reagents are also available in the market, include tetraethylammonium hydroxide (TEAH) and tetramethylammonium hydroxide (TMAH) etc. As TBAH is available at our research laboratory, the same is used for the preparation of the mobile phase to hold the negatively charged species in the column. TBAH in the mobile phase could hold the negatively charged species by decreasing the charge to mass ratio. Due to the presence of basic ion pair reagent in the mobile phase, the hydrophobic property of negatively charged species increases, resulting in longer retention times. Based on the literature, the mobile backup phase was prepared at basic pH by using TBAH [26-28]. The development work was carried out in columns which could withstand basic mobile phase pH such as Gemini C18 (250×4.6 mm, 5 µm) make: Phenomenex, Inertsustain C18 (250×4.6 mm, 5 µm) (make: GL

Sciences), X-Bridge C18 (250×4.6 mm, 5 µm) (Make: Waters), Zorbax extend C18 (250 ×4.6 mm, 5 µm) (make: Agilent). Of all the mentioned columns, the optimum separation between the impurities was achieved in the Inertsustain C18 column with dimensions (250×4.6 mm, 5 µm) (make: GL Sciences). Along with columns, different mobile phase pH, compositions, column temperatures and flow rates were also evaluated. Some of the method development trials for mobile phase composition, pH and column oven temperature by keeping other chromatographic conditions constant were given in table 1.

Table 1: Method development trials to improve the resolution between SNP related impurities

Buffer pH	Composition*	Temp** (°C)	Observation
6.0	58:42	40 °C	Poor resolution between ferricyanide and SNP
7.1	70:30	40 °C	Poor resolution between ferrocyanide and unknown impurity.
8.0	62:38	40 °C	Resolution between ferricyanide and SNP was not satisfactory.
8.0	65:35	40 °C	Resolution between ferricyanide and SNP was not satisfactory.
8.5	58:42	35 °C	Resolution between ferrocyanide and unknown impurity was not satisfactory.
8.5	58:42	40 °C	Resolution between ferrocyanide and unknown impurity was not satisfactory.
8.5	62:38	35 °C	Resolution between ferricyanide and SNP was not satisfactory.
8.5	60:40	35 °C	Resolution between ferrocyanide and unknown impurity was not satisfactory.
8.5	60:40	40 °C	Resolution between ferrocyanide and unknown impurity was not satisfactory.
8.6	65:35	30 °C	Peak shapes and resolutions between all degradants found satisfactory.
8.8	65:35	30 °C	Peak shapes and resolutions between all degradants found satisfactory.

*Mobile phase composition (Buffer: Acetonitrile) % v/v, **Column oven temperature

Response factor calculation for both ferricyanide and ferrocyanide

The percentage of known impurities were calculated against their corresponding standard solutions. Out of four known impurities, ferrocyanide and ferricyanide are readily interconvertible [Fe (CN)6]3−+e− ⇌ Fe (CN)6]4− [29]. During the stress study, it was observed that the presence of peroxides in diluent is facilitating the conversion of ferricyanide to ferrocyanide. Ferricyanide and ferrocyanide redox reaction could be controlled by purging the diluent with inert gas (Nitrogen/Helium) from the bottom of the flask to remove the residual oxygen. Even after taking such precautions, ferricyanide is slightly converting to ferrocyanide based on time. In addressing the issue of quantifying the known impurities and degradation impurities, two different standards were proposed. The first standard consists of nitrite, nitrate, ferrocyanide and SNP, whereas the second standard consists of ferricyanide. Actual areas of ferrocyanide and ferricyanide from both the standards even after slight interconversion were determined by using the response factor (RF).

Ferricyanide easily converts to ferrocyanide in the presence of peroxides. Hence, RF for ferrocyanide and ferricyanide redox conversion was determined using area lost to the area gain by using 2% hydrogen peroxide to facilitate the redox reaction. RF determination for ferrocyanide and ferricyanide redox conversion was given in table 2.

Table 2: Determination of response factor for redox couple by using 2% hydrogen peroxide

Degradation time* (h)	Area from peroxide stress sample				Response factor (RF)	
	Ferricyanide	Ferrocyanide	Area of ferricyanide lost (A)	Area of ferrocyanide gained (B)	Ferricyanide RF (B/A)	Ferrocyanide RF (A/B)
0	5509424	458792	NA	NA	NA	NA
1	5468329	595608	41095	136816	3.33	0.30
2	5417119	777561	92305	318769	3.45	0.29
5	4987023	2065594	522401	1606802	3.08	0.33
8	4392412	4234070	1117012	3775278	3.38	0.30
12	3976131	5566206	1533293	5107414	3.33	0.30
Average response factor					3.3	0.3

*Ferricyanide degradation time after addition of 2% hydrogen peroxide, Actual area of ferrocyanide and ferricyanide from both the standard solutions were calculated with the help of RF. The details of the calculation were given in table 3.

Table 3: Area calculations

Calculation for determination of actual ferrocyanide area from standard-1				
A_1 Ferrocyanide area from standard-1	B_1 Ferricyanide area from standard-1	RF_1 (3.3) Response factor [To convert area of ferricyanide (B_1) to ferrocyanide (C_1)	$C_1 = B_1 \times RF_1$ Area of ferrocyanide lost	C_1+A_1 Actual area of ferrocyanide from standard-1
Calculation for determination of actual ferricyanide area from standard-2				
A_2 Ferricyanide area from standard-2	B_2 Ferrocyanide area from standard-2	RF_2 (0.3) Response factor (To convert area of ferrocyanide (B_2) to ferricyanide (C_2)	$C_2 = B_2 \times RF_2$ Area of ferricyanide lost	C_2+A_2 Actual area of ferricyanide from standard-2

System suitability

The finalized related substances method was capable of quantifying all known and other possible degradation impurities in SNP injection formulation. As a part of the system suitability, two different standard solutions are adopted to find the exact areas of ferrocyanide and ferricyanide.

Standard-1 contains 10 µg/ml of each nitrite, nitrate, ferrocyanide and SNP and Standard-2 contains 10 µg/ml of ferricyanide. Chromatograms of both standards were given in fig. 2 and 3.

System suitability results were calculated from both the standards to check the ability of the analytical instrument and method. The system suitability results from both the standards were given in table 4.

System suitability results show the peak asymmetry, USP plate count, resolution between the closely eluting impurities nitrite and nitrate are meeting the acceptance criteria.

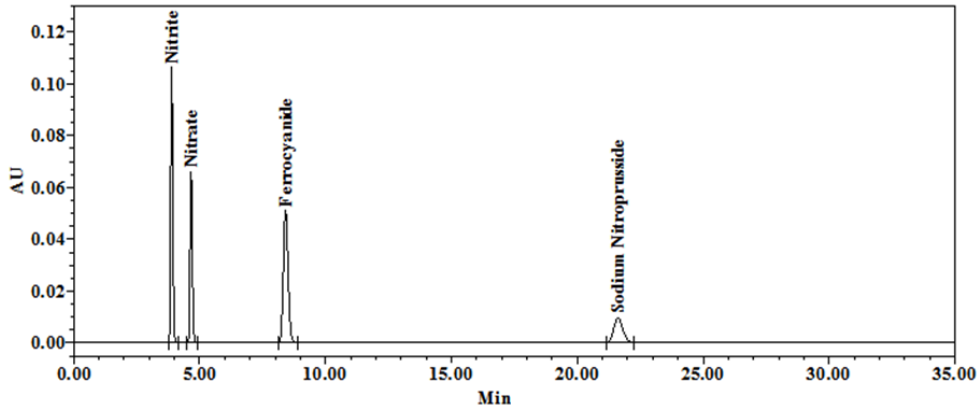

Fig. 2: Representative chromatogram of standard-1 containing nitrite, nitrate, ferrocyanide and SNP

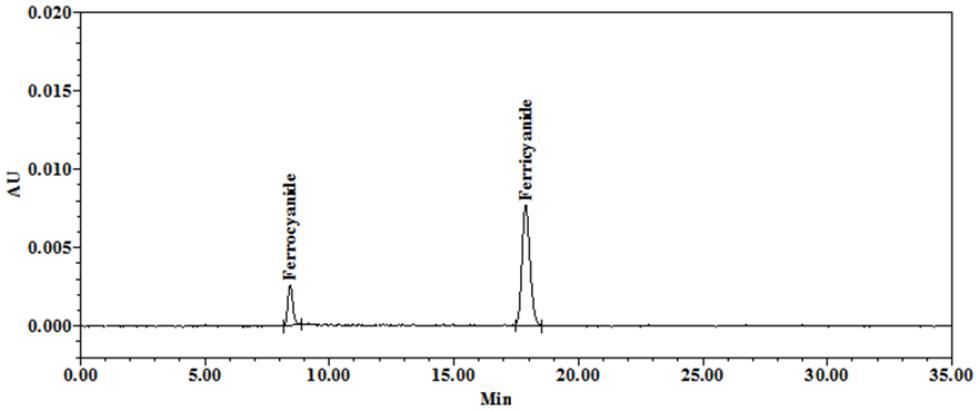

Fig. 3: Representative chromatogram of standard-2 containing ferricyanide and its redox counterpart ferrocyanide

Table 4: System suitability results obtained from both standard-1 and 2

S. No.	Components	RT*	RRT**	Area	Resolution#	Peak asymmetry	USP plate count
1	Nitrite	3.92	0.17	598085	---	1.2	11172
2	Nitrate	4.71	0.21	414971	5.0	1.2	13163
3	Ferrocyanide	8.76	0.39	668677	---	1.1	9739
4	Ferricyanide	18.74	0.83	172022	---	1.2	15500
5	SNP	22.49	1.00	237932	---	1.1	18059

*Retention time (min), **Relative retention time (RT of impurity/RT of SNP), #Resolution between closely eluting impurities nitrite and nitrate from standard-1

Method validation

The proposed LC method for quantification of known and other possible degradation impurities obtained during stress studies of SNP was validated as per ICH guidelines Q2 (R1).

Specificity

Specificity indicates the analytical method's ability to distinguish accurately and specifically each analyte of interest in the presence of other components such as blank, placebo matrix and other possible impurity peaks related to SNP.

The specificity experiment was conducted by injecting blank, individual impurities, and known impurity spiked samples in LC equipped with a photodiode array (PDA) detector to check the peak purity. The SNP injection formulation does not have any placebo matrix; hence placebo is not injected. Peak purity was calculated for known impurities and SNP from the spiked sample, and the data were given in table 5. Typical chromatograms of the blank, control sample and spiked sample were given in fig. 4-6

Selectivity

Selectivity indicates the ability of an analytical method to distinguish all possible degradation impurities that are going to be generated in any sample over a period of time. To make sure the method selectivity SNP injection was exposed to different stress conditions such as hydrolysis, thermal, oxidation and photolytic [30-32]. Degraded samples were chromatographed by using HPLC equipped with PDA detector to establish method selectivity. Chromatograms of these stress conditions were given in fig. 7-11. From these stress conditions, peak purity of the SNP was measured to make sure the co-elution of any degradation impurity. Peak purity results of the SNP from degraded samples were given in table 6.

Acid degradation

Into a 10 ml volumetric flask containing 2 ml of SNP injection, 1 ml of 1 mol hydrochloric acid was added and the solution was kept at room temperature for 3 h. The degraded sample was neutralized with 1 ml of 1 mol sodium hydroxide, made up to 10 ml mark with diluent and chromatographed. The resultant chromatogram was given in fig. 7.

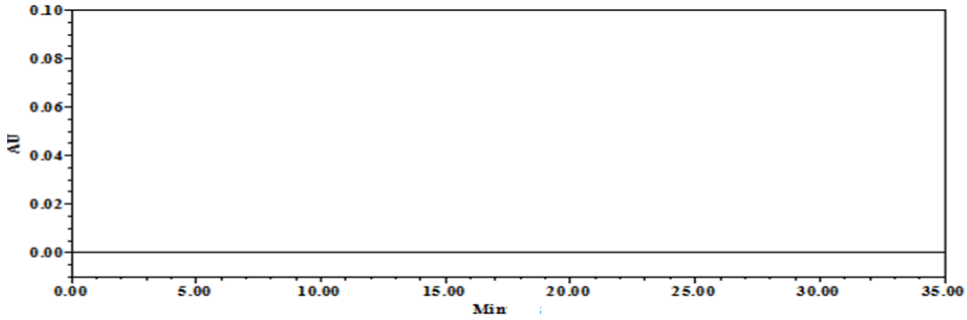

Fig. 4: Blank chromatogram

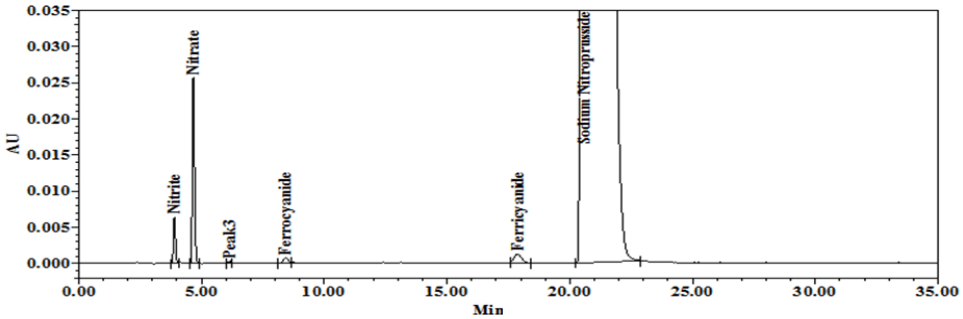

Fig. 5: Control sample chromatogram

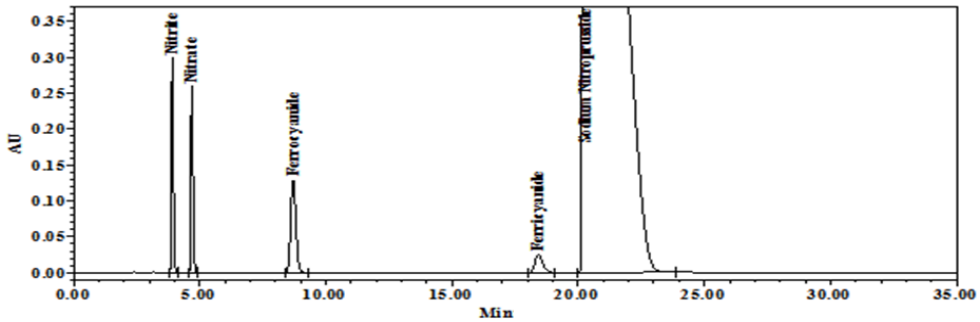

Fig. 6: Chromatogram of spiked sample with known impurities at 0.5% level (25 µg/ml)

Table 5: Peak purity results of known impurities and SNP from spiked sample

Component	RT	RRT	Peak purity*	
			Purity angle	Purity threshold
Nitrite	3.915	0.19	0.069	0.246
Nitrate	4.689	0.23	0.051	0.265
Ferrocyanide	8.701	0.43	0.055	0.243
Ferricyanide	18.417	0.91	0.228	0.478
SNP	20.268	1.00	0.069	0.292

*To consider any peak is pure, purity angle should be less than purity threshold

Base degradation

Into a 10 ml volumetric flask containing 2 ml of SNP injection, 1 ml of 1 mol sodium hydroxide was added and the solution was kept at room temperature for 1 h. The degraded sample was neutralized with 1 ml of 1 mol hydrochloric acid, made up to 10 ml mark with diluent and chromatographed. The resultant chromatogram was given in fig. 8.

Oxidative degradation

Into a 10 ml volumetric flask containing 2 ml of SNP injection, 1 ml of 30% hydrogen peroxide was added and the solution was kept at room temperature for 2 h. The degraded sample was made up to 10 ml mark with diluent and chromatographed. The resultant chromatogram is given in fig. 9.

Thermal degradation

The SNP injection sample is kept in a hot air oven at 85 °C for 72 h. After attaining the room, temperature sample was prepared as per the test method by taking 2 ml of thermal degradation sample and chromatographed. The resultant chromatogram is given in fig. 10.

Photolytic degradation

The SNP injection sample was kept in the photolytic chamber and exposed to white fluorescent light, 1.2 million Lux hours and UV light 200-watt hours/square meter. After attaining room, temperature the sample was prepared as per the test method by taking 2 ml of photolytic degradation sample and chromatographed. The resultant chromatogram was given in fig. 11.

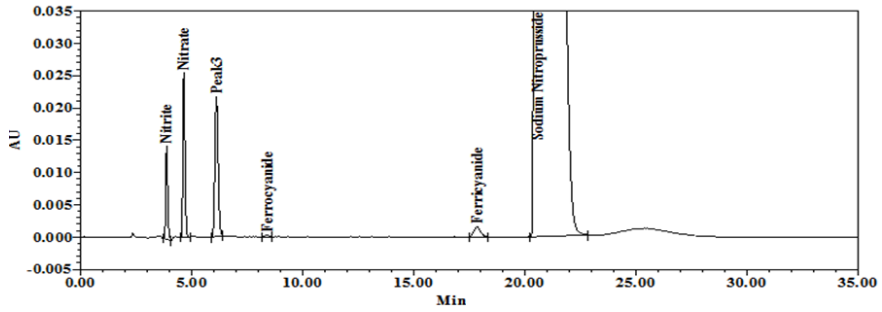

Fig. 7: Acid degradation chromatogram of SNP injection formulation

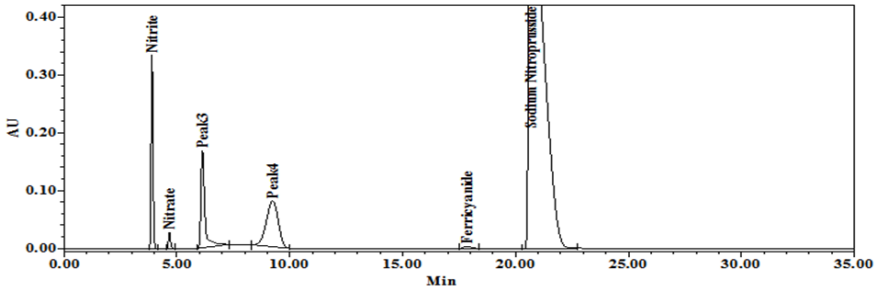

Fig. 8: Base degradation chromatogram of SNP injection formulation

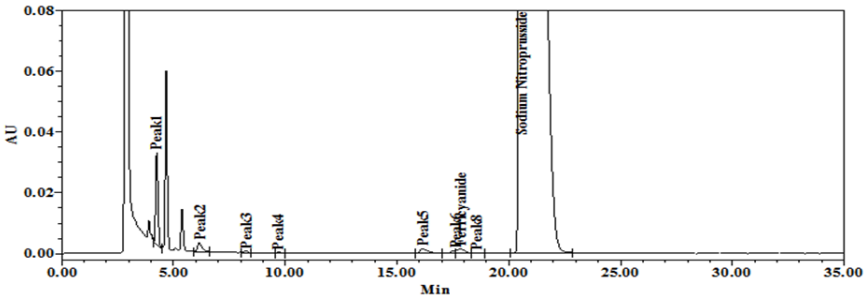

Fig. 9: Oxidative degradation chromatogram of SNP injection formulation

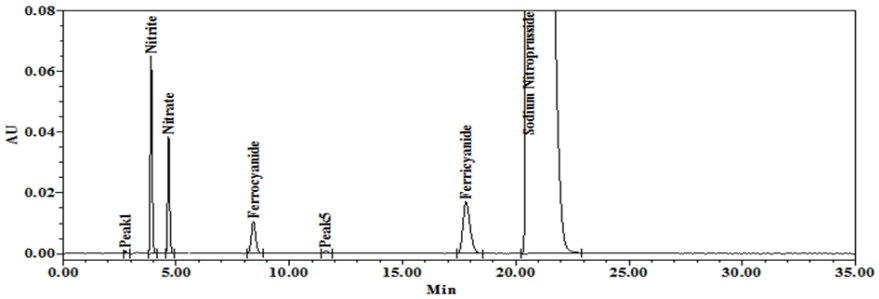

Fig. 10: Thermal degradation chromatogram of SNP injection formulation

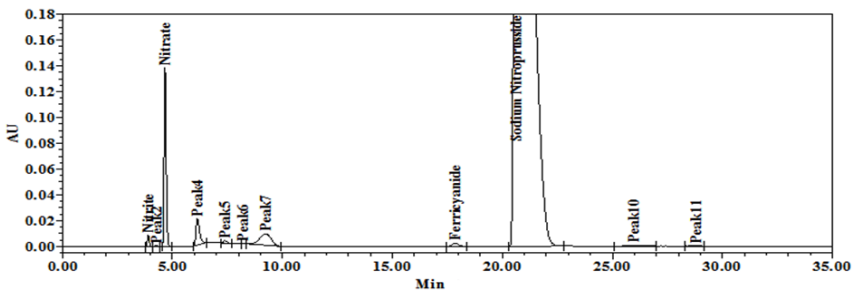

Fig. 11: Photolytic degradation chromatogram of SNP injection formulation

Table 6: Peak purity results of SNP from degradation samples

Degradation	Area of SNP	% of degradation	Peak purity of SNP	
			Purity angle	Purity threshold
Control sample	35895014	NA	0.106	0.303
Acid degradation	35932047	Nil	0.022	0.303
Base degradation	30010842	16.4	0.027	0.284
Peroxide degradation	35471819	1.2	0.027	0.314
Thermal degradation	34400144	4.2	0.105	0.393
Photolytic degradation	32324418	10.5	0.075	0.282

Precision

The precision of an analytical method expresses the closeness of agreement between a series of injections prepared from a single homogenized stock solution. It indicates the reproducible capability of an analytical method.

Method precision was evaluated by preparing six different spiked samples on the same day, and intermediate precision was evaluated by preparing six different spiked samples on a different day. Each precision sample was prepared by spiking known impurities at a 0.5% level to SNP injection formulation and chromatographed as per the proposed method. The obtained precision results were given in table 7.

Table 7: Results from both method precision and intermediate precision.

Preparation	Nitrite	Nitrate	Ferrocyanide	Ferricyanide
Method precision (n=6)*				
Mean (%w/v)	0.505	0.493	0.495	0.525
SD	0.0008	0.0008	0.0015	0.0008
%RSD	0.2	0.2	0.4	0.2
Intermediate precision (n=6)*				
Mean (%w/v)	0.495	0.491	0.493	0.512
SD	0.0021	0.0025	0.0021	0.0015
%RSD	0.04	0.4	0.4	0.2
Cumulative precision (n=12)#				
Mean (%w/v)	0.500	0.492	0.494	0.519
SD	0.0056	0.0020	0.0023	0.0069
%RSD	1.1	0.4	0.5	1.3

*Number of experiments done (n):6, #Number of experiments done (n):12 (cumulative results of both precision and intermediate precision)

Linearity and sensitivity

The linearity of an analytical method indicates its ability to obtain test results which are directly proportional to the concentration in a specific range. Linearity was performed for all four known impurities and SNP from 0.05 µg/ml to 38 µg/ml. Linearity solutions were evaluated by linear regression analysis and calculated by the least square method and studied. Limit of detection (LOD) and Limit of quantification (LOQ) results for four known impurities and the SNP were calculated from linearity solutions by using the slope of the calibration curve. Based on the sensitivity results in six different preparations of known impurities and SNP were prepared at their LOD and LOQ to check the precision. Precision results at their lowest detection and quantification level were given in table 8.

LOD = 3.3 x Standard deviation of the response/Slope of the calibration curve

LOQ = 10 x Standard deviation of the response/Slope of the calibration curve

Table 8: Linearity and sensitivity results of known impurities and SNP

Parameter	Nitrite	Nitrate	Ferrocyanide	Ferricyanide	SNP
Range (µg/ml)	0.25-37.68	0.25-37.53	0.25-37.50	1.00-37.74	0.75-37.27
Regression equation*	69738x+1266.5	50744x+2176.5	78543x-4930.4	23284x-5515.8	29661x-1238.2
Correlation**	0.9999	0.9999	0.9999	0.9999	0.9999
LOD (µg/ml)	0.084	0.083	0.083	0.332	0.248
LOQ (µg/ml)	0.253	0.252	0.252	1.005	0.752
LOD precision (n=6)#					
Mean area	7132	4334	6150	6781	7424
%RSD	1.5	0.8	1.6	2.7	2.2
LOQ precision (n=6)#					
Mean area	18925	13423	19325	21378	22294
%RSD	0.4	0.1	0.8	0.7	1.2

*Y is the peak area and X is the concentration injected, **Correlation coefficient, #Number of experiments done (n):6

Accuracy

The accuracy of an analytical method indicates the closeness of agreement between an actual value and obtained value at a particular range. Accuracy for all known impurities was evaluated at levels LOQ, 50%, 100%, and 150% by spiking to the SNP injection formulation. At each level, triplicate solutions were prepared by spiking individual impurities from their respective stock solutions and chromatographed. Accuracy results for known impurities were given in table 9 and 10.

Table 9: Accuracy results of known impurities at their minimum quantification level (LOQ)

Component	Amount added (%w/w)	Amount found (%w/w)	% recovery	Mean recovery	SD*	% RSD
	n=3#					
	0.0051	0.0058	113.7			
Nitrite	0.0051	0.0058	113.7	113.7	0.000	0.0
	0.0051	0.0058	113.7			
	0.0051	0.0052	102.0			
Nitrate	0.0051	0.0053	103.9	102.6	1.096	1.1
	0.0051	0.0052	102.0			
	0.0051	0.0051	100.0			
Ferrocyanide	0.0051	0.0050	98.0	98.7	1.154	1.1
	0.0051	0.0050	98.0			
	0.0198	0.0199	100.5			
Ferricyanide	0.0198	0.0197	99.5	100.2	0.577	0.6
	0.0198	0.0199	100.5			

*Standard deviation, #Number of experiments done (n):3

Table 10: Accuracy results of known impurities

% of specification	Amount added (%w/w)	Amount found (%w/w)	% of recovery	% RSD±SD	% of recovery	% RSD±SD
	n=3#				n=9#	
Nitrite						
50%	0.253	0.256	101.2	0.0±0.000		
100%	0.506	0.505	99.7	0.1±0.115	100.5	0.6±0.638
150%	0.759	0.763	100.5	0.1±0.057		
Nitrate						
50%	0.251	0.251	100.0	0.0±0.000		
100%	0.503	0.493	97.9	0.1±0.115	98.7	1.0±0.961
150%	0.754	0.741	98.3	0.2±0.173		
Ferrocyanide						
50%	0.251	0.249	99.2	0.0±0.000		
100%	0.503	0.495	98.4	0.2±0.200	99.5	1.1±1.048
150%	0.754	0.760	100.8	0.1±0.058		
Ferricyanide						
50%	0.253	0.266	105.0	0.6±0.611		
100%	0.506	0.525	103.7	0.1±0115	104.4	0.6±0.621
150%	0.758	0.792	104.5	0.1±0.058		

Number of experiments done at each level (n):3, cumulative recovery for all three levels (n):9

Robustness

Robustness of the analytical method was evaluated by deliberately altering some of the critical method parameters to check the method capability on system suitability results and provides an indication of its reliability during normal usage. Robustness was checked for flow rate (±10%), column oven temperature (±5 °C), pH of the buffer (±0.2 units), organic composition in the mobile phase (±2% absolute), and wavelength (±5 nm). The obtained robustness results were given in table 11 to 15.

Table 11: Robustness impact on nitrite peak from system suitability solution

Parameter	Variation	RRT	USP tailing	USP plate count
Control	-	0.19	1.2	11172
Flow rate (±10%)	0.72 ml/min	0.19	1.2	11686
	0.88 ml/min	0.19	1.2	10654
Column oven temperature(±5 °C)	25 °C	0.18	1.2	10593
	35 °C	0.21	1.2	11425
Buffer pH (±0.2 units)	8.4	0.18	1.2	11346
	8.8	0.18	1.2	11397
Mobile phase composition (±2%)	67:33	0.15	1.2	11637
	63:37	0.22	1.2	10726
Wave length (±5 nm)	215 nm	0.19	1.2	11168
	225 nm	0.19	1.2	11185

Table 12: Robustness impact on nitrate peak from system suitability solution

Parameter	Variation	RRT	Resolution*	USP Tailing	USP plate count
Control	-	0.23	5.0	1.2	13163
Flow rate (±10%)	0.72 ml/min	0.23	5.0	1.2	13258
	0.88 ml/min	0.23	4.8	1.2	12415
Column oven	25 °C	0.21	5.2	1.2	11759
temperature(±5 °C)	35 °C	0.25	4.6	1.2	12966
Buffer pH (±0.2 units)	8.4	0.22	4.9	1.2	10949
	8.8	0.22	4.9	1.2	11513
Mobile phase composition	67:33	0.18	5.6	1.2	13876
(±2%)	63:37	0.26	4.1	1.3	7933
Wave length (±5 nm)	215 nm	0.23	4.9	1.2	13094
	225 nm	0.23	4.9	1.2	13151

*Resolution between nitrite and nitrate

Table 13: Robustness impact on ferrocyanide peak from system suitability solution

Parameter	Variation	RRT	USP Tailing	USP plate count
Control	-	0.43	1.1	9739
Flow rate (±10%)	0.72 ml/min	0.43	1.1	10401
	0.88 ml/min	0.43	1.1	9136
Column oven temperature(±5 °C)	25 °C	0.39	1.1	8977
	35 °C	0.47	1.1	10392
Buffer pH (±0.2 units)	8.4	0.43	1.1	10122
	8.8	0.43	1.1	9972
Mobile phase composition (±2%)	67:33	0.42	1.1	10980
	63:37	0.44	1.1	9105
Wave length (±5 nm)	215 nm	0.43	1.1	9667
	225 nm	0.43	1.1	9761

Table 14: Robustness impact on ferricyanide peak from system suitability solution

Parameter	Variation	RRT	USP Tailing	USP plate count
Control	-	0.91	0.2	15500
Flow rate (±10%)	0.72 ml/min	0.91	0.2	15611
	0.88 ml/min	0.91	0.2	14532
Column oven temperature (±5 °C)	25 °C	0.88	0.2	14482
	35 °C	0.93	0.2	15477
Buffer pH (±0.2 units)	8.4	0.93	0.2	14932
	8.8	0.92	0.2	15568
Mobile phase composition (±2%)	67:33	0.94	0.2	15664
	63:37	0.91	0.2	14484
Wave length (±5 nm)	215 nm	0.91	0.2	15060
	225 nm	0.91	0.2	15048

Table 15: Robustness impact on SNP peak from system suitability solution

Parameter	Variation	RRT	USP Tailing	USP plate count
Control	-	1.0	1.1	18059
Flow rate (±10%)	0.72 ml/min	1.0	1.2	18262
	0.88 ml/min	1.0	1.2	17037
Column oven temperature(±5 °C)	25 °C	1.0	1.2	16900
	35 °C	1.0	1.1	18174
Buffer pH (±0.2 units)	8.4	1.0	1.1	17438
	8.8	1.0	1.1	17433
Mobile phase composition (±2%)	67:33	1.0	1.2	17974
	63:37	1.0	1.1	17589
Wave length (±5 nm)	215 nm	1.0	1.1	17358
	225 nm	1.0	1.1	18311

Solution stability

Solution stability is necessary to assess the stability of standard and sample solutions during product development and stability data evaluation. Sample solution stability was evaluated by spiking all known impurities on SNP injection formulation and injected at different time intervals by keeping the solution at controlled room temperature (~25 °C). Similarly, standard solutions were injected periodically. The % difference in an area of each impurity and SNP was calculated, and the results were given in table 16, 17.

Table 16: Standard solution stability results from standard-1 and 2

Component	0 h	2 h	6 h	12 h	24 h	48 h
Nitrite	NA	0.0	0.1	0.3	0.5	1.0
Nitrate	NA	0.4	0.1	0.1	0.6	0.9
Ferrocyanide	NA	0.1	0.3	0.3	0.6	0.2
Ferricyanide	NA	1.5	2.0	2.2	2.4	1.9
SNP	NA	0.4	0.2	0.6	1.0	1.7

Table 17: Spiked sample solution stability results

Component	0 h	2 h	6 h	12 h	24 h	48 h
Nitrite	NA	0.2	0.1	0.3	0.8	1.2
Nitrate	NA	0.2	0.1	0.3	0.9	1.4
Ferrocyanide	NA	0.6	1.2	1.6	1.3	1.3
Ferricyanide	NA	0.6	1.8	2.9	4.3	4.1
SNP	NA	0.1	0.1	0.1	0.7	1.0

CONCLUSION

A simple RP-HPLC method for quantification of known impurities and other possible degradation impurities in the SNP injectable formulation was successfully developed and validated. The proposed methodology is specific, precise, rugged, linear, accurate, stable, and robust for the quantification of related impurities of SNP injection. Moreover, the developed method has the lowest detectable and quantification capability. The degradation data shows, the SNP is sensitive to photolytic and base hydrolysis, moderately sensitive to thermal oxidation, and stable in acid hydrolysis. The degradation impurities formed during stress study are well resolved; hence any degradation impurity formed during the product life cycle above the identification threshold could be quantified. Based on the above advantages, the proposed method could be useful for monitoring possible degradation impurities of SNP in its injectable formulation in quality control departments of manufacturing units.

ACKNOWLEDGEMENT

The authors are thankful to the management of the APL Research Centre (A division of Aurobindo Pharma Limited, Hyderabad) for providing chemicals, standards and all other essential facilities to do the research work and the school of chemistry, Andhra University, Visakhapatnam for their guidance.

FUNDING

Nil

AUTHORS CONTRIBUTIONS

Mr. Murali Krishnam Raju has generated the research activity and prepared the manuscript. Dr. Venkata Narayana, Dr. Shyamala have given guidance and supervision to carry out this research work. Mr. Kondra Srinivasu and Mr. HSN Raju Dantuluri supported in acquiring validation data and its compilation.

CONFLICT OF INTERESTS

The authors confirm that this article content has no conflict of interest.

REFERENCES

1. Sodium nitroprusside, USP Monographs, official (USP42-NF37), 4045; 2020.
2. Sodium Nitroprusside-European Pharmacopoeia 10.3, (01/2016:0565). Available from: https:// www.pharmacopoeia.com/bp-2020/monographs/sodium-nitroprusside [Last accessed on 15 Jul 2020].
3. NITROPRESS® (sodium nitroprusside injection) product information leaflet, Hospira, Inc., Lake Forest, IL 60045 USA, Document Name: QEN-3406v4. qxp, Reference ID: 3442071; 2013.
4. Taylor HT, Styles M, Lamming JA. Sodium nitroprusside as a hypotensive agent in general anesthesia. Br J Anuesth 1970;42:859-64.
5. Moraca PP, Bitte ME, Hale ED, Wasmuth EC, Poutasse FE. Clinical evaluation of sodium nitroprusside as a hypotensive agent. Anesthesiology 1962;23:193-9.
6. Cottrell EJ, Casthely P, Brodie DJ, Patel K, Klein A, Turndorf H. Prevention of Nitroprusside-induced cyanide toxicity with hydroxocobalamin. N Engl J Med 1978;298:809-11.
7. Sodium Nitroprusside, USP Monographs, official (USP42-NF37), 4045; 2020.
8. Sodium Nitroprusside-European Pharmacopoeia 10.3, (01/2016:0565). Available from: https://www. pharmacopoeia.com/bp-2020/monographs/sodium-nitroprusside [Last accessed on 15 Jul 2020].
9. Gerritse GR. Rapid simultaneous determination of nitrate and nitrite by high-performance liquid chromatography using ultraviolet detection. J Chromatogr A 1979;171:527-9.
10. Chou SS, Chung JC, Hwang DF. A High-performance liquid chromatography method for determining nitrate and nitrite levels in vegetables. J Food Drug Anal 2003;11:233-8.
11. Lim HS, Hwang JY, Choi EA, Lee G, Yoon SS, Kim MK. Development and validation of HPLC method for the determination of ferrocyanide ion in food-grade salts. Food Chem 2018;239:1167-74.
12. Frank JM, Johnsonx BJ, Rubin HS. Spectrophotometric determination of sodium nitroprusside and its photodegradation products. J Pharm Sci 1976;65:45-8.
13. David MB, Marilyn DS, Nancy K, James EC. High-performance liquid chromatographic determination of sodium nitroprusside. J Chromatogr A 1981;212:339-46.
14. Mahony C, Brown EJ, Stargel WW, Verghese PC, Bjornsson DT. *In vitro* stability of sodium nitroprusside solutions for intravenous administration. J Pharm Sci 1984;73:838-9.
15. Iram F, Iram H, Iqbal A, Husain A. Forced degradation studies. J Anal Pharm Res 2016;3:1-5.
16. Blessy M, Ruchi DP, Prajesh NP, Agrawal YK. Development of forced degradation and stability indicating studies of drugs-a review. J Pharm Anal 2014;4:159-65.
17. Reynolds WD, Facchine LK, Mullaney FJ. Available guidance and best practices for conducting forced degradation studies. Pharm Technol 2002;26:48-56.
18. International Conference on Harmonization (ICH) Harmonized Tripartite Guidelines, Validation of Analytical Procedures, Text, and Methodology, Q2 (R1), Parent Guidelines on Methodology Dated; 1996. p. 6.
19. Inert Search TM for LC, Inertsil ® Applications, Analysis of Potassium ferricyanide and Potassium ferrocyanide, Data No. LB018-0811; 2020.
20. Chou SS, Chung JC, Hwang DF. A high-performance liquid chromatography method for determining nitrate and nitrite levels in vegetables. J Food Drug Anal 2003;11:233-8.
21. Sodium nitroprusside, USP Monographs, official (USP42-NF37), 4045; 2020.
22. Sodium Nitroprusside-European Pharmacopoeia 10.3, (01/2016:0565). Available from: https://www. pharmacopoeia.com/bp-2020/monographs/sodium-nitroprusside [Last accessed on 15 Jul 2020].
23. Loenen CVA, Kemper HW. Stability and degradation of sodium nitroprusside. Pharm Weekbl 1979;1:424-36.
24. Frank JM, Johnsonx BJ, Rubin HS. Spectrophotometric determination of sodium nitroprusside and its photodegradation products. J Pharm Sci 1976;65:45-8.
25. Chou SS, Chung JC, Hwang DF. A high-performance liquid chromatography method for determining nitrate and nitrite levels in vegetables. J Food Drug Anal 2003;11:233-8.
26. David MB, Marilyn DS, Nancy K, James EC. High-performance liquid chromatographic determination of sodium nitroprusside. J Chromatogr A 1981;212:339-46.
27. InertSearch TM for LC, Inertsil ® Applications, Analysis of Potassium ferricyanide and Potassium ferrocyanide, Data No. LB018-0811. Ion pair reagents for HPLC (Article No: A1084E 20190913); 2020.
28. https://www.tcichemicals.com/assets/brochure-pdfs/Brochure_A1084_E.pdf [Last accessed on 15 Jul 2020]
29. Huang HT, Salter G, Kahn LS, Gindt MY. Redox titration of ferricyanide to ferrocyanide with ascorbic acid: illustrating the nernst equation and beer–lambert law. J Chem Educ 2007;84:1461-63.
30. Jahanvee KT, Chirag JP, Patel MM. RP-HPLC method development and validation of macitentan with its known and unknown degradation impurities in its tablet dosage form. Int J Appl Pharm 2018;10:81-9.
31. Adison F, Sanjay Pai PN. A validated stability-indicating rp-hplc method for estimation of tolfenamic acid in the presence of its pharmacopoeial impurities. Int J Appl Pharm 2019;11:264-70.
32. International Conference on Harmonization (ICH), Stability testing of new drug substances and products, Q1A (R2); 2003.