Determination Of Nitrite Content In Pharmaceutical Products By High-Performance Liquid Chromatography With Fluorescence Detection

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Abstract: Nitrite is present everywhere including water, solvents, and the environment. When it combines with amides and secondary amines, leads to generation of N-nitrosamines, that behave as a carcinogenic agent. During the production drug or products may have these impurities such as N-nitrosamines due to the employment of reagents, catalysts, solvents, or raw ingredients, which has amides or secondary amines groups during. Therefore, determination of nitrite content is very important at initial stage to find out the possible of N-nitrosamines. Different approaches have been used to determine the nitrite content in the various samples. During this research, an optimized and improved method for the determination of nitrite content was utilized. 0.1 ppm limit for nitrite concentration in pharmaceutical products can be found using the Reverse Phase HPLC technique with Fluorescence Detection. This process produces 2,3-naphthotriazole (NAT) by pre-column derivatization of nitrite with 2,3-Diaminonapthalene (DAN) in an acidic medium. Chromatographic separation was accomplished on an Xbridge BEH C8 (150 x 4.6) mm, 2.5 µm column using a gradient elution technique based on buffer pH 7.0 and acetonitrile as the mobile phase to determine NAT. At 415 nm for emission and 375 nm for excitation, the absorbance of NAT was measured. This technique could be very crucial for the detection of nitrite substances, that might lead to production of carcinogenic substances such as N-nitrosamines. In this way, we can avoid usage of these substances in pharmaceutical industry.

Keywords: Nitrite; Nitrosamine impurities; Method Development; High-performance liquid chromatography

1. Introduction

Molecules having a nitroso functional group are members of the N-nitrosamines, or nitrosamine, class of chemical substances. The "cohort of concern" is a collection of highly potent mutagenesis carcinogens, and these N-nitroso compounds are part of it. When N-nitroso diethylamine (NDEA) was produced by reacting sodium nitrite and dimethylamine hydrochloride in the nineteenth century, nitrosamines were first identified. On the other hand, before 1954, these compounds were not given much attention by scientists. The U.S. Food and Drug Administration (FDA) released a public health alert in July 2018 about the presence of N-nitroso dimethylamine (NDMA) in the medication Valsartan. Since then, metformin, ranitidine, and nizatidine have also been linked to NDMA. According to FDA advice, in acidic environments, nitrosamines can develop when secondary, tertiary, or quaternary amines are present along with nitrite salts as presented in figure 1 [1].
Another source of secondary amines that can react to produce nitrosamines is amide solvents. Dimethylamine, for instance, might be an impurity in dimethylformide. Additionally, secondary amines found in trimethylamine can produce nitrosamines. Additionally, the FDA has noted: 1) Contamination of fresh solvents with nitrosamine. 2) The initial material's contamination with sodium nitrite. 3) Certain raw materials include secondary amines as contaminants. 4) Nitrosamines and cross-contamination. Additionally, according to the FDA, nitrite impurities in excipients can react with secondary and tertiary amines to produce nitrosamines [2].

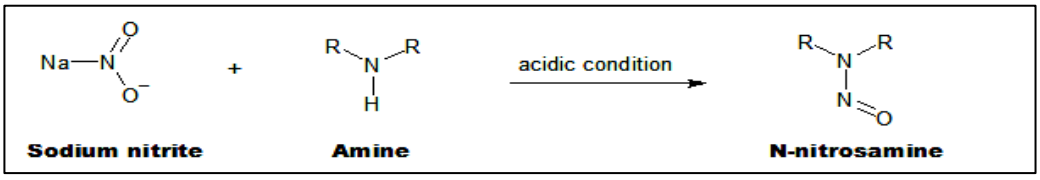

Figure 1. This is a figure. Schemes follow the same formatting.

The detection of nitrite and nitrate, two persistent oxidation products of nitric oxide (NO), is a useful approach for investigating NO formation in vivo and in cell cultures. Nitric oxide (NO) has a variety of physiological and pathological roles in the circulatory, immune, and neurological systems have led to the development of various methods for determining NO synthesis [1,3]. Because NO is a free radical molecule released by cells in picomolar to nanomolar ranges and has a very short half-life [4], a direct measurement of its production is difficult. Thus, the analysis of nitrite and nitrate, the stable products of NO oxidation, is often performed to estimate NO synthesis in biological systems and cell cultures [2].

The commonly employed methods for nitrite determination have included the Griess colorimetric assay [5], the chemiluminescence analysis [6], and the fluorometric method [7]. For nitrate analysis, it is converted to nitrite either by reducing metals such as cadmium or by nitrate reductase [8]. The Griess assay is based on the two-step diazotization reaction in which nitrite is chemically transformed into a colored azo dye [9]. Although the Griess reaction is simple, the detection limit for nitrite and nitrate by a UV–VIS spectrophotometer is only 1–2 μM [10]. The lack of sensitivity severely restricts the application of this colorimetric method for quantifying submicromolar levels of nitrite and nitrate in biological samples.

The chemiluminescence test was initially developed to assess NO in air samples. It utilises the interaction of NO with ozone to generate light for detection by a photomultiplier tube, and fewer than 10 pmol NO may be detected [11]. This method can be adapted to analyze nitrite and nitrate in aqueous solutions after the release of NO from nitrite or nitrate under acid–iodide or stronger reducing conditions [12]. The chemiluminescence assay, however, requires an expensive and bulky apparatus, which is not available in most laboratory settings, and is interfered by N-G-nitro-L-arginine (a commonly used inhibitor of NO synthase) and some nitroso compounds (e.g., S-nitrosothiols and nitrosodiphenylamine) [13].

The batch fluorometric assay is based on the reaction of nitrite with 2,3-diaminonaphthalene (DAN) under acidic conditions to yield the highly fluorescent product 2,3-naphthotriazole (NAT) [14], and can be used to detect 10–20 nM nitrite and nitrate. However, there is a great difficulty in employing the batch fluorometric method to detect picomole levels of nitrite and nitrate in cell culture medium and biological samples, because of high blank values as well as the fluorescence quenching and interference by biological components and colorimetric chemicals.

Several ion-exchange and reversed-phase ion-paired HPLC methods have been developed for measuring nitrite and nitrate in biological systems, with detection by either UV–VIS absorbance or conductivity [15,16]. Most HPLC methods require several purification steps to remove interfering substances such as chloride and biogenic amines. These additional preparative steps may cause variable recovery and introduce contamination by environmental nitrite and nitrate. In addition, the HPLC methods with UV–VIS or conductimetric detection suffer from low sensitivity compared with fluorescence and chemiluminescence assays. Thus, quantification of picomole levels of nitrite and nitrate in cell culture medium and biological samples is still a challenge. The objective of this study was to develop a rapid, sensitive and specific HPLC method for measuring nitrite and nitrate. This method involves the reaction of nitrite with DAN to form NAT, the chromatographic separation of NAT, and the fluorescence detection of NAT. Thus, our HPLC method offers high sensitivity and specificity as well as easy automation for determining picomolar levels of nitrite and nitrate in cell culture medium and biological samples.

2. Materials and Methods

A Waters Alliance (Model number 2996) with a Fluorescence Detector was used for Nitrite detection. Empower 3 software is used for complete system control and data handling.

2.1. Reagents and chemicals

Commercially available reagents used in this study were of analytical reagent grade (Merck, Spectrochem, Qualigens, Advent, and Sigma-Aldrich) and these reagents were used without further purification. HPLC-grade methanol and acetonitrile were used as solvents. Distilled, de-ionized water was used in all sample preparation procedures. Different mobile phase compositions in various mixture ratios using methanol (Advent), acetonitrile (Qualigens), and DDW were used to elute the prepared analyte standards.

2.2. Preparation of Buffer solution and mobile phase

Transfer 2 mL of Triethylamine into a beaker containing 1000 mL water, sonicate to dissolve and adjust the pH of the solution to 7.00 ± 0.05 with orthophosphoric acid. Filter the solution through a 0.45 um PVDF membrane filter. Moreover, for the preparation of mobile phase A and B, buffer solution and acetonitrile was mixed

in the ratio of 90:10 (v/v) and 10:90 (v/v), respectively. The prepared mobile phases were degassed before the use. All the other solution was prepared using HPLC grade water as diluent.

2.3. Preparation of 2,3-Diaminonapthalene Solution

Accurately weigh and transfer 5 mg of 2,3-Diaminonapthalene to a 50 mL amber volumetric flask. Add 35 mL of 0.68 M Hydrochloric acid solution, sonicate to dissolve, and make up the volume with 0.68 M Hydrochloric acid solution.

2.4. Preparation of Blank Solution

Take 2 mL of methanol into a 15 mL centrifuge tube or test tube and add 6 mL of water and mix well. Add 1 mL of 2,3-Diaminonapthalene solution and mix thoroughly, keep the solution at room temperature for 120 minutes. Add 1 mL of 2.8 N Sodium hydroxide solution and mix thoroughly (do not make up the volume). Filter the solution through 0.22 µm Nylon-66 Prefilter (Make- Nupore, Catalogue No. GNSY25) after discarding the first 1 mL of filtrate.

2.5. Preparation of Standard Stock Solution

Weigh and transfer about 30 mg of sodium nitrite standard to a 200 mL volumetric flask. Add 100 mL of diluent and sonicate for 2 minutes until dissolved. Make up the volume with diluent and mix. Transfer 5 mL of this solution into a 200 mL volumetric flask, dilute up to the mark with diluent, and mix it properly. Further, dilute 2 mL of the above prepared solution into a 100 mL volumetric flask and dilute up to the mark with diluent and mix.

2.6. Preparation of Standard Solution

Take 1 mL of the standard stock solution into a 15 mL centrifuge tube or test tube, add 2 mL of methanol and 5 mL of water and mix well. Add 1 mL of 2,3-Diaminonapthalene solution and mix thoroughly, keep the solution at room temperature for 120 minutes. Add 1 mL of 2.8 N Sodium hydroxide solution and mix thoroughly (do not make up the volume). Filter the solution through 0.22 µm Nylon-66 Prefilter (Make- Nupore, Catalogue No. GNSY25) after discarding the first 1 mL of filtrate.

2.7. Preparation of Sample Solution

Accurately weigh and transfer about 500 mg sample into a 15 mL centrifuge tube or test tube, add 2 mL of methanol and shake thoroughly to dissolve the sample. Add 6 mL of water and mix well. Add 1 mL of 2,3-Diaminonapthalene solution and mix thoroughly, keep the solution at room temperature for 120 minutes. Add 1 mL of 2.8 N Sodium hydroxide solution and mix thoroughly (do not make up the volume). Filter the solution through 0.22 µm Nylon-66 Prefilter (Make- Nupore, Catalogue No. GNSY25) after discarding the first 1 mL of filtrate.

2.8. Chromatographic Parameters

All the parameters used for chromatography is presented in table 2.

Table 2. Details of the parameters used for chromatography experiment.

Column	Xbridge BEH C8 (150 x 4.6) mm, 2.5 µm
Detector	Fluorescence detector
Excitation Wavelength	375 nm
Emission Wavelength	415 nm
Injection Volume	10 µl
Run time	30 minutes
Column oven temperature	50°C
Sample tray temperature	25°C

Flow rate	1.0 mL/min
Needle wash solvent	Water and methanol in a ratio of 50:50 (v/v)
Needle wash type	Extended

Table 3. Detail of the gradient programme and mobile phase used for chromatography.

Time (minutes)	Mobile phase A (%)	Mobile phase B (%)
00	90	10
14	82	18
15	00	100
20	00	100
22	90	10
30	90	10

3. Results

During the calculation of nitrite content, area counts of NAT peak in the sample chromatogram and however standard chromatogram was diluted to 0.1 ppm in the entire calculations. The potency/Purity of Sodium Nitrite was taken as a working standard. All, the values were calculated as per standard calculation methods.

3.1. Analytical method validation

Validation is an important feature after the development of any analytical method because it is closely related to the quality of the results. All analytical methods, whether qualitative or quantitative are required to be validated. The degree of validation varies for the type of method and its application. For several years, method validation studies, guidelines, and procedures have focused mainly on quantitative analysis methods. Validation is an imperative activity in the process of impurities profiling where the developed analytical method used for the determination of genotoxic impurities in drug substances is validated to establish that the method is suitable for its aimed purpose. The analytical methods are validated with linearity, precision, accuracy, ruggedness, robustness and LOQ/LOD parameters by ICH Harmonized Guidelines.

3.4. Precision

The system precision was verified by injecting six replicate injections of standard solution. Calculated the percent relative standard deviation (%RSD) of area counts of NAT peak.

Table:4 NAT peak area data and relative standard deviation for sample

Summary of System Precision Data	
Injection No.	**NAT Peak Area**
1	1845578
2	1860815
3	1850013
4	1847021
5	1864048
6	1858241
Mean	1854286
Std. Dev.	7751.57
%RSD	0.42

The method precision was verified by injecting six replicate injections of sample solution. Calculated the per-cent relative standard deviation (%RSD) of area counts of NAT peak.

Table:5 NAT peak area data and relative standard deviation for reference sample

Summary of Method Precision Data	
Injection No.	**NAT Peak Area**
1	555354
2	556321
3	554269
4	558572
5	557013
6	556495
Mean	556337
Std. Dev.	1464.26
%RSD	0.26

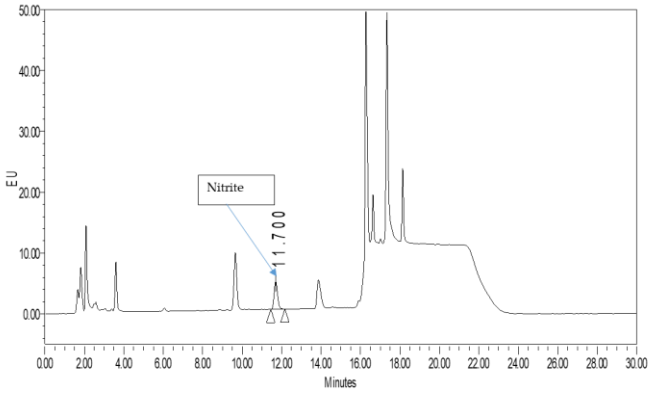

Fig-2 Nitrite analysis in sample medium with fluorescence detection

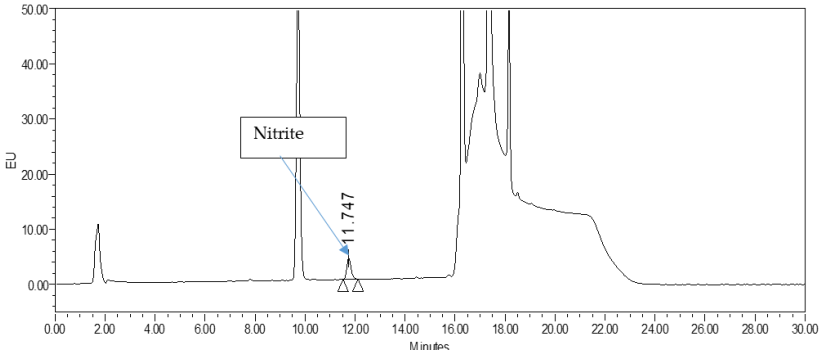

Fig-3 Nitrite analysis in reference medium with fluorescence detection

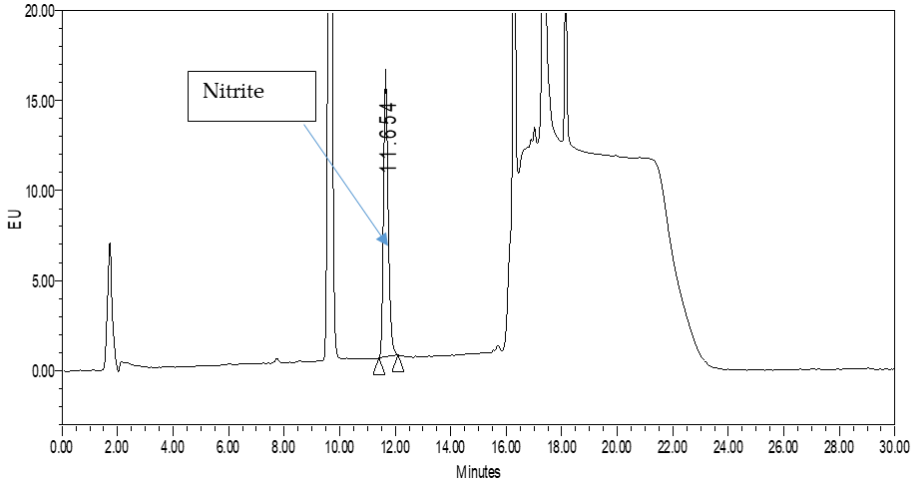

Fig-4 Nitrite analysis in standard medium with fluorescence detection

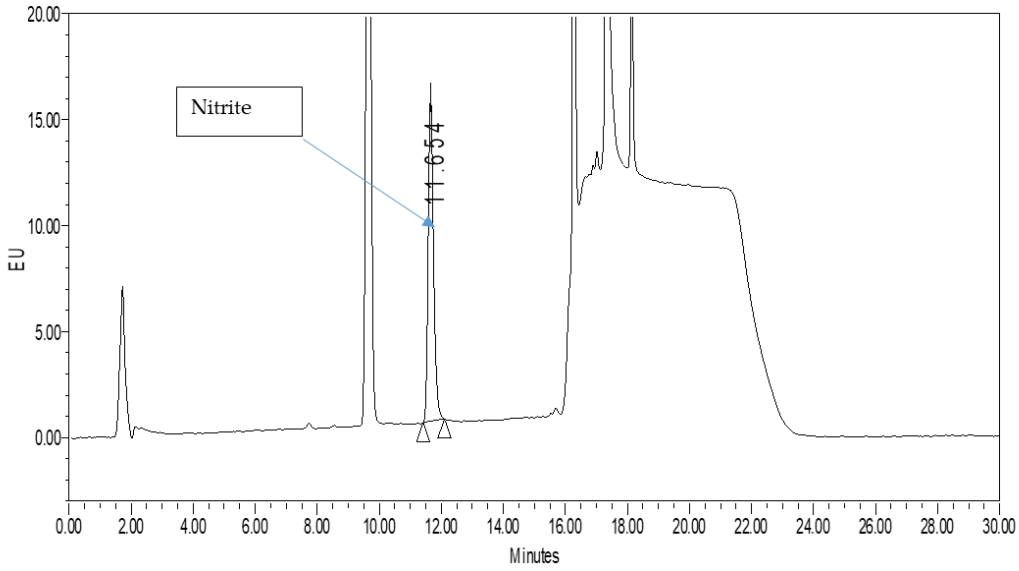

Fig-5 Nitrite spiked in sample medium with fluorescence detection

3.5. Linearity

In the linearity parameter, the concentration of NAT response was determined in the range of 10%-150% of the standard solution. Calibration curves were plotted between analyte concentration and peak response. The slope, intercept, and coefficient of correlation were calculated using MS Excel. The calibration data of NAT is given below:

Table:6 Calibration data of NAT standard solution ranging from 10-150%

Limit	ppm WRT sample	Conc. (µg/mL)	Area Counts	Corrected Area Counts	Limits
10	0.010	0.001	564517	336970	
25	0.026	0.001	766160	538613	
50	0.052	0.003	1044038	816491	
100	0.105	0.005	1836736	1609189	
150	0.157	0.008	2740254	2512707	
Slope				**296282122.7297**	

Intercept	**123746.4213**	
Correlation Coefficient (r)	**0.99701**	**NLT 0.99000**

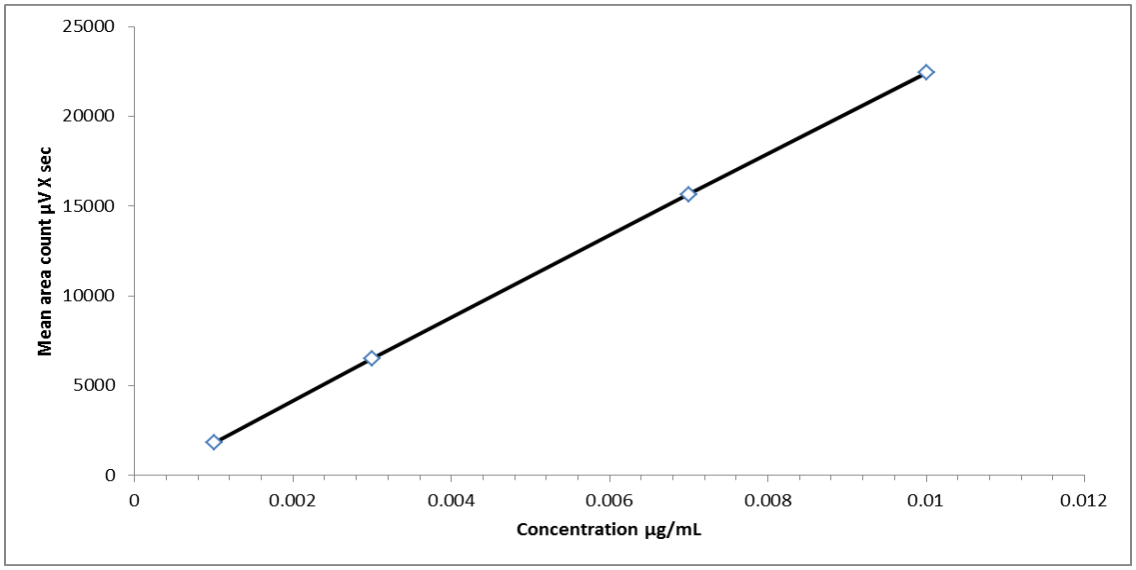

Fig-6 Calibration curve for NAT concentration

3.6. Limit of Quantification

The limit of quantification is the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy. The limit of quantification was calculated by using signal to noise ratio approach, LOQ = 10(SD)/S, where SD = Standard deviation of response (peak area) and S = the slope of the calibration curve.

Table-7 Limit of quantification(LOQ) for NAT peak area

Summary of LOQ Data	
Injection No.	**NAT Peak Area**
1	584308
2	583145
3	572071
4	592613
5	592816
6	573445
Mean	583066
Std. Dev.	8957.79
%RSD	1.5

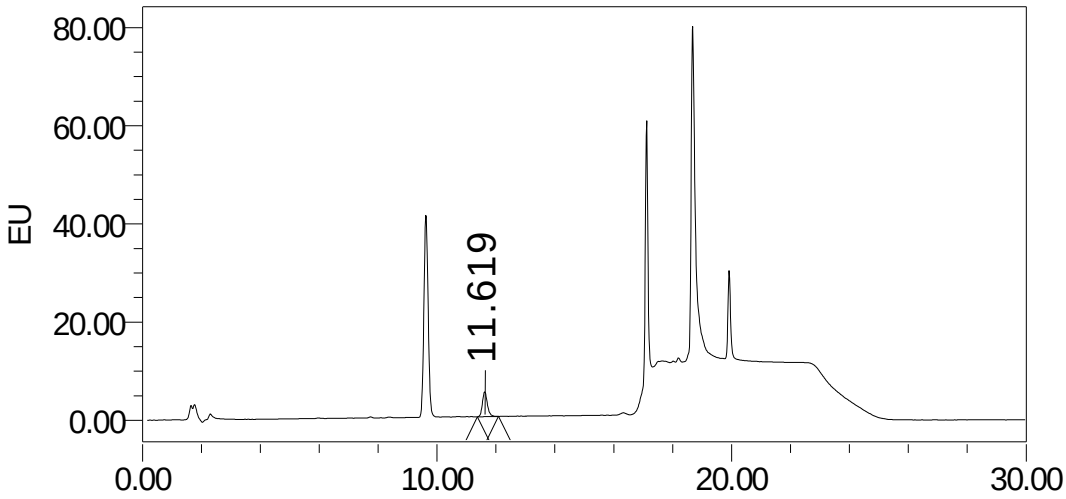

Fig-7 Vhromatogram for NAT peak area *3.7. Accuracy*

The accuracy was determined by adding the known quantities of the analyte to the sample. A 3-fold measurement at 50% (Level-1), 100% (Level-2) and 150% (Level-3) of sample concentration was carried out.

Table:8 Recovery of sample concentration and percentage mean recovery

Level	Sample	% Recovery	% Mean Recovery
50%	1	93.77	92.9
	2	97.71	
	3	87.16	
100%	1	89.49	92.8
	2	95.14	
	3	93.66	
150%	1	93.80	95.7
	2	91.19	
	3	102.13	

4. Discussion

During the entire study of various samples, this has been reported that the correlation coefficient for NAT (Nitrite) was found to be 0.99701 between the 10%-150% range of the target concentration of an analyte. Precisely the %RSD was found 0.42 for system precision and 0.26 was found for repeatability study. However, percentage recovery for NAT (Nitrite) was found to be 92.9%, 92.8%, and 95.7% at three levels (50%, 100% and 150%). However, researchers have developed different methods to identify and quantify nitrite content of water, environmental samples as well as biological samples. Drug substances or products may have these impurities of nitrite due to the regular use of reagents, catalysts, solvents as well as raw ingredients in the manufacturing process. However, there is a need to quantify the nitrite content in pharmaceutical samples which may be accurate, precise and validated. During the entire work, we have developed and optimized an effective and validated process for the determination of nitrite content in pharmaceutical products.

5. Conclusions

The gradient HPLC method for the simultaneous determination of Nitrite content in different pharmaceutical products has been developed. This method is improved and much more precise than that of the previously observed methods. This method is validated as per the standard guidelines of the ICH. This method has been developed using 2,3-diamino naphthalene (DAN) which is cost-effective, time-shaving, and can be used for routine analysis of different pharmaceutical products used in industries.

Author Contributions:
Ankit Kumar: Data curation, original draft preparation, methodology, software, and validation;
Sanjay Kumar: Review and writing, analysis
Subrahmanya Kumar Kukkupuni: Review and editing, supervision and conceptualization
Mohit Kumar: Review and writing, analysis
Vivek Kohli: Review and writing, analysis
Raman Thakur: Review and writing, analysis
Santosh Kumar Mishra: Review and editing, supervision and conceptualization

Conflicts of Interest:
The authors declare no conflicts of interest

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