

Development and Validation of Comprehensive UV-visible Spectroscopy Method for Sensitive Detection of Nitrite Levels in Pharmaceuticals

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ABSTRACT

Introduction: Nitrosamines are known to damage DNA and induce cancer, a characteristic that classifies them as genotoxic and carcinogenic. This research presents an innovative methodology designed for the quantification of nitrite concentrations in active pharmaceutical ingredients, excipients, and finished drug products. This approach mitigates the shortcomings of existing methodologies, which are often characterized by limited sensitivity and elevated costs. **Materials and Methods:** A novel ultraviolet spectroscopy technique, utilizing the Griess reagent, was developed to facilitate the precise quantification of trace nitrite levels. The method was validated following ICH guidelines, confirming acceptable linearity, sensitivity, accuracy, precision, and robustness. **Results:** The resulting method offers a cost-effective and easily adaptable solution for the routine monitoring of nitrite in pharmaceutical products, thereby supporting quality control initiatives and ensuring compliance with regulatory standards. The method is validated to meet ICH Q2(R1) and USP<1469> standards, method reliably or accurately exhibited outstanding linearity ($r^2=0.995-0.999$) across a range of concentrations for each API, excipient, and marketed formulation. Notably, this method surpasses traditional approaches by providing a comprehensive tool for quality assurance, which is essential for monitoring nitrosamine formation. **Conclusion:** The implementation of this method represents a significant advancement in nitrite detection capabilities, providing an essential means for ensuring the safety and quality of pharmaceutical products.

Keywords: N-Nitrosamines, Nitrites, UV-visible spectroscopy, Method Validation.

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INTRODUCTION

The presence of N-nitrosamines which are classified as probable human carcinogens, in pharmaceutical products has raised considerable concerns. These compounds are often formed through an acid-catalyzed reaction between a secondary amine and a nitrosating agent like nitrite.¹ Pharmaceutical excipients are among the various sources that can introduce nitrites. Consequently, the pharmaceutical industry needs a thorough understanding of how nitrites contribute to nitrosamine impurities to effectively minimize the associated risks.² Nitrosamines typically form when nitrites and amines react in acidic conditions. This process involves the generation of nitrous acid from nitrite salts, which then reacts with secondary or tertiary amines, resulting in the formation of nitrosamines.³

Excipients are a notable source of nitrites in pharmaceutical formulations.^{2,3} The concentration of nitrite can vary significantly depending on supplier and kind of excipient, which influences the potential for nitrosamine formation in the final drug product. Some active pharmaceutical ingredients naturally contain secondary or tertiary amines susceptible to nitrosation.^{4,5} However, tertiary amines generally exhibit lower reactivity toward nitrosation compared to secondary amines. This difference stems from the fact that tertiary amines require an additional dealkylation step to form a nitrosamine, a process that is significantly slower than the direct nitrosation of a secondary amine.

This difference arises because tertiary amines require an additional dealkylation step to generate a nitrosamine, a process that occurs at a considerably slower rate than the direct nitrosation of a secondary amine. However, exceptions occur, such as when an electron-rich aromatic ring is linked to the N-nitrosatable group, which can increase the reactivity of tertiary amines.⁶ The various factors can also affect the kinetics of nitrosamine formation, like presence of chemical species, temperature and pH.



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The pharmaceutical industry has adopted various analytical methods for quantifying nitrite, as detailed in Table 1. The main techniques currently employed include ion chromatography with conductivity detection. The Griess method is another important way to find nitrites using UV and liquid chromatography. Ion chromatography with conductivity detection can also quantify nitrite. The alternative methods entail the reduction of nitrate to nitrite before the Griess reaction, facilitating nitrate detection.⁷

The Griess reagent is a key element in many methods for detecting nitrite ions. This chemical reagent reacts with nitrites to produce a colored azo dye, which is then quantified using spectrophotometry. The reagent consists of sulfanilic acid and ethylenediamine dihydrochloride, which undergo a diazotization reaction when exposed to nitrite, resulting in a pink or reddish-purple color. This color's intensity is directly correlated with the sample's nitrite concentration. The Griess reagent is widely used in environmental monitoring due to its high sensitivity and specificity, clinical diagnostics, and food analysis for accurately determining nitrite concentrations. It is also frequently used to detect nitrite ions in pharmaceutical samples.^{18,19} The Griess reagent typically includes:

- **Sulfanilamide:** An aniline derivative that forms a diazonium salt when it reacts with nitrite.
- **N-ethylenediamine dihydrochloride:** A coupling agent that interacts with the diazonium salt to produce a colored azo dye.

The detection of nitrite using the Griess reagent involves:

- **Diazotization Reaction:** When sulfanilamide is added to a sample containing nitrite, a diazonium salt is produced.
- **Azo Coupling Reaction:** After treating with NED, diazonium salt produces pink-red azo dye. Nitrite concentration in the sample is directly correlated with the intensity of the resultant color.

Quantitative analysis of nitrite levels is achieved through spectrophotometric measurements of the colored solution, typically at a wavelength of 540 nm.²⁰ UV-visible spectroscopy is an analytical method employed for measuring the absorption of ultraviolet and visible light by chemical compounds. It is a key method in fields like chemistry, biology, and environmental science for both qualitative and quantitative analyses.²¹ The light and matter interact with each other and this is the fundamental principle of UV-visible spectroscopy. When a sample is exposed to visible or ultraviolet light, its molecules absorb particular wavelengths. This absorption causes electronic transitions, where electrons move from ground state to an excited state.²² The quantity of light absorbed is related to energy difference between

ground and excited states, which is unique to each molecule depending on its electronic structure.^{23,24}

A spectroscopic analytical method using the Griess reagent has been developed and validated for determining nitrite content in pharmaceuticals. This method quantifies nitrite levels in active pharmaceutical ingredients, excipients and finished products. The method has been applied to various samples and the results demonstrate the quantified amounts of nitrite.

MATERIALS AND METHODS

Materials

All chemicals and reagents utilized in this study were obtained from reputable vendors. Specifically, N-Ethylenediamine dihydrochloride solution, sulfanilic acid solution, sodium nitrite, and glacial acetic acid conforming to pharmacopoeial and analytical standards and procured from JP and company, Pune. The excipients microcrystalline cellulose (Asahi Kasei), stearic acid, Kollitab DC 87L (BASF), maltodextrin (Roquette) and croscarmellose sodium (IFF) were received as a gift sample. Pharmaceutical samples, including Varenicline, Ranitidine, Valsartan, Paracetamol, and Metformin tablets, were procured from Shree Medical in Nigdi, Pune.

Methods

The Griess method involves preparation of three components, and its preparation is described as follows.

Component 1: Acetic Acid Solution

In a 300.0 mL volumetric flask, 45.0 mL of glacial acetic acid has been added to create an acetic acid solution. To produce a homogenous solution, distilled water has been carefully blended into flask until it reaches calibration point.

Component 2: N-Ethylenediamine Dihydrochloride (NED) Solution

A 100.0 mL amber volumetric flask containing 0.14 g of N-Ethylenediamine Dihydrochloride was used to prepare the N-Ethylenediamine dihydrochloride solution. N-Ethylenediamine dihydrochloride was completely dissolved after 100.0 mL of prepared acetic acid solution has been added and mixture had been shaken. After adjusting volume of solution with acetic acid solution, it was completely mixed.

Component 3: Preparation of Sulfanilic Acid Solution

A solution of sulfanilic acid has been prepared by placing 0.34 g of sulfanilic acid into 100.0 mL amber volumetric flask. Subsequently, 100.0 mL of acetic acid solution was introduced and flask was stirred until sulfanilic acid was fully dissolved. The solution was thereafter adjusted to the requisite volume with the acetic acid solution and stirred until homogeneity was attained.

Preparation of Nitrite Standard Stock Solution

The nitrite standard stock solution was prepared by drying sodium nitrite at 110°C for 1 hr, followed by cooling in a desiccator. 15.0 mg of dried Sodium Nitrite have been meticulously weighed or placed in 100.0 mL volumetric flask. After adding 60.0 mL of distilled water, the flask was shaken to dissolve the sodium nitrite. The solution was subsequently diluted to specified volume with distilled water than properly mixed, yielding a stock solution to a nitrite content of 1000.0 µg/mL. Subsequently, 1 mL of stock solution has been transferred into 100.0 mL volumetric flask, diluted to mark with distilled water and agitated to achieve a homogenous mixture.²⁵

Method Validation

According to ICH requirements, the UV-spectrophotometric technique for nitrites was developed and validated to thoroughly assess criteria like linearity, specificity, selectivity, accuracy, ruggedness, stability, LOD, and LOQ.

Specificity and Selectivity

The UV spectra of both nitrite solutions and blank Griess reagent were scanned across a spectrum of wavelengths from 800 nm to 200 nm. This procedure has been performed to detect any interference caused by the solvent at the wavelength where nitrite exhibits maximum absorbance.

Determination of Linearity Range Responses

Linearity ranges, specifically from 0.01 to 0.1 µg/mL for nitrite, have been assessed in triplicate utilizing a stock solution, with absorbance readings recorded at 540 nm. A calibration curve has been built by graphing absorbance against concentration, and a linear regression equation was then calculated. The procedure involved transferring nitrite solution volumes ranging from 1.0 mL to 10.0 mL into 100.0 mL volumetric flasks. Following this, 2.5 mL of sulfanilic acid solution was introduced, or the mixture was agitated until it reached homogeneity, followed by a 5-min incubation period. Subsequently, 2.5 mL of N-Ethylenediamine dihydrochloride solution has been added and solution has been diluted to the marked volume using distilled water. The resultant solution featured nitrite concentrations spanning from 0.01 µg/mL to 0.1 µg/mL. Absorbance measurements were conducted at the wavelength of maximum absorbance once the UV-visible spectrophotometer had achieved its operational readiness (Model: [Jasco V 630], Wavelength range: [540 nm]). The solution containing different nitrite samples in various concentrations was evaluated for regression (r²) guidelines.

Precision Analysis

System Precision

To assess system precision, six replicates of nitrite solutions at concentrations of 0.1 µg/mL and 0.05 µg/mL were prepared or

analyzed. The relative standard deviation was then calculated from resulting data.

Intraday Precision

Intraday precision was evaluated by preparing nitrite solutions at concentrations of 0.01, 0.05, and 0.1 µg/mL in triplicate from the stock solution. These samples were analyzed on the same day, and RSD was computed at various time intervals to determine the precision within a single day.

Interday Precision

Interday precision was assessed over 3 consecutive days. Nitrite solutions at concentrations of 0.01, 0.05, and 0.1 µg/mL have been prepared in triplicate each day from the stock solutions, analyzed, and RSD was computed at various intervals to evaluate the precision across multiple days.

Ruggedness

Ruggedness was evaluated by introducing deliberate changes to the analytical procedure, specifically by using different instruments and analysts. Nitrite solutions at concentrations of 0.01, 0.05, and 0.1 µg/mL have been prepared in triplicate, and their absorbance was measured at 540 nm by different analysts and with different instruments. The RSD was then calculated to quantify the impact of these variations on the results.

Robustness

To determine robustness, the wavelength was altered. Nitrite solutions at concentrations of 0.01, 0.05, and 0.1 µg/mL have been prepared in triplicate, and their absorption was measured at both 525 nm and 540 nm. The % RSD was then computed to assess the method's sensitivity to small changes in wavelength.

Standard Stock Solution Stability

The stability of standard stock solution has been assessed by comparing freshly prepared stock solutions against older ones. Dilutions of 0.01, 0.05, and 0.1 µg/mL have been prepared from a fresh stock solution, and their absorbance has been measured. The RSD has been calculated as a percentage or compared to that of old stock dilutions.

LOD and LOQ

The Limit of Detection and Limit of Quantification have been calculated using statistical methods applied towards the linear curve, according to established formulas.²⁶

$$LOD = \frac{3.3 \times Y - \text{intercept}}{\text{Slope of the calibration curve}}$$

$$LOQ = \frac{10 \times Y - \text{intercept}}{\text{Slope of the calibration curve}}$$

Table 1: Comparative study of diverse analytical methodologies for nitrite determination.

Techniques	Mechanism	Detection Methods	Sample Preparation	Sensitivity
Ion Chromatography (IC) ⁸	Separation via anion-exchange column	UV, conductivity, MS	Minimal; no derivatization	µg/L level sensitivity
GC-MS ⁹	GC separation + MS identification via derivatized nitrite	Fluorescent derivative (e.g., DAN → NAT)	Derivatization required	As low as 0.02 pg/mL
LC-MS ¹⁰	LC separation followed by MS quantification	NAT fluorescence	Protein ppt., SPE, derivatization	0.2-5.0 µg/g (LOD ~0.5 ng/mL)
HS-GC-MS ¹¹	Volatile nitrite extracted from sample headspace	GC-MS	Extensive cleanup; volatile extraction	~0.05 mg/kg (ppm)
HPLC-UV ¹²	Separation + UV detection at specific wavelength	UV absorbance	Standard filtration; azo dye formation (Griess)	0.006 µg/mL (LOQ: 0.012 µg/mL)
Colorimetric (Auto) ^{13,14}	Nitrate → Nitrite → Azo dye (Griess)	Visual or spectrophotometric color change.	Filtration (0.45 µm), mild derivatization	0.05-10.0 mg/L
LPME Techniques ¹⁵	Liquid phase extraction + micro-spectrophotometry	GC-MS, LC-MS	Extraction + derivatization	LOD ~1.5 µg/L
Fluorometric HPLC ^{16,17}	Nitrite + DAN → NAT → separated by RP-HPLC	Fluorescence intensity of NAT	Alkaline condition; derivatization	~25 nM (0.025 µg/mL), linear up to 800 nM

Nitrite determination by using Griess reagent

Nitrite levels in APIs, excipients, and marketed formulations were quantified through the application of the Griess reagent assay.

Active Pharmaceutical Ingredients (API)

50 mg of the substance had dissolved in 95 mL of ultrapure water. Subsequently, 2.5 mL of sulfanilic acid and 2.5 mL of NED solution were added. The resulting mixture underwent agitation via an orbital shaker at 170 rpm for a duration of 30 min, followed by centrifugation at 3900 rpm for 10 min. The supernatant has been collected or subjected to filtration through a 0.45-mm PTFE syringe filter.

Excipients

In preparing excipient sample, 1 g of the substance was dissolved in 95 mL of ultrapure water, after which 2.5 mL of sulfanilic acid and 2.5 mL of NED solution were introduced. The resulting solution was mixed utilizing an orbital shaker at a speed of 170 rpm for 30 min, subsequently centrifuged at 3900 rpm for 10 min. A 0.45-mm PTFE syringe filter was then used to collect and filter the supernatant. UV absorbance measurements were conducted on the filtered solution to quantify the concentration of nitrite.

Marketed Formulations

For the preparation of various marketed formulations, individual tablets of varenicline, ranitidine, valsartan, paracetamol, and

metformin were separately dissolved in 95 mL of ultrapure water. Following dissolution, 2.5 mL of sulfanilic acid and 2.5 mL of NED solution have been added to each distinct sample. The resultant solutions were mixed via an orbital shaker at 170 rpm for 30 min, followed by centrifugation at 3900 rpm for 10 min. The supernatant from each sample has been collected or filtered through a 0.45-mm, PTFE syringe filter. Ultimately, UV absorbance of each filtered solution was measured to ascertain the nitrite content.

RESULTS AND DISCUSSION

Method Validation

Specificity and Selectivity

UV spectra of solvent water or Griess reagent were obtained, revealing no solvent interference. The absence of solvent interference observation indicates the suitability and purity solvents used for your analysis.

Linearity and Range Responses

Linearity is determined by evaluating the scatter (variance) of data points around the regression line. This line is mathematically derived from analytical results obtained from samples containing varying concentrations of nitrite. The correlation coefficient (r), and particularly the coefficient of determination (R^2) serves as the key parameter in assessing linearity, with the (R^2) value confirming the suitability of the calibration curve.

The concentration values were incrementally increased to generate a calibration curve. The r^2 values for the majority of analytes approached 1, demonstrating a robust linear correlation within concentration or instrument response. This indicates that calibration curves are dependable for nitrite quantification, thereby facilitating the efficient analysis of samples with unknown concentrations using the generated calibration data (Figure 1). This finding demonstrates the method's capability for accurate quantification of nitrite within the specified concentration range.

Precision

A precision underpinned by the essential linear relationship observed between concentration and analytical response. A precision approach can also be referred to as repeatability or reproducibility because it was carried out by the same analyst more than once in a shorter period of time. The method's precision was confirmed by calculating the %RSD for nitrite at different levels (system, intraday, and interday). The %RSD based on absorbance was below 2% (Table 2). The outcome demonstrates that the suggested methodology is reliable.

Ruggedness

Method ruggedness for nitrite was checked by changing analyst or instrument. The %RSD has been below 2% (Table 3).

Robustness

Method robustness was tested by changing the wavelength. The %RSD was below 2%, showing the method is robust (Table 3).

Standard Stock Solution Stability

Comparing fresh and 5-day-old standard stock solutions gave %RSD values below 2%, proving the standard stock solutions are stable (Table 3).

LOD and LOQ

The nitrite Limit of Detection is 0.009 $\mu\text{g/mL}$ and Limit of Quantification is 0.027 $\mu\text{g/mL}$.

Comparative Evaluation of Active Pharmaceutical Ingredients, Excipients and Marketed Products

The absorbance of all API excipients and marketed formulations was analyzed at 540 nm, followed by an evaluation of nitrite concentrations (Table 4).

Table 2: Precision studies.

Concn ($\mu\text{g/mL}$)	System Precision ^a (n=6)		Intraday Precision ^b (n=3)				Interday Precision ^b (n=3)			
			0 hr		3 hr		1 st day		2 nd day	
	UV Abs	% RSD	UV Abs	% RSD	UV Abs	% RSD	UV Abs	% RSD	UV Abs	% RSD
0.01	--	--	0.01	1.601	0.01	0.461	0.01	1.601	0.01	0.8
0.05	0.052	0.21	0.052	0.157	0.052	0.567	0.052	0.157	0.052	0.928
0.1	0.108	0.07	0.108	0.076	0.105	0.55	0.108	0.076	0.103	0.734
^a Average of 6 estimates										
^b Average of 3 estimates										

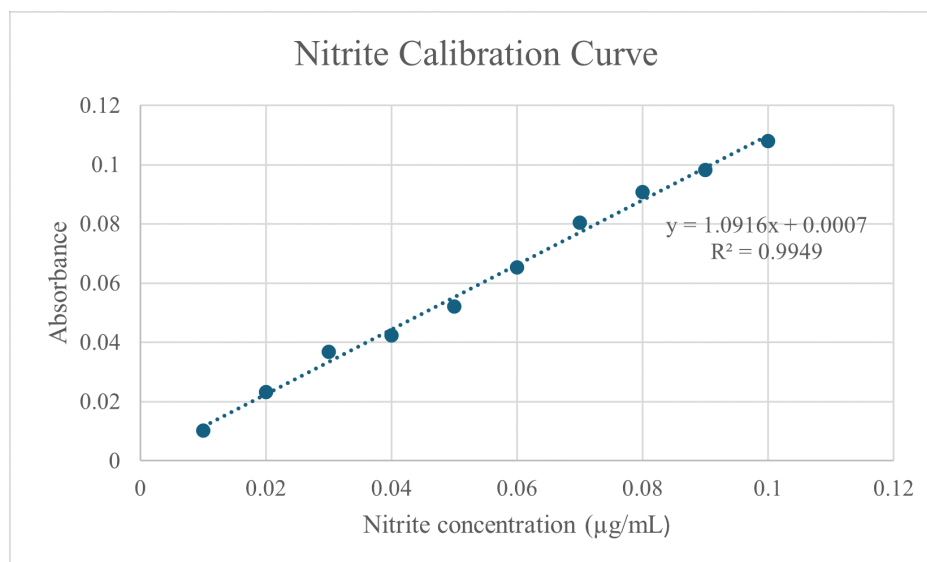


Figure 1: Calibration curve of nitrite standard solution at 540 nm.

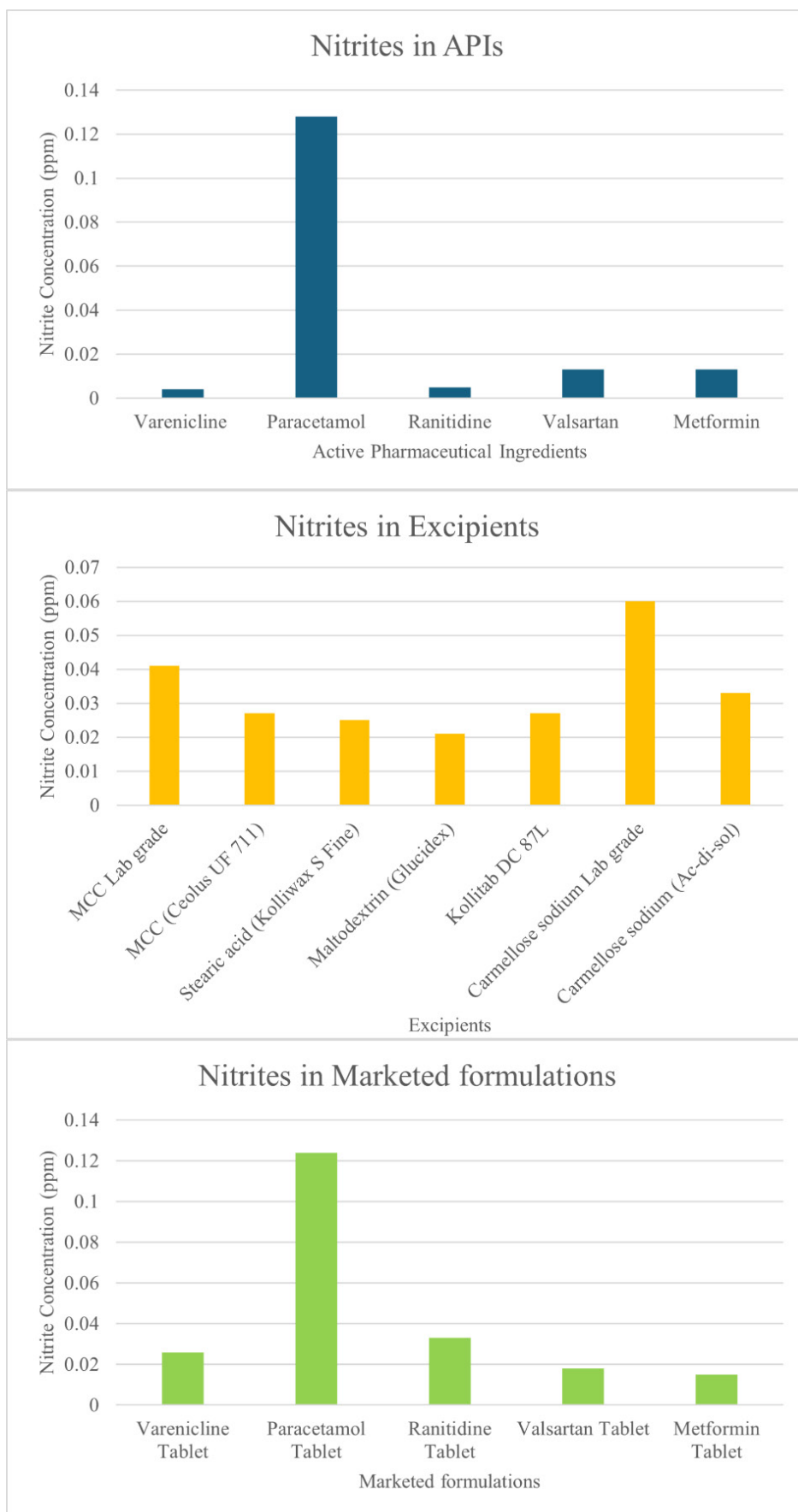


Figure 2: Comparative analysis of nitrite levels in API, excipients and marketed formulations.

Table 3: Ruggedness, Robustness and Solution Stability studies.

Concn (µg/ mL)	Ruggedness ^b				Robustness ^b				Standard Solution Stability ^b			
	Analyst 1		Analyst 2		540 nm		525 nm		1 st day		5 th day	
	UV Abs	% RSD	UV Abs	% RSD	UV Abs	% RSD	UV Abs	% RSD	UV Abs	% RSD	UV Abs	% RSD
0.01	0.01	1.601	0.01	1.807	0.01	1.601	0.01	1.268	0.01	1.601	0.093	1.517
0.05	0.052	0.157	0.052	0.452	0.052	0.157	0.050	0.654	0.052	0.157	0.049	1.594
0.1	0.108	0.076	0.108	0.151	0.108	0.076	0.101	0.168	0.108	0.076	0.1	1.564

^b Average of 3 estimates**Table 4: Experimental Results from Sample Analysis.**

Sr. No.	Particulars	UV absorbances (n=3)			Absorbance (mean)	Nitrite concentration (ppm) [#]
APIs						
1.	Varenicline	0.0031	0.0042	0.0058	0.0044	< LOQ
2.	Paracetamol	0.1262	0.1291	0.1290	0.1281	0.128
3.	Ranitidine	0.0020	0.0059	0.0080	0.0053	< LOQ
4.	Valsartan	0.0134	0.0140	0.0158	0.0144	< LOQ
5.	Metformin	0.0145	0.0138	0.0156	0.0146	< LOQ
Excipients						
1.	MCC Lab grade	0.0415	0.0414	0.0416	0.0415	0.041
2.	MCC (Ceolus UF 711)	0.0279	0.0278	0.0280	0.0279	0.027
3.	Stearic acid (Kolliwax S Fine)	0.0258	0.0257	0.0259	0.0258	< LOQ
4.	Maltodextrin (Glucidex)	0.0216	0.0215	0.0217	0.0216	< LOQ
5.	Kollitab DC 87L	0.0262	0.0281	0.0300	0.0281	0.027
6.	Carmellose Sodium Lab grade	0.0644	0.0602	0.0620	0.0622	0.060
7.	Carmellose sodium (Ac-di-sol)	0.0298	0.0336	0.0410	0.0348	0.033
Marketed Formulations						
1.	Varenicline tablet	0.0272	0.0264	0.0322	0.0286	< LOQ
2.	Paracetamol tablet	0.1204	0.1246	0.1248	0.1230	0.124
3.	Ranitidine Tablet	0.0250	0.0334	0.0400	0.0328	0.033
4.	Valsartan Tablet	0.0158	0.0189	0.0220	0.0159	< LOQ
5.	Metformin Tablet	0.0150	0.0158	0.0166	0.0158	< LOQ

[#]Values below the limit of quantification (LOQ, 0.027 µg/mL) were reported as <LOQ

Paracetamol presents the highest nitrite levels when compared to other active pharmaceutical ingredients. The nitrite concentrations of other APIs are markedly lower, typically in the range of 0.004 to 0.013 ppm. The analysis revealed that Carmellose sodium presented the greatest nitrite concentration among the tested excipients. Moreover, excipients, as a category, tend to possess elevated nitrite levels relative to active pharmaceutical ingredients. Similar to Paracetamol API, Paracetamol tablets exhibit the highest nitrite concentration, aligning with the API results. The remaining formulations demonstrate moderate nitrite concentrations, potentially attributable to the combined influence of both the API and excipients (Figure 2).

CONCLUSION

This study focused on the urgent requirement for a thorough approach to identify possible nitrite in pharmaceuticals. The existing methods of nitrite determinations have some disadvantages in terms of sensitivity and expense. We developed a novel UV method using Griess reagent to achieve sensitivity for nitrite. The approach exhibited outstanding linearity ($r^2=0.995-0.999$) across range of concentrations for each API, excipient, and marketed formulation. The method was thoroughly tested to ensure accuracy, precision, and robustness, meeting international pharmaceutical standards. This illustrates method's capacity to accurately

quantify nitrites at trace concentrations. This unique UV-visible spectroscopy approach provides an effective instrument for quality control. It offers a thorough methodology for identifying a wide range of nitrites, facilitating nitrosamine synthesis. The technique was verified to comply with ICH Q2(R1) and USP<1469> standards, ensuring reliability and precision.

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ABBREVIATIONS

API: Active pharmaceutical ingredient; **Min:** Minute; **mL:** Millilitre; **g:** Gram; **mg:** Milligram; **µg:** Microgram; **ng:** Nanogram; **NMT:** Not more than; **RSD:** Relative standard deviation; **LOD:** Limit of Detection; **LOQ:** Limit of Quantification; **UV Abs:** UV absorbance; **Concn:** Concentration.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

SUMMARY

The method's specificity and selectivity were confirmed, with no solvent interference observed. Linearity was demonstrated with r^2 values approaching 1, showing a strong correlation between concentration and instrument response. The method also exhibited good precision, with %RSD values below 2% for system, intraday, and interday analyses. Ruggedness and robustness were confirmed by varying analysts, instruments, and wavelengths, with %RSD values remaining below 2%. Standard stock solution stability were also demonstrated, with %RSD values below 2% when comparing fresh and 5-day-old solutions. The limit of detection has been determined to be 0.009 µg/mL or limit of quantification has been 0.027 µg/mL. The highest nitrite levels were among the APIs, while Carmellose sodium showed the greatest nitrite concentration among the tested excipients. Excipients generally had higher nitrite levels than APIs. Paracetamol tablets also showed the highest nitrite concentration among the tested formulations, consistent with the API results.

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