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Nota de aplicación

Reliable LC/UV Analysis of Nitrite and Nitrate Using the Arc™ Premier System With an Atlantis™ Premier BEH™ C_{18} AX Column

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Abstract

Nitrite and nitrate are considered as potential precursors in the formation of nitrosamine impurities, carcinogenic compounds that can cause cancer. Herein, we present a reverse phase method developed for the analysis of nitrite and nitrate using an Arc Premier System with UV detection and an Atlantis Premier BEH C_{18} AX Column. The method is based on direct injection, eliminating the need for a derivatization step prior to analysis. The limits of quantitation (LOQ) for nitrite and nitrate were found to be 0.025 µg/mL and 0.075 µg/mL, respectively. The method demonstrated excellent repeatability, precision, and linearity and was found to be suitable for the determination of nitrite and nitrate in the polyvinylpyrrolidone (PVP) excipient with accurate quantitative performance with 91–100% recovery.

Benefits

- Successful retention of the nitrite and nitrate ions under reverse phase conditions using an Atlantis Premier BEH C_{18} AX Column
- Fast and easy screening method by direct injection, eliminating the need for a complex sample derivatization step prior to analysis

Introduction

Nitrite and nitrate are naturally occurring nitrogen compounds in soil, water, air, and plants.[1] They are also used in food preservation, some pharmaceutical drugs, and in the production of explosives.[1] Recent publications have indicated that nitrite and nitrate are considered precursors or nitrosating agents in the formation of N-nitrosamine compounds, potential human carcinogens.[2–6] Nitrosamines are generally formed by the reaction of secondary or tertiary amines in the pharmaceutical active pharmaceutical ingredients (APIs) with the residual nitrite during the drug product manufacturing process. Nitrate is also recognized as nitrosating agent as it can be reduced to nitrite under certain conditions.[4] Formulation ingredients, including excipients, can contain nitrosating precursors, which may contribute to nitrosamine contamination in drug products.[4]

The most widely utilized methods for nitrate and nitrite determination involve the use of ion chromatography (IC) with conductivity detection, liquid chromatography (LC) with UV detection, and the Griess test.[4–6] While effective, these methods allow the determination of a single analyte, nitrite, or nitrate, and often require different sample preparation procedures. The Griess test is specific for nitrite and cannot be used to measure nitrate content.[4] Considering the possible conversion of nitrate to nitrite upon reduction, there is a desire to measure both compounds.

Herein, a single liquid chromatography (LC) method with UV detection is described for the analysis of both nitrite and nitrate, based on direct injection. This reverse phase method offers easy and fast screening for nitrite and nitrate in polyvinylpyrrolidone excipient.

Experimental

High purity LC/MS grade acetonitrile, ammonium formate, and formic acid were purchased from Fisher Chemicals. Sodium nitrate ($NaNO_3$) and sodium nitrite ($NaNO_2$) analytical grade quality were purchased from Sigma. Polyvinylpyrrolidone (PVP) K 15 was obtained from Sigma.

Standard Solutions

Nitrite and nitrate stock solutions were prepared by dissolving sodium nitrite and sodium nitrate salts in water at 5.0 mg/mL. Stock solutions were diluted with water to make a mixture standard solution containing nitrite and

nitrate at 0.1 mg/mL concentration. Calibration standards were prepared by diluting the mixture standard solution with water.

Polyvinylpyrrolidone (PVP) Sample Solutions

Test sample solutions were prepared in water at 1 mg/mL and dissolved by sonication for 20 minutes. After extraction, sample solutions were centrifuged for ten minutes at 3000 rpm and filtered through a PVDF 0.2 µm syringe filter (p/n: WAT200806 <https://www.waters.com/nextgen/us/en/shop/sample-preparation--filtration/wat200806-acrodisc-syringe-filter-pvdf-13-mm-02--m-aqueous-100-pk.html>) prior to analysis.

Method Conditions

LC system:	Arc Premier System, column manager with active pre-heating, PDA Detector
Vials:	LC/MS Maximum Recovery 2 mL volume, p/n: 600000670CV
Detection:	UV at 210 nm
Column:	Atlantis Premier BEH C_{18} AX, 2.1 x 100 mm, 2.5 µm (p/n: 186009392)
Column temperature:	30 °C
Sample temperature:	15 °C
Injection volume:	40.0 µL
Flow rate:	0.5 mL/min
Mobile phase A:	5 mM Ammonium formate in water with 0.1% formic acid in water

Mobile phase B:	Acetonitrile with 0.1% formic acid
Separation:	Isocratic separation with 70:30 mobile phase A/mobile phase B

Data Management

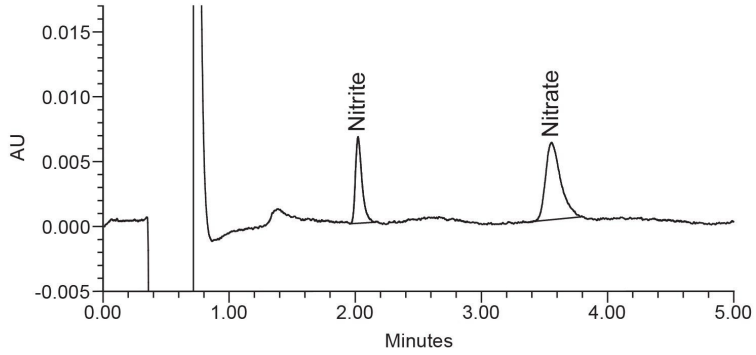
Chromatography software:	Empower™ 3 Feature Release 5 Service Release 5 (FR5 SR5)

Data acquisition and analysis performed using Empower Software. Summary reports generated using the report templates are available in the Empower project.

Results and Discussion

Various column chemistries, organic solvents, mobile phase additives, and pH were explored during the method development to ensure chromatographic retention and separation of nitrite and nitrate ions. The Atlantis Premier BEH C_{18} AX Column successfully retained and separated nitrite and nitrate under reverse-phase conditions (Figure 1A). The replicate injections of the 0.1 µg/mL standard demonstrated excellent repeatability of the Arc Premier System for nitrite and nitrate analysis with relative standard deviation (RSD) for peak areas of 0.94 and 3.06%, respectively (Figure 1B).

A.



B.

System Suitability_Report

Sample Set ID: 8579 Processed Channel Descr.: 2998 Ch1 210nm@4.8nm

	Name	RT	Area	K*
1	Nitrite	2.022	24116	2.0
2	Nitrite	2.022	23970	2.0
3	Nitrite	2.022	23785	2.0
4	Nitrite	2.023	23704	2.0
5	Nitrite	2.025	23547	2.0
Mean		2.022	23825	2.0
Std. Dev.		0.001	223.119	
% RSD		0.06	0.94	

	Name	RT	Area	USP Rs	K*
1	Nitrate	3.553	51244	9.7	4.2
2	Nitrate	3.556	48039	9.9	4.2
3	Nitrate	3.556	49384	10.0	4.2
4	Nitrate	3.557	47967	9.9	4.2
5	Nitrate	3.559	47621	10.0	4.2
Mean		3.556	48851	9.9	4.2
Std. Dev.		0.002	1497.152		
% RSD		0.06	3.06		

Figure 1. Nitrite and nitrate standard at 0.1 µg/mL run on an Arc Premier System with an Atlantis Premier BEH C_{18} AX Column (A) and system suitability results for replicate injections (n=5).

Limits of Detection and Quantitation (LOD and LOQ)

The minimum concentration levels at which nitrite and nitrate can be reliably detected and quantified using the UV method were determined using the signal-to-noise (S/N) criteria of 3:1 and 10:1, respectively. Calculation of S/N ratio was performed using Empower software by comparing the measured signal for nitrite and nitrate in the standard solution to the blank solution. Data from ten replicate injections of standard solutions was used to establish and assess performance of the method at the LOD and LOQ levels.

Representative chromatograms showing the analysis of nitrite and nitrate LOD and LOQ solutions are shown in Figure 2. The method performance at the LOD and LOQ levels is shown in Figure 3. The LOD and LOQ for nitrite

was found to be 0.01 and 0.025 µg/mL, respectively. The LOD and LOQ for nitrate was at 0.05 and 0.075 µg/mL, respectively. The data from ten replicate injections at the LOQ levels demonstrated excellent repeatability of the method, with RSD of peak areas at 4.30% for nitrite and 5.52% for nitrate (Figure 3).

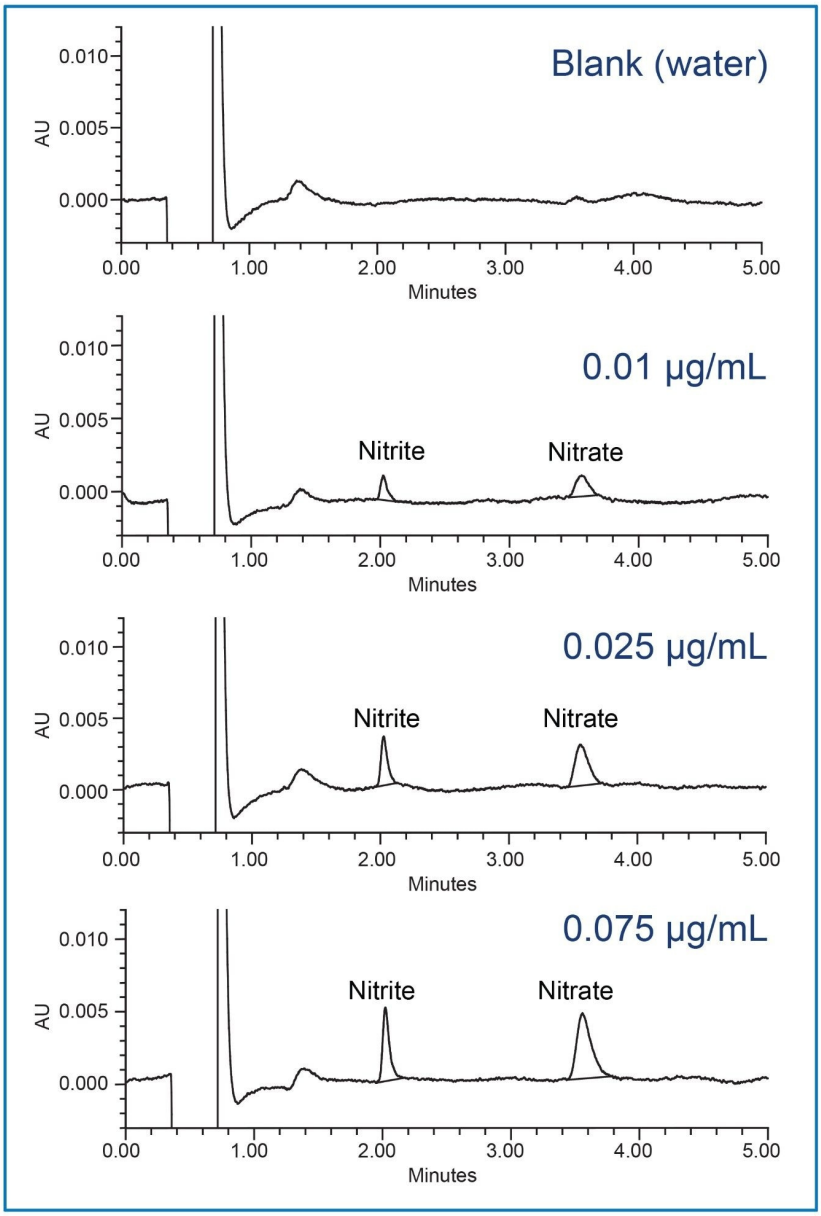


Figure 2. Representative chromatograms of nitrite and nitrate LOD and LOQ solutions, UV at 210 nm.

Nitrite (NO_2)

LOD: 0.01 µg/mL

	RT	Area	USP s/n
1	2.020	1970	4
2	2.022	2155	4
3	2.022	2375	4
4	2.023	2271	4
5	2.024	2328	4
6	2.025	2186	4
7	2.027	2416	4
8	2.032	2347	3
9	2.033	2054	3
10	2.038	2562	3
Mean	2.027	2266	4
Std. Dev.	0.006	177.578	
% RSD	0.28	7.84	

LOQ: 0.025 µg/mL

	RT	Area	USP s/n
1	2.020	5658	10
2	2.021	5534	10
3	2.022	5137	10
4	2.023	5481	10
5	2.023	5668	10
6	2.024	5541	10
7	2.025	5217	10
8	2.025	5236	10
9	2.026	5797	8
10	2.027	5187	8
Mean	2.024	5446	10
Std. Dev.	0.002	234.425	
% RSD	0.10	4.30	

Nitrate (NO_3)

LOD: 0.05 µg/mL

	RT	Area	USP s/n
1	3.552	23513	3
2	3.553	21191	3
3	3.555	22444	3
4	3.555	20874	3
5	3.556	27695	4
6	3.557	22186	3
7	3.561	26177	3
8	3.561	22398	3
9	3.562	26379	3
10	3.566	19151	3
Mean	3.558	23201	3
Std. Dev.	0.004	2735.710	
% RSD	0.12	11.79	

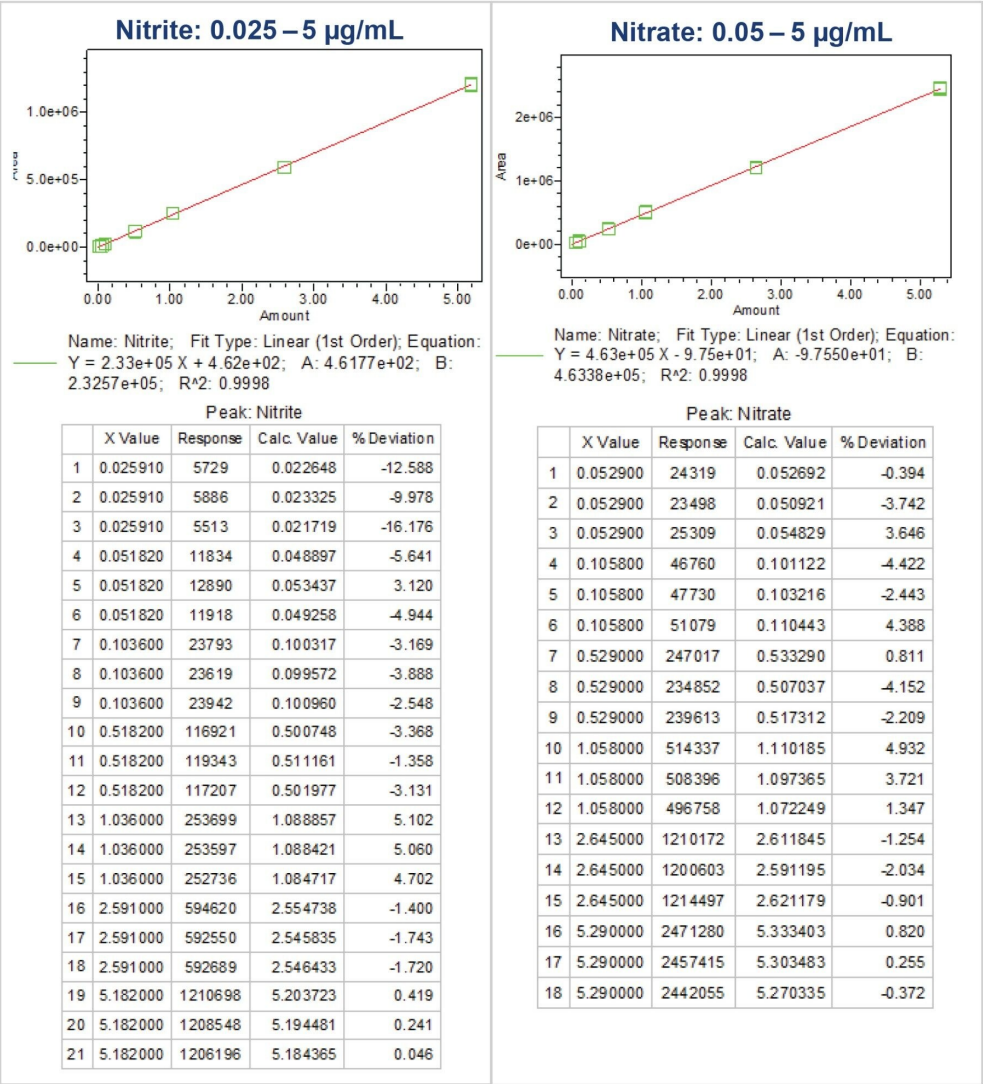
LOQ: 0.075 µg/mL

	RT	Area	USP s/n
1	3.550	34429	10
2	3.552	36517	10
3	3.553	33639	10
4	3.554	33332	10
5	3.555	34830	10
6	3.556	35810	10
7	3.558	38732	10
8	3.560	38149	10
9	3.560	33453	10
10	3.560	37001	10
Mean	3.556	35589	10
Std. Dev.	0.003	1964.001	
% RSD	0.10	5.52	

Figure 3. Method performance at the LOD and LOQ levels. UV at 210 nm.

Linearity and Concentration Range

The calibration plot for nitrite and nitrate was constructed in the range of 0.025 to 5 µg/mL and 0.05 to 5 µg/mL, respectively. The calibration curve of the peak areas versus concentration produced a linear relationship with a correlation coefficient (R^2) ≥0.999 (Figure 4).



Peak: Nitrite

	X Value	Response	Calc. Value	% Deviation
1	0.025910	5729	0.022648	-12.588
2	0.025910	5886	0.023325	-9.978
3	0.025910	5513	0.021719	-16.176
4	0.051820	11834	0.048897	-5.641
5	0.051820	12890	0.053437	3.120
6	0.051820	11918	0.049258	-4.944
7	0.103600	23793	0.100317	-3.169
8	0.103600	23619	0.099572	-3.888
9	0.103600	23942	0.100960	-2.548
10	0.518200	116921	0.500748	-3.368
11	0.518200	119343	0.511161	-1.358
12	0.518200	117207	0.501977	-3.131
13	1.036000	253699	1.088857	5.102
14	1.036000	253597	1.088421	5.060
15	1.036000	252736	1.084717	4.702
16	2.591000	594620	2.554738	-1.400
17	2.591000	592550	2.545835	-1.743
18	2.591000	592689	2.546433	-1.720
19	5.182000	1210698	5.203723	0.419
20	5.182000	1208548	5.194481	0.241
21	5.182000	1206196	5.184365	0.046

Peak: Nitrate

	X Value	Response	Calc. Value	% Deviation
1	0.052900	24319	0.052692	-0.394
2	0.052900	23498	0.050921	-3.742
3	0.052900	25309	0.054829	3.646
4	0.105800	46760	0.101122	-4.422
5	0.105800	47730	0.103216	-2.443
6	0.105800	51079	0.110443	4.388
7	0.529000	247017	0.533290	0.811
8	0.529000	234852	0.507037	-4.152
9	0.529000	239613	0.517312	-2.209
10	1.058000	514337	1.110185	4.932
11	1.058000	508396	1.097365	3.721
12	1.058000	496758	1.072249	1.347
13	2.645000	1210172	2.611845	-1.254
14	2.645000	1200603	2.591195	-2.034
15	2.645000	1214497	2.621179	-0.901
16	5.290000	2471280	5.333403	0.820
17	5.290000	2457415	5.303483	0.255
18	5.290000	2442055	5.270335	-0.372

Figure 4. Calibration curve for nitrite and nitrate. UV at 210 nm.

Polyvinylpyrrolidone Analysis

Polyvinylpyrrolidone, also known as povidone or PVP, is a synthetic polymer used in the pharmaceutical industry as a binder in tablet and capsules manufacturing, a film former for ophthalmic solutions, to aid in flavoring liquids and chewable tablets, and as an adhesive for transdermal systems.[7]

The PVP test samples, prepared at 1 mg/mL in water, were analyzed using the developed method.

Representative chromatograms of the PVP sample and the PVP sample spiked with nitrite and nitrate are shown in Figure 5. The method effectively separated the highly concentrated PVP peak from the nitrite and nitrate. In the PVP test sample, no nitrite was detected, but residual levels of nitrate were found, at a concentration of 0.04 µg/mL.

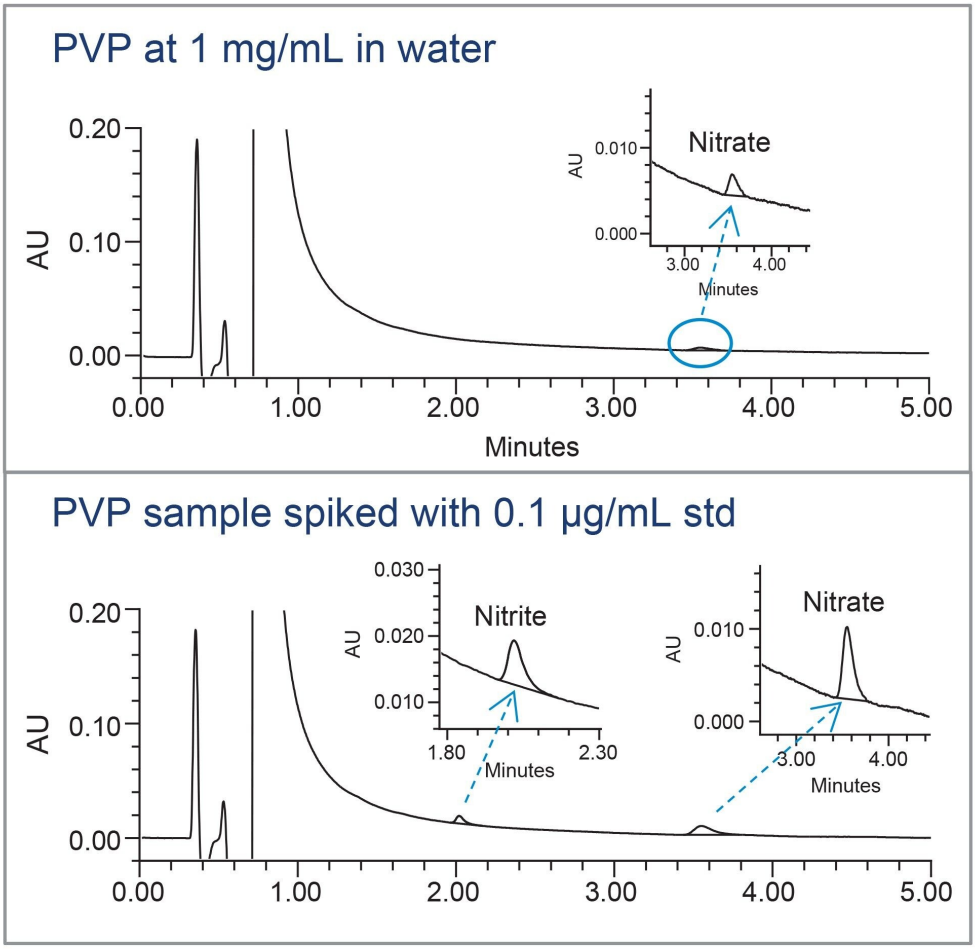


Figure 5. Analysis of polyvinylpyrrolidone (PVP) test sample solutions. UV at 210 nm.

Method Accuracy

The accuracy of the method was assessed by determining recovery of the nitrite and nitrate spiked into PVP samples at three levels including 0.05, 0.1, and 0.5 µg/mL. For nitrate recovery, calculations were corrected for

the amount already present in the sample. Recoveries for nitrite and nitrate (n=6 per each level) ranged from 93 to 100% and 91 to 100% (Figure 6), respectively, within the acceptance criteria range of 90–110%.

% Recovery at 0.05 µg/mL

	SampleName	Nitrite
1	PVP 1 mg +0.05ug	96.8
2	PVP 1 mg +0.05ug	95.6
3	PVP 1 mg +0.05ug	98.2
4	PVP 1 mg +0.05ug	95.3
5	PVP 1 mg +0.05ug	93.2
6	PVP 1 mg +0.05ug	96.4
Mean		96
Std. Dev.		1.685
% RSD		1.76

% Recovery at 0.1 µg/mL

	SampleName	Nitrite	Nitrate
1	PVP 1 mg +0.1ug	93.8	95.6
2	PVP 1 mg +0.1ug	95.6	90.6
3	PVP 1 mg +0.1ug	94.7	91.9
4	PVP 1 mg +0.1ug	98.3	96.2
5	PVP 1 mg +0.1ug	100.0	91.9
6	PVP 1 mg +0.1ug	99.7	91.9
Mean		97	93
Std. Dev.		2.666	2.326
% RSD		2.75	2.50

% Recovery at 0.5 µg/mL

	SampleName	Nitrite	Nitrate
1	PVP 1 mg +0.5ug	97.6	99.8
2	PVP 1 mg +0.5ug	98.2	95.2
3	PVP 1 mg +0.5ug	100.1	95.4
4	PVP 1 mg +0.5ug	98.7	96.0
5	PVP 1 mg +0.5ug	98.4	96.4
6	PVP 1 mg +0.5ug	100.3	98.1
Mean		99	97
Std. Dev.		1.076	1.793
% RSD		1.09	1.85

Figure 6. Method accuracy showing percent (%) recovery for nitrite and nitrate spiked to PVP test samples.

Conclusion

A single LC/UV with direct injection method was developed for the analysis of both nitrite and nitrate, utilizing the Arc Premier System and an Atlantis Premier BEH C_{18} AX Column. The method demonstrated excellent performance at the LOQ levels of 0.025 µg/mL and 0.075 µg/mL for nitrite and nitrate respectively. The method has been demonstrated as suitable for the analysis of polyvinylpyrrolidone excipient, with recoveries of nitrite and nitrate between 91 to 100%. The LC-UV method offered a quick and easy solution for screening nitrite and nitrate in the PVP K15 excipient or raw materials.

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720008083, October 2023