IJPPR
INTERNATIONAL JOURNAL OF PHARMACY & PHARMACEUTICAL RESEARCH
An official Publication of Human Journals
ISSN 2349-7203
HUMAN

Human Journals
Research Article
July 2018 Vol.:12, Issue:4

Determination of Hydroxylamine by HPLC, a Mutagenic Impurity in Febuxostat Drug Substance

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ISSN 2349-7203
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V.Saradhi Venkata Ramana[1], K. Durga Raja[1], K.Raghu Babu[2], M. Padma[2], Vundavilli Jagadeesh Kumar[1*], K. S. R. Pavan Kumar[1], Hemant Kumar Sharma[1]

[1] Analytical Research Department, Aurobindo Pharma Limited Research Centre-II, Indrakaran (V), Kandi (M), Sangareddy Dist-502329, Telangana, India.

[2] Department of Engineering Chemistry, Andhra University, Vishakapatnam-530003, Andhra Pradesh, India.

Submission: 25 June 2018
Accepted: 1 July 2018
Published: 30 July 2018

HUMAN JOURNALS
www.ijppr.humanjournals.com

Keywords: Febuxostat, Hydroxylamine, mutagenic impurity, Validation, HPLC.

ABSTRACT

A sensitive, derivatized procedure by HPLC technique was developed and validated for the determination of hydroxylamine, a mutagenic impurity at the low level in Febuxostat drug substance according to ICH guidelines. The HPLC method was developed and optimized on Sunfire C18, 250 mm × 4.6 mm, 5µm column with oven temperature maintaining at 40°C. Phosphate buffer (0.01M) pH 2.5 was selected as mobile phase A and acetonitrile were selected as mobile phase B in gradient reverse phase mode. Chromatographic parameters i.e flow rate: 1.5 ml/min, wavelength detection: 254 nm, injection volume: 20µl and run time: 40 min. Based on validation data, the method is found to be specific, sensitive, accurate and precise. The established limits of Limit of detection and Limit of quantification for this impurity are found to be 1.7µg/g and 5.0µg/g respectively. The average recovery obtained was 101.6% at four levels in twelve determinations for hydroxylamine in Febuxostat drug substance. This method can be used as the good quality control tool for quantization of hydroxylamine at the low level. The experimental results are discussed in detail in this research paper.

INTRODUCTION

Febuxostat is chemically known as 2-[3-cyano-4-(2-methylpropoxy)phenyl]-4-methyl thiazole-5-carboxylic acid (or) 2-(3-cyano-4-isobutoxyphenyl)-4-methylthiazole-5-carboxylic acid, its molecular formula is $C_{16}H_{16}N_2O_3S$ and molecular weight is 316.37. Febuxostat, a novel non-purine selective inhibitor of *xanthine oxidase*, is a potential alternative to allopurinol for patients with hyperuricemia and gout [1]. Febuxostat displayed potent mixed-type inhibition of the activity of purified bovine milk XO, with Ki and Ki′ values of 0.6 and 3.1 nM respectively, indicating inhibition of both the oxidized and reduced forms of XO indicating that Febuxostat is potent non-purine [2].

Febuxostat has been granted marketing authorization by the European Commission in early 2008 for the treatment of chronic hyperuricemia and gout. Febuxostat is the first major treatment alternative for gout in more than 40 years and is a promising alternative to allopurinol, although continued long-term surveillance on safety and efficacy is required [3]. Febuxostat is marketed under the trade name ULORIC [4], For treatment of hyperuricemia in patients with gout, ULORIC is recommended at 40 mg or 80 mg once daily. The recommended starting dose of ULORIC is 40 mg once daily. For patients who do not achieve a serum uric acid (sUA) less than 6 mg/dL after two weeks with 40 mg, ULORIC 80 mg is recommended [4] and duration of treatment is >10 years to the lifetime as the usage is for chronic management of hyperuricemia in patients with gout. Chemical structure of Febuxostat is shown in Fig.1.

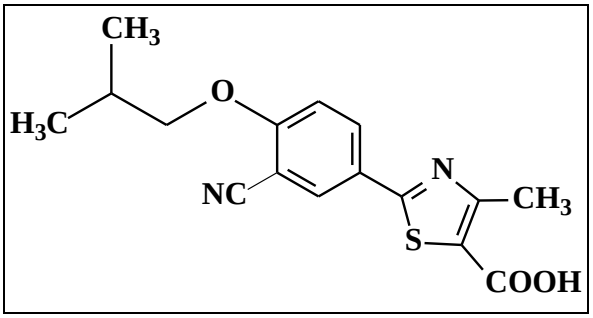


Figure 1: Chemical structure of Febuxostat

Hydroxylamine and its salts are commonly used as reducing agents in myriad organic and inorganic reactions. They can also act as antioxidants for fatty acids; it is used to prepare oximes, an important functional group. It is also an intermediate in biological nitrification. In the synthesis of Febuxostat, hydroxylamine hydrochloride reagent is used in the Febuxostat ethyl ester intermediate preparation from formyl febuxostat ethyl ester. Hydroxylamine hydrochloride was reported to be mutagenic in the mouse lymphoma *tk* mutation assay, with and without metabolic activation [5], but the data do not convincingly meet the up-to-date criteria for positive results in this assay [6]. It is considered that hydroxylamine induces tumors via a mode of action with a threshold (i.e., hemosiderosis of the spleen). An increase in tumors was observed in male rats at ≥ 5 ppm or 0.2 mg/kg/day for hemangiosarcomas and females at the high dose of 80 ppm or 6.2 mg/kg/day (hemangiosarcomas and hemangiomas). [7] By referring available literature, ICH M7(R1) –step-2 [8], concluded that the lowest observed adverse effect level (LOAEL) in the 2-year rat study was 0.2 mg/kg/day in males and according to this NOEL value lifetime PDE of hydroxylamine is 2µg/day. By considering this PDE information and the 80mg maximum daily dose of Febuxostat, the allowed TTC limit of hydroxylamine in this drug substance is 25µg. Either some of the analytical methods for the determination of related substances, assay in Febuxostat drug substance or its pharmaceutical products have been reported in the literature by using spectroscopic, RP-HPLC and LCMS techniques [9-13]. However, in accordance with the literature, there is no method available in the literature for determination of hydroxylamine in Febuxostat. The goal of this research study is to develop a sensitive, selective, accurate, reproducible and simple method to analyze Hydroxylamine in Febuxostat drug substance. Several analytical techniques have been reported for the determination of hydroxylamine. These include spectrophotometry [14-16], indirect potentiometry [17], polarography [18], HPLC [19], and gas chromatography [20]. But in this research paper, we have done hydroxylamine quantification with benzaldehyde derivatization procedure by HPLC. The derivatization mechanism is shown in Figure 2.

To the best of our knowledge, this procedure has not been reported in the literature to date. This paper describes the development, optimization of HPLC method for the determination of hydroxylamine and method validated accordance with ICH guidelines [21].

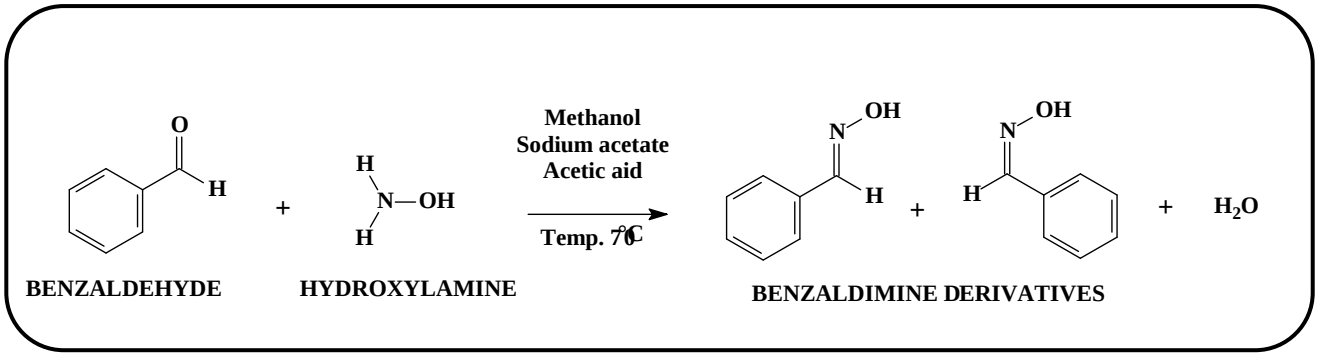


Fig 2: Reaction mechanism of Derivatization procedure

EXPERIMENTAL

Chemicals, reagents, and samples

Febuxostat drug substance and its related substances were procured from APL Research Centre-II (A division of Aurobindo Pharma Ltd., Hyderabad). Hydroxylamine hydrochloride (reagent grade) was procured from Sigma Aldrich, Potassium dihydrogen orthophosphate (Analytical grade), Orthophosphoric acid (GR grade), Acetic acid (AR grade), Benzaldehyde (AR grade), Sodium acetate (AR grade), Acetonitrile (HPLC grade) and Methanol (HPLC grade) were procured from Merck and highly pure milli-Q water was obtained by using a Millipore purification system.

Instrumentation and Chromatographic conditions

Chromatographic separations were performed on HPLC (High-Performance Liquid Chromatograph) system with Alliance waters 2695 separation module with 2996 PDA detector using Empower software. The mobile phase A was a prepared by dissolving 1.36 g of Potassium dihydrogen orthophosphate in 1000 ml of water. Adjust pH 2.5±0.05 with orthophosphoric acid. The mobile phase B was Acetonitrile. A degassed mixture of methanol and water in the ratio of 80:20 v/v was used as diluent. The analysis was carried out on a stainless steel column 250 mm long, 4.6 mm internal diameter filled with Octadecyl silane chemically bonded to porous silica particles of 5 µm diameter (Sunfire C18, 250mm × 4.6mm, 5µm particle diameter column, Make: Waters), maintained at temperature 40°C. The mobile phase was running through the column at a flow rate of 1.5 ml/min and a pump was in gradient mode. The gradient program was as follows: Time (min)/ A (v/v): B(v/v); $T_{0.01}$/75:25, T_{15}/75:25, T_{17}/20:80,T_{30}/20:80,T_{32}/75:25, T_{40}/75:25. The runtime was 30 min.

The injection volume was 20 µl and the analyte was monitored at 254 nm. The retention time of hydroxylamine as benzaldimine derivative peak-1 is about 9.5 min and that of benzaldimine derivative peak-2 is about 10 min.

Preparation of solutions

During the derivatization procedure, it is recommended that while heating the volumetric flask to be kept inside the water bath and ensure the reaction solution should completely dip in the water.

Standard solution (0.00016 *mg/ml*)

Accurately weigh and transfer about 80 mg of Hydroxylamine hydrochloride reference standard into a 50 ml clean, dry volumetric flask, add 25 ml of water and sonicate to dissolve. Makeup to volume with methanol. Dilute 5 ml of this solution to 100 ml with diluent. Further, dilute 5 ml of this solution to 100 ml with diluent.

Transfer 4 ml of this solution to 100 ml clean, dry volumetric flask, add 20 ml of methanol, 300 mg of sodium acetate and 40 ml of diluent and sonicate to dissolve. Add 5 ml of acetic acid and 80 mg (~80 µl) of benzaldehyde, shake well and heat the solution at 70°C for 30 min by using a water bath. Allow the volumetric flask to cool to room temperature and makeup to volume with diluent.

Blank solution

Take 10 ml of methanol into a 50 ml clean, dry volumetric flask, add 150 mg of sodium acetate, 20 ml of diluent and sonicate to dissolve. Add 2.5 ml of acetic acid and about 40 mg (~ 40 µl) of benzaldehyde shake well and heat the solution at 70°C for 30 min by using a water bath. Allow the volumetric flask to cool to room temperature and make up to volume with diluent.

Sample solution (3 *mg/ml*)

Accurately weigh and transfer about 150 mg of sample into a 50 ml clean, dry volumetric flask, add 10 ml of methanol and sonicate to dissolve. Add 150 mg of sodium acetate and 20 ml of diluent and sonicate to dissolve. Add 2.5 ml of acetic acid and 40 mg (~ 40 µl) of

benzaldehyde, shake well and heat the solution at 70°C for 30 min by using water bath Allow the volumetric flask to cool to room temperature and make up to volume with diluent.

System suitability criteria

The column efficiency as determined from the benzaldimine derivative peak-2 is not less than 5000 USP plate count and USP tailing for the same peak is not more than 2.0. RSD for the average area of the sum of benzaldimine derivative peak-1 and benzaldimine derivative peak-2 areas of six injections of the standard solution is not more than 5.0%.

RESULTS AND DISCUSSION

Method validation

By using instrumentation and chromatographic conditions as mentioned in above sections, the developed and optimized method was then validated for its specificity, linearity, LOD and LOQ, accuracy, the stability of solutions and precision to demonstrate that the method is suitable for its intended use per regular sample analysis.

Specificity

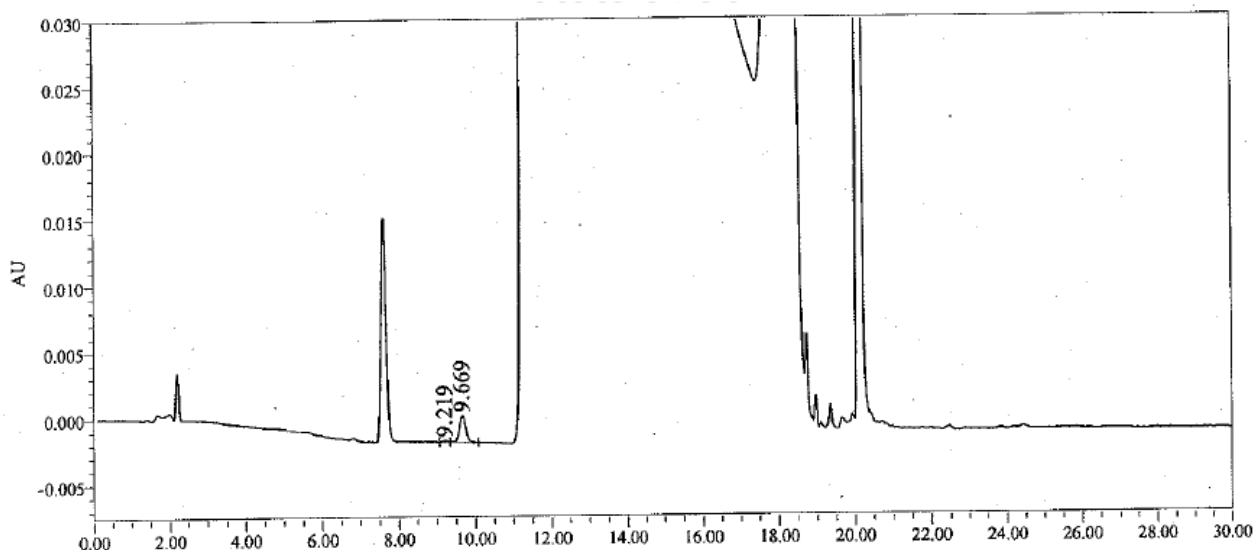
Specificity of the method is its ability to detect and separate all relates substances present in the Febuxostat drug substance. Specificity of the method is demonstrated in terms of spectral as well as peak purity data of the drug and its impurities are present in the drug and achieved the peak purity test. The diluent, Febuxostat drug substance spiked with Hydroxylamine (*control sample*) and Febuxostat drug substance spiked with all known related substances including Hydroxylamine (*spiked sample*) were injected to confirm any co-elution with a benzaldimine derivative peak from any known related substances. Peak purity for Benzaldimine derivative peak-1 & 2 was established by using waters Empower software and found to be passed (Purity angle should be less than purity threshold). No peak is observed at the retention time of Benzaldimine derivative peak-1 & 2 in the diluent chromatogram. Further, the peak purity data of Benzaldimine derivative peak-1 & 2 from the control sample and spiked sample indicated that the peaks were homogeneous and have no co-eluting peaks. Based on the above observations, it can be concluded that there is no interference due to listed known related substances for the determination of Hydroxylamine content in Febuxostat drug substance.

Hence, this method is specific and selective. Typical HPLC chromatograms of Febuxostat spiked with Hydroxylamine and Febuxostat spiked with all known related substances including Hydroxylamine are shown in Figure 3. The specificity experiments data is given in Table 1. Hence, it can be concluded that there is no interference due to listed known related substances for the determination of Hydroxylamine content in Febuxostat drug substance.

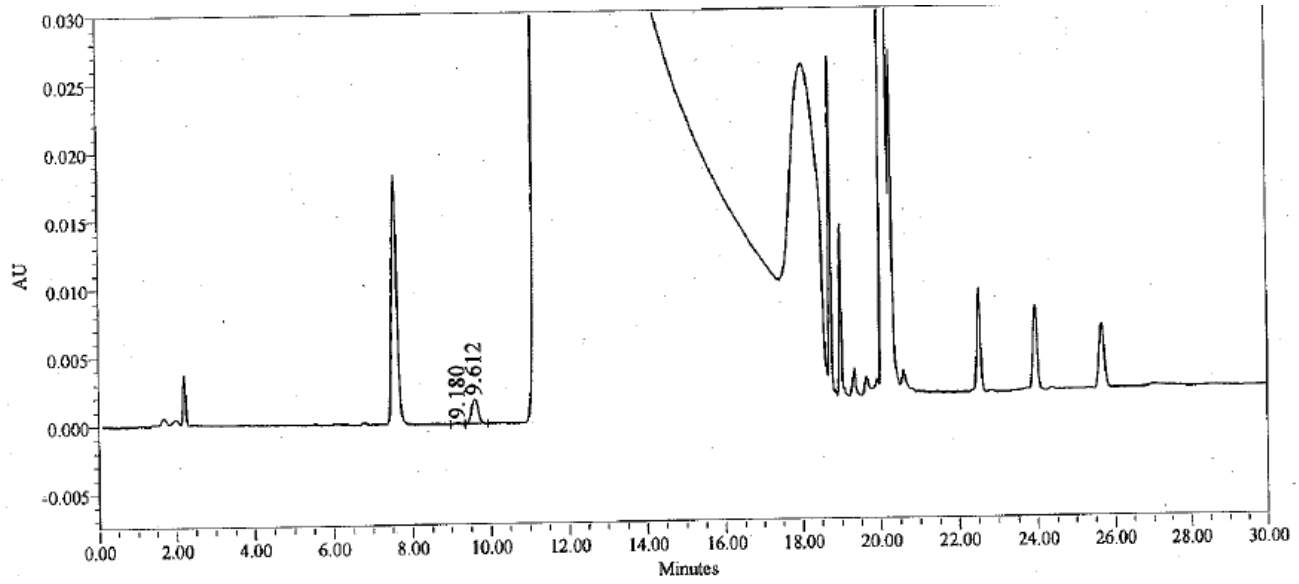
Table 1: Specificity data

Name		Retention time (min)	Peak purity	
			Purity angle	Purity threshold
Control sample	Benzaldimine derivative peak-1	9.219	11.480	64.965
	Benzaldimine derivative peak-2	9.669	0.918	3.489
Spiked sample	Benzaldimine derivative peak-1	9.180	14.334	32.616
	Benzaldimine derivative peak-2	9.612	0.869	1.923

Table 1: Specificity data



A control sample (spiked with Hydroxylamine)



Spiked sample (spiked with Hydroxylamine and known related substances)

Fig 3: Typical HPLC chromatograms of specificity experiment

LOD and LOQ

The sensitivity for detection can be demonstrated by determining the limit of detection (LOD) and limit of quantitation (LOQ). LOD/LOQ values of hydroxylamine were determined from based on the response of analyte. The predicted concentrations of LOD and LOQ for hydroxylamine were verified for precision by preparing the solutions containing Benzaldimine (after derivitization) at about predicted concentrations. Each of these solutions six times was injected into HPLC.

Linearity

The linearity of the method was checked by preparing solutions at seven concentration levels from LOQ to 150% of specification level by prepared using hydroxylamine standard solution and each solution was injected into HPLC. Linearity was established by using concentration (µg/ml) on the X-axis, area on Y-axis and calculated statistical values like slope, intercept, the residual sum of squares and the correlation coefficient. The linearity, LOD and LOQ experiments data is shown in Table 2.

Table 2: LOD/LOQ and Linearity experiments data

Concentration (µg/mL)	Area *	Statistical analysis	
0.015	5248	Slope	315817
0.019	6153	Intercept	284
0.038	12279		
0.057	18142	STEYX	194
0.076	24122		
0.095	30551	Correlation coefficient	0.9998
0.114	36243		
LOD & LOQ			
LOD	1.7 µg/g	5.6 (% RSD)	
LOQ	5.0 µg/g	2.6 (% RSD)	

**Sum area of Benzaldimine derivative peak-1 and 2*

Accuracy

The accuracy of the method was performed by recovery experiments using standard addition technique. Sample solutions were prepared in triplicate by spiking hydroxylamine at levels of LOQ, 50%, 100% and 150% of specification limit as per test method and injected each solution into HPLC as per methodology and the percentage recoveries were calculated. The fully validated recovery results are shown in Table 3.

Table 3: Accuracy data

LOQ level							
% Level / Sample ID		**Amount Added (µg/g)**		**Amount Found (µg/g)**		**% Recovery**	
LOQ Level Sample - 1		5.07		4.91		96.8	
LOQ Level Sample - 2		5.07		4.72		93.1	
LOQ Level Sample - 3		5.09		4.85		95.3	
Statistical Analysis							
Mean	95.1	SD	1.86	% RSD	2.0	95% Confidence Interval (±)	4.6
(50% to 150% level)							
Concentration / Sample ID		**Amount Added (µg/g)**	**Amount Found (µg/g)**	**% Recovery**	**Statistical Analysis**		
50% Level Sample 1		12.6	13.5	107.1	Mean	101.0	
50% Level Sample 2		12.6	12.5	99.2	SD	5.39	
50% Level Sample 3		12.6	12.2	96.8	% RSD	5.3	
100% Level Sample 1		25.3	25.7	101.6	Mean	103.2	
100% Level Sample 2		25.3	26.5	104.7	SD	1.55	
100% Level Sample 3		25.3	26.1	103.2	% RSD	1.5	
150% Level Sample 1		37.8	39.8	105.3	Mean	107.1	
150% Level Sample 2		38.0	41.2	108.4	SD	1.61	
150% Level Sample 3		38.0	40.9	107.6	% RSD	1.5	
Overall Statistical Analysis							
Mean	103.8	SD	3.95	% RSD	3.8	95% Confidence Interval (±)	3.0

Precision

System precision was demonstrated by preparing the standard solution of hydroxylamine as per methodology and analyzed by injecting six replicates. Method precision experiment was demonstrated by preparing six sample solutions individually using a single batch of

Febuxostat drug substance spiked with hydroxylamine at specification level and determined the hydroxylamine content by HPLC. Similarly, intermediate precision was demonstrated by preparing six sample solutions individually using a single batch of Febuxostat drug substance (same sample used for method precision experiment) spiked with hydroxylamine at specification level and determined the hydroxylamine content by different HPLC, another analyst, another lot column on a different day. Achieved results like % RSD and a 95% confidence interval for six determinations are summarized in Table 4.

Table 4: Precision experiments data

System Precision	**Injection ID**	**Sum area of Benzaldimine derivative -1 and 2**	**Statistical Analysis**	
	1	23700		
	2	23597		
	3	23524	Mean	23677
	4	23899	SD	126
	5	23680	% RSD	0.5
	6	23664	95% Confidence Interval (±)	132

Method Precision	**Sample**	**Hydroxylamine (µg/g)**	**Statistical Analysis**	
	1	24		
	2	25		
	3	24	Mean	24
	4	24	SD	0.5
	5	24	% RSD	2.1
	6	25	95% Confidence Interval (±)	0.5

Intermediate Precision	**Sample**	**Hydroxylamine (µg/g)**	**Statistical Analysis**		
	1	25			
	2	25		For ruggedness	overall
	3	25	Mean	25	25
	4	25	SD	0.0	0.5
	5	25	% RSD	0.0	2.0
	6	25	95% Confidence Interval (±)	0.0	0.3

Solution stability

To evaluate stability of the standard and sample solutions, standard solution and sample solution spiked with hydroxylamine at specification level were prepared as per methodology and analyzed initially and at different time intervals by keeping the solution at room temperature (25°±2°C) The % difference in the peak area obtained at initial and after 15 hours time interval was found to be less than 1.7 for standard solution and 0.6 for sample solution at room temperature (25°±2°C). These stability results concluded that the standard and sample solutions are stable for at least 15 hours at 25°±2°C. Generated data has been presented in Table 5.

Table 5: Solution stability experiments data

		Sum area of Benzaldimine derivative -1 and 2	**% Difference**
Standard (at 25°±2°C)	Initial	24761	1.7
	After 15 hours	25173	
Sample (at 25°±2°C)	Initial	23159	0.6
	After 15 hours	23304	

CONCLUSION

The HPLC method was developed, optimized and validated for the determination of hydroxylamine content in Febuxostat drug substance and the results of various validation parameters proved that the method is specific, sensitive, precise and accurate and the method can be introduced into routine testing.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the management of APL Research Centre-II (A Division of Aurobindo Pharma Ltd.), for allowing us to carry out the present work. The authors are also thankful to the colleagues of the Analytical Research Department and Chemical Research Department for their co-operation.

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