Development And Validation Of N-Nitroso Atomoxetine Impurity (Ndsri) Determination Method In Atomoxetine Hydrochloride With Uflc-Ms/Ms (Esi).

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KEYWORDS
NDSRI; Nitrosamine; LC-MS; ESI; Analytical methods & Validation; Atomoxetine Hydrochloride; Impurities

ABSTRACT
Now a day's nitrosamine impurities major concern for the pharmaceutical industries due to the cancer-causing potential, they can impact on health risk, recall the products and withdrawals, process modification, financial and operational impact. Multiple regulatory authority requirements for Pharmaceuticals for Human Use has established guidelines for nitrosamine impurities, which classify them as a cohort of concern (CoC) and present potential genotoxic risks. Therefore, controlling these impurities at lower levels during the quantification process in active pharmaceutical ingredients (API) and products is crucial to ensure safe human consumption. This research paper presented a simple, fast, and trace-level quantification by LC-ESI-MS-MS analytical method for N-Nitroso atomoxetine (NDSRI) in atomoxetine hydrochloride with a LOQ of 0.02 ppm and LOD 0.006 ppm. Chromatographic separation is achieved using HPLC column Kromasil 100-5C8 (250 mm x 4.6 mm x 5µ) and mobile phase A 1mL formic acid in water and mobile phase B acetonitrile. Gradient mode with 0.8 ml/min using a total run time of 23 min. Multiple reaction monitoring (MRM) with Electron Spray Ionization (ESI) is used to identification and quantify NDSRI in a positive mode and validate regulatory guidelines. MS diverter valve to avoid MS contamination from API. Validated method is specific, linear in the range of 0.02 ppm – 0.27 ppm with regression coefficient >0.99, Accurate & Precise. A triplicate sample preparation is used to establish method recoveries, which are found to be satisfactory within the range of 70% to 130%. The N-Nitroso atomoxetine impurity in atomoxetine hydrochloride can be regularly detected using this method

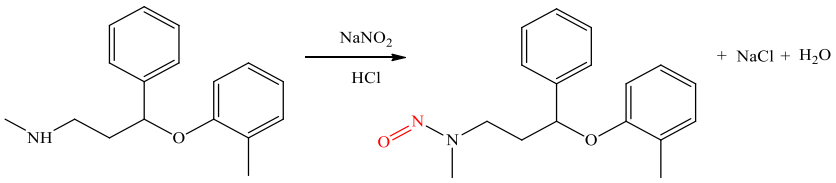
INTRODUCTION

Atomoxetine hydrochloride drug is prescribed for adults and children above the age of six for the symptomatic treatment of attention-deficit hyperactivity disorder (ADHD); this is the first non-stimulant substitute prescription[1-2]. The chemical name, molecular formula, and molecular weight for atomoxetine hydrochloride and N-nitroso atomoxetine hydrochloride are represented in figure 1. Since mid-2018, the identification and detection and control strategy of genotoxic nitrosamine impurities has become mandatory quality and safety considerations for various drugs[3-9]. Regulatory requirements demand the accurate determination and quantification at lower level of nitrosamine impurities in drugs, with the challenge of establishing sensitive analytical methods due to the strict low detection and specification limits[3-9]. The primary objective behind limiting these impurities' safe daily dose consumption and ensuring their absence in the molecular structure is to safeguard human health and prevent potential risks of exposure to carcinogenic substances. Therefore, rigorous efforts are made to develop sensitive and reliable analytical techniques to measure and control nitrosamine impurities in pharmaceutical products accurately. By adhering to these stringent regulations and

employing advanced analytical approaches, the pharmaceutical industry aims to prioritize the safety and well-being of consumers. N-nitrosamine and nitrosamine drug substance-related impurities (NDSRIs) are a class of complex chemicals having a chemical structure with a nitroso group attached to an amine (secondary, tertiary), and they are unwanted impurities that may be generated during the synthesis of drug substances, drug products, or degradation and can also increase or be generated during stability, excipient or contaminant interactions, or contaminations from the manufacturing process[4,17-21]. Amines (secondary or tertiary) containing APIs or API fragments (process impurities, degradation impurities) that are treated with nitrosating substances or reagents (sodium nitrite, nitrous acid), in residual levels of nitrites in excipients, are enough to produce the nitrosamine impurities[17]. The acceptable intake (AI) limit of NDSRIs can be determined based on study data (typically from animal studies), Carcinogenicity and mutagenicity are widely not available[4,8]. The ICH M7 clearly stated that the trace level quantification required to complete elimination the cancer-causing effect in human consumption, due to the category of Cohort of Concern and potential genotoxic impurities[3]. The pharmaceutical industry has seen a surge in drug recalls across different therapeutic categories by regulatory bodies U.S. Food and Drug Administration (USFDA), European Medicines Agency (EMA), Health Canada, and Therapeutic Goods Administration (TGA) and many more due to the presence of various nitrosamine impurities above acceptable limits[5-11]. This highlights the crucial need to eliminate these impurities to ensure safe consumption by humans. The USFDA and other regulatory bodies have published guidelines for the management of nitrosamine impurities in drug substances and drug products[4-11]. Nitrosamine impurities are eliminated or controlled during the manufacturing process, starting ingredients or materials (KSM or ORM), solvents used, and the stability of the drug substance and final product, nitrosamine impurities are examined. Several drug products and drug substances were recalled by regulatory authorities as well as by manufacturers due to the higher level of N-nitroso impurity[12-16]. Several analytical techniques have been developed by regulatory agencies and research to detect trace-level nitrosamines and NDSRIs in various pharmaceutical ingredients and medications, Due to the lower level of detection, analytical methods make more challenges, similar to the API structure of NDSRIs chromatographic separation, which are more challenging [22-30].

Formation of N-Nitroso Atomoxetine

N-Nitroso Atomoxetine is generated during the manufacturing process of Atomoxetine hydrochloride. As mentioned in USP general chapter 1469, potential sources of nitrosating agents from Solvents, H_2O, Excipients, Drug substances, Manufacturing processes, Drug products (storage & stability), and packing materials. In the Manufacturing process of Atomoxetine Hydrochloride, no nitrosating reagent was used, and the only possibility to generate N-Nitroso Atomoxetine in the residual level presence of sodium nitrite ($NaNO_2$) (source from water) in acidic environment[17-20].



Atomoxetine N-Nitroso Atomoxetine

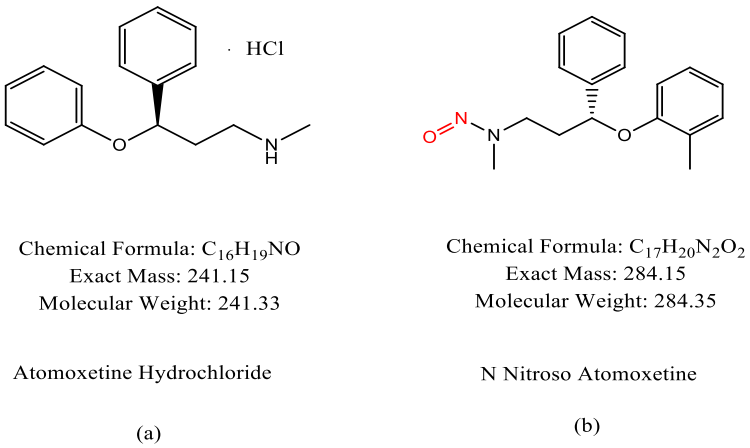
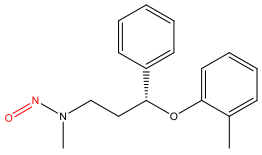


Figure 1. (a) Atomoxetine Hydrochloride; (b) N-Nitroso Atomoxetine

QSAR Prediction and Limit establishment based on Acceptable intake (AI):

In chemistry and biology, a computational method called QSAR methodology is used to predict a chemical compound's properties or activity based only on its molecular structure within a very short time. As per ICH M7, classification QSAR predictions play a significant role in hazard characterization. Nexus 2.5.1 used QSAR prediction for the atomoxetine, and N-Nitroso atomoxetine structures, Following ICH M7 guidelines, the classification was done using the ICH classification model[21-22]. The QSAR results are assumed in table 1. The QSAR study results of impurity, Derek is plausible, Sarah is a CoC and positive (18%). The "CoC" group is more potent than most carcinogenic compounds, so according to ICH M7 guidelines, it cannot be quantified by the TTC limit. Atomoxetine hydrochloride's maximum daily dose (MDD) was 100 mg/day. Based on published EDQM guideline (15/02/2024, EMA/72902/2024 /Rev. 3, Non-clinical Working Party (NcWP)), The CPCA category of N-Nitroso atomoxetine is 100 ng/day. The limit in ppm can be derived from the following calculation formula and the **Limit (ppm) = AI/MDD (MDD is the maximum daily dose of an API (mg)**. Therefore, the limit of the N Nitroso atomoxetine is 1 ppm (100 ng/ day) / (100 mg/day)). Still, the considering worst com worst scenario for nitrosamine impurities AI is 18 ng/day (18ng/day - 1500 ng/day) and based on this considering 0.18 ppm limits for N Nitroso atomoxetine for the detection.

Table 1. QSAR results.

Structure	ICH M7 Class	Cohort of Concern	Derek Prediction	Sarah Prediction	Experimental Data	Similarity to API	Overall In Silico
	Class 3	Yes (N-Nitroso compound)			Carc: Unspecified Ames: Unspecified	Alert(s) not found in API	Positive

EXPERIMENTAL

Material and Reagents:

Development and validation activity performed by using LCMS-grade solvents and reagents which are of the highest purity >99.8%. Acetonitrile and Methanol was purchased from J. T. Baker (LLC, USA), Formic acid was procured from Fischer Chemicals (Czech Republic), and water used from Milli-Q. N-Nitroso Atomoxetine impurity (Potency: 99.92%) was procured from SynZeal

Research Private Limited, Ahmedabad, India, and Atomoxetine hydrochloride used inhouse, Cohance Lifesciences Limited, API R&D Unit-II, Ankleshwar, India.

Mobile Phase and diluent Preparation

Mobile phase A was done by mixing 1mL of formic acid in 1000mL water and Acetonitrile (LCMS grade) was used as mobile phase B. Degassed the mobile phase and kept it at room temperature for use. Acetonitrile was used as a diluent.

Preparation of Standard Solutions

First stock of impurity done by 100 ppm in methanol and final standard solution having concentration 0.01 ppm containing 10 % water in acetonitrile.

Sample Preparation:

Weigh and transfer 556 mg of sample into centrifuge tube, add 5 mL of diluent, shake well and centrifuge at 1000rpm for 5 minutes. Filter this solution with 0.22µ PVDF syringe filter into 10 mL volumetric flask, add 1 mL of water and make up volume with diluent and mix well.

Operating Conditions of LC-MS/MS

UFLC (Shimadzu, Japan) equipped with a binary pump (LC-20AD), Autosampler (SIL-20ACHT), photodiode array detector (SPD-M20A), and Valve unit (FCV-20AH2) and coupled with an LCMS-8040 (Shimadzu, Japan) LCMS-MS triple quadrupole with ESI (Electrospray ionization) interface. Kromasil (AkzoNobel) 100-5 C8 (250 mm x 4.6 mm x 5µ) column was used to separate the N-Nitroso Atomoxetine impurity and Atomoxetine. A gradient mode with 0.8 mL/min is used for the chromatographic separation to elution and separation of analytes peak and API with 1 mL formic acid in 1000mL water Mobile phase A and Acetonitrile as mobile phase B and a run time of 23 min. The column oven and auto sampler temperature were set at 40 ˚C and 15 ˚C respectively, and an injection volume of 90 µl was used. The gradient program used is as follows (time in min/%B): 0.00/60, 05.00/60, 10.00/85, 15.00/85, 16.00/60, 23.00/60. A MS of N-Nitroso Atomoxetine impurity in the form of protonated molecular ions $(M + H)^+$ at *m/z* 285.15 in ESI source (positive polarity). Three transitions for MRM used for the quantification and identification of impurity, 285.15 > 177.05 (Quantifier), 285.15 > 117.10 (reference) and 285.15 > 73.00 (reference). The ionization source was operated, desolvation line (DL) temperature 250˚C, Heat block temperature 300°C, Drying gas (N_2) flow 15 L/min, Nebulizer gas (N_2) flow 3.0 L/min. To control LC and MS parameters LabSolution software version 6.72 was used.

Method Validation

The analytical method validation was carried out successfully using optimized conditions according to the guidelines recommended by ICH in terms of Specificity, lower detection (LOD), lower quantification (LOQ), Linearity and range (LOQ to 150%), Precision (at LOQ and specification level), Accuracy (three different concentrations, LOQ to 150%), and solution stability. All the essential parameters were determined to demonstrate the effectiveness of the method. To establish the specificity and system precision of the method six injections of standard solution 0.001 ppm (0.18 ppm wrt to Atomoxetine Hydrochloride 55600 ppm), sample solution (55600 ppm), and spiked sample (0.18 ppm wrt to Atomoxetine Hydrochloride 55600 ppm) preparation injected. Next, LOD and LOQ and linearity and Range of the method were evaluated from 0.001 ppm to 0.015 ppm (0.02 ppm to 0.27 ppm wrt to Atomoxetine Hydrochloride 55600 ppm) using six different concentration levels. Solution stability is established by injecting sample solutions and spiked sample solutions at different time intervals.

RESULTS AND DISCUSSION

Method Development

The goal of this research is to represent and develop a sensitive, accurate, precise and specific analytical technique that could quantify nitrosamine impurities in atomoxetine hydrochloride API at the trace level. Several trials, in terms of mobile phase pH conditions and gradient composition, were evaluated to achieve good peak shapes and critical separation between Atomoxetine Hydrochloride and nitrosamine impurity. Finally, 1 mL formic acid in 1000 mL water as mobile phase A and Acetonitrile as Mobile phase B suitable. For the column selection, we tried Inertsil ODS-3V (250 x 4.6) mm, 5µ but in this column, The separation between N Nitroso-Atomoxetine and Atomoxetine Hydrochloride was found satisfactory but the peak shape of N Nitroso-Atomoxetine not good. Different column category involves to achieving the good peak shape and separation and in Kromasil 100-5 C8 (250 × 4.6) mm, 5µ found the suitable. The major concern of this study was the recovery of N nitroso impurity, so for recovery and accuracy different diluent approaches applied and finally, acetonitrile and water (9:1) were suitable. The retention times of N-Nitroso Atomoxetine Impurity were observed to be 11.9 and Atomoxetine Hydrochloride eluted at 3.3 min. The injection volume was optimized to 90µL. To avoid the contamination of the mass detector, the sample diverter valve was used for the first 0.00 minutes, and after 9 minutes the solution was diverted to waste, because of the huge amount concentration of sample solution contaminated the Mass detector and its effect on the quantification of impurity. Three MRM transitions were selected for N-Nitroso atomoxetine. The most intense transition was used for quantification, while the two others were employed for identification in Table 1.

Optimization of MSMS Parameters

To determine the N-nitroso atomoxetine impurity in atomoxetine hydrochloride API, mass spectrometric conditions optimization was used. The goal was to create a straightforward, robust, selective, and highly sensitive method. For the MSMS method development 0.01ppm impurity standard solution was used in the scanning range of mass range 100 to 1000 *m/z*, impurity ionization in positive mode with *M+1H* in figure 2. Compound specific parameters such as capillary Precursor m/z, Product m/z, Dwell time, Q1 pre-bias, collision energies (CE), and Q3 Pre-Bias were fine-tuned to achieve the desired response for impurity, as outlined in Table 2. Additionally, CE were adjusted by testing various voltages in collagen cell to establish reliable and sensitive MRM for the N-Nitroso Atomoxetine impurity.

Figure 2: Mass spectrum of N-Nitroso Atomoxetine

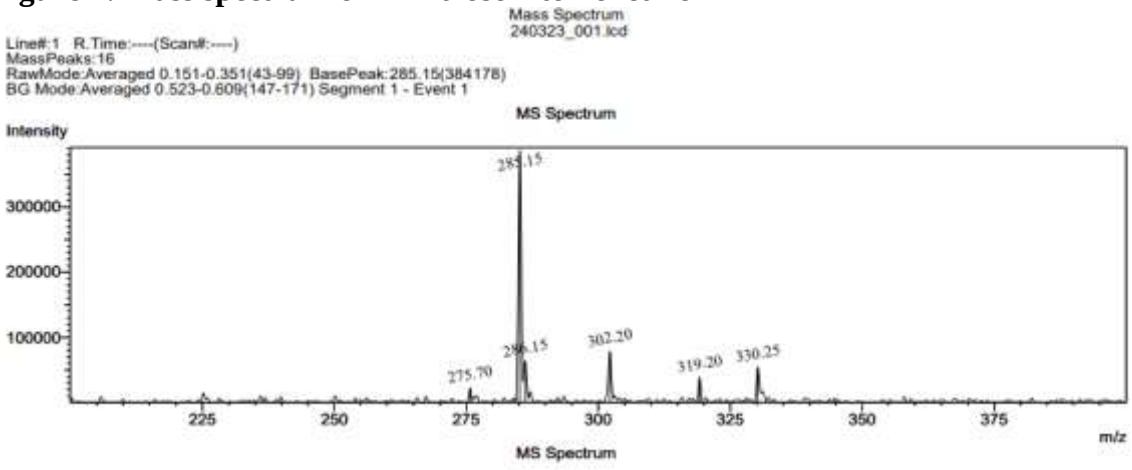


Table 2. Optimized MSMS parameters by MRM for N-Nitroso Atomoxetine impurity in positive ion mode.

Precursor m/z	Product m/z	Dwell time	Q1 Pre Bias	CE	Q3 Pre Bias
285.15	177.05	100	-30	-7	-20
285.15	117.10	100	-30	-15	-22
285.15	73.00	100	-30	-18	-30

Method Validation

The ICH and USP guidelines were followed in the successful completion of the method validation, and all critical and necessary parameters were evaluated and established to demonstrate the analytical method capability.

Specificity

To establish specificity, A injection of blank solution, Sample solution, and spiked sample solution (at specification level) with N-Nitroso atomoxetine impurity was prepared and injected into LCMS-MS analysis. There is no blank interference at the retention times of N-Nitroso atomoxetine in blank solution and sample solution. Therefore, the method is considered as specific. The chromatogram of the standard and spiked sample solution was captured in Figure 3 & 4.

Figure 3: Standard solution

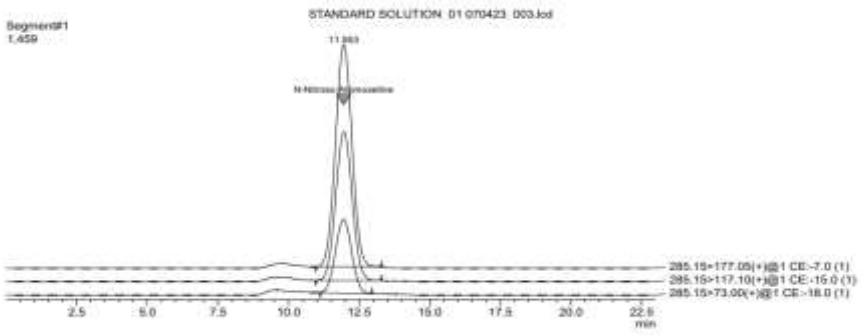
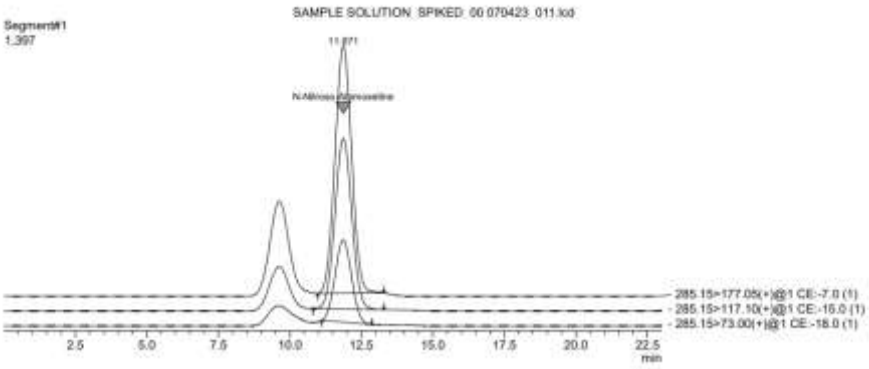


Figure 4: Spiked sample solution



LOD and LOQ and LOQ precision

The Signal to Noise ratio (S/N) and visual detection method were employed for the Limit of detection (LOD) (3.0) and Limit of Quantification (LOQ) (10.0) values for N-Nitroso atomoxetine impurity; the known concentration diluted standard was injected, and the results are in Table 3. Six replicate

injections at LOQ level concentration for LOQ precisions were injected and evaluated and result comply and within the acceptance criteria.

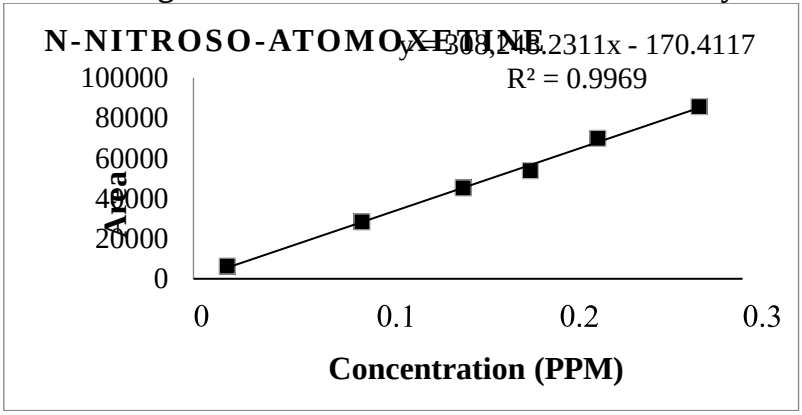
Linearity

Linearity was established from 0.001 ppm to 0.015 ppm (0.02 ppm – 0.27 ppm) for N-Nitroso Atomoxetine impurity. The least squares linear regression analysis was used to calculate the regression coefficient, slope, and intercept values for the peak areas plotted against impurity concentration. Table 3 illustrates the correlation between peak areas and impurity concentrations, while Figure 5 depicts the calibration curves.

Table 3. Linearity ranges interpretation, LOD-LOQ values for N-Nitroso Atomoxetine impurity.

Sr. No.	Impurity	Linearity-Range (wrt. test ppm)	Correlation coefficient (R)	Correlation coefficient (R^2)	RMS-based S/N ratios LOQ	RMS-based S/N ratios LOD
1.	N-Nitroso Atomoxetine	0.02 – 0.27	0.9984	0.9969	245	137

Figure 5. Calibration curve for N-Nitroso Atomoxetine impurity from 0.02 ppm to 0.27 ppm wrt. 56.5 mg/ml test concentration of Atomoxetine Hydrochloride.



Accuracy and Recovery

Accuracy (as recovery) was assessed in triplicate at three different lower to higher concentrations (LOQ, 100%, and 150%) at levels of 0.02 ppm, 0.18 ppm, and 0.27 ppm in Atomoxetine Hydrochloride API, and LOQ recovery was established at 0.02ppm which is about 5% of the specification limit. The recovery criteria set for this study are 70% to 130% and found as per acceptance criteria. The percentage recoveries in Table 3.

Table 3. Recoveries of N-Nitroso Atomoxetine

% Level	Preparation	Impurity (ppm) Added	Impurity (ppm) Found	Recovery (%)	Mean Recovery (%)	% RSD
LOQ (0.02 ppm)	1	0.0180	0.0193	107.22	103.15	15.99
	2		0.0153	85.00		
	3		0.0211	117.22		
100% 0.18 ppm	1	0.1835	0.1853	100.98	101.03	0.09
	2		0.1853	100.98		
	3		0.1856	101.14		
150% 0.27 ppm	1	0.2770	0.2798	101.01	101.15	0.34
	2		0.2795	100.90		
	3		0.2813	101.55		

Method Precision (Repeatability)

Method Precision was established by analyzing six spiked samples with 0.18 ppm (0.03 ppm wrt. test concentration) and % RSD were calculated for the found value of impurity. The % RSD of the recovered individual impurity of six sample preparations (Spiked at specification level) should not be more than 20.0 set and found 0.55 %, which was in acceptable limit and captured in Table 4.

Table 4. Repeatability data for N-Nitroso Atomoxetine impurity at 10 ng/mL (Wrt 0.18 ppm).

Sample No.	Set-1	Set-2	Set-3	Set-4	Set-5	Set-6	**Mean**	**% RSD**
Impurity found (ppm)	0.1853	0.1853	0.1856	0.1861	0.1870	0.1839	**0.1855**	**0.55**

Solution Stability

The Samples were prepared by spiking N-Nitroso atomoxetine at a limit level wrt sample conc. sample solution and Spiked sample solution were stored at 15 °C for 12 hours and area ration calculated with a initial area of spiked sample and time interval, area and area ration tabulated in Table 5. There were significant changes observed after 10 h for N-Nitroso atomoxetine impurity area ration. Therefore, we confirmed the impurity in the sample and spiked the sample solution to stable for at least 10 hours.

Table 5. Area ratio of Spiked solution stability

Spiked sample solution		
Time (HR: MIN)	**N-Nitroso Atomoxetine**	
	Area	**Area ratio**
Initial	53355	NA
5:31	57283	1.07
10:15	60711	1.14
12:14	62367	1.17

Validated Method and Their Pharmaceutical Application:

Using the validated UPLC-MS/MS method for quantification of the N-nitrosamine, we accurately quantified atomoxetine impurity across three different samples. The estimated amount of N Nitroso atomoxetine impurity ranged from 0.0056 ppm to 0.0062 ppm, which is below the limit of detection (LOD). This work tends to be the first in-line method to determine the determination of N-nitrosamine atomoxetine impurity in atomoxetine hydrochloride which would help pharmaceuticals evaluate the possible presence of N-nitrosamine in atomoxetine hydrochloride.

CONCLUSION

To sum up, we have created a highly sensitive LC-MS/MS technique that allows us to simultaneously determine the N-nitroso atomoxetine impurity in the drug substance atomoxetine hydrochloride. We could set up every crucial validation parameter to determine the method's performance. There is no LC-MS/MS method available for the determination of N-Nitroso Atomoxetine impurity in Atomoxetine Hydrochloride. The extremely low determined LOQ and LOD values demonstrate the method's sensitivity performance. For routine quantification of N-nitroso atomoxetine impurity in atomoxetine hydrochloride at a concentration of 1 ppb (0.02 ppm wrt test), the validated method can be used.

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CONFLICT OF INTERESTS
The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS
All the authors contributed significantly to this manuscript, participated in reviewing/editing, and approved the final draft for publication. The research profile of the authors can be verified from their ORCID IDs, given below:

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