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Research Article

Analytical Method Validation Of N-Nitroso Methoxyphenamine Impurity By Lc-Ms/Ms In Methoxyphenamine Hcl

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ABSTRACT

Sensitive LC-MS/MS techniques were developed and verified for N-Nitroso Methoxyphenamine impurity in the active pharmaceutical ingredient (API) of Methoxyphenamine HCl. Ultra Performance Liquid Chromatography hyphenated with triple Quadrupole LC-MS/MS Mass Spectrometer was employed in the method. Method development and validation procedures were carried out as per ICH guidelines. The developed method was found to be robust, accurate, linear, and specific. Over a predefined concentration range the calibration curves demonstrated satisfactory linearity; the correlation coefficient was 0.999. The limit of quantification (LOQ) and limit of detection (LOD) were 0.25 ppm and 0.083 ppm respectively. Analytical test method for determination of N-nitroso methoxyphenamine in methoxyphenamine hydrochloride by LC-MS/MS was validated for System Suitability, Specificity, Precision, Accuracy and Linearity

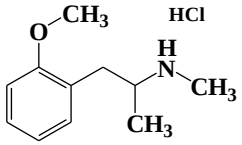
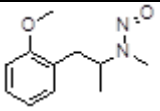
Keywords: LC-MS/MS, MRM, methoxyphenamine, Validation, ICH.

Introduction:

In recent years, the presence of nitrosamine impurities in pharmaceutical products[1-6] has raised significant concerns due to their potential carcinogenicity and adverse health effects. Nitrosamines, a class of compounds with a nitrogen-nitroso functional group (–NO) attached to an amine group, are well-known for their toxicological risks. Their formation, particularly in pharmaceutical manufacturing, has become a central issue for regulators, manufacturers, and public health authorities. Growing awareness of the health hazards posed by nitrosamines has led to increased scrutiny of their presence in medicinal products, prompting global initiatives to monitor, identify, and mitigate their formation. Nitrosamine formation is not exclusive to pharmaceuticals; it can also occur in other industries, including food production, cosmetics, and environmental settings. The widespread presence of nitrosamines in such a variety of products is primarily attributed to their ability to form under relatively mild conditions from secondary and tertiary amines in the presence of nitrosating agents like nitrites or other nitrogenous compounds. However, in the context of pharmaceuticals, the discovery of nitrosamine impurities in commonly used medications such as angiotensin II receptor blockers (ARBs) like valsartan and ranitidine has triggered a significant regulatory response, including product recalls and heightened concerns about consumer safety. The first major public recognition of the risks associated with nitrosamine contamination in drugs occurred in 2018 when nitrosamine impurities were found in several batches of valsartan, an antihypertensive medication. This discovery resulted in the suspension of valsartan and related medications, as well as global investigations into the presence of nitrosamines in drugs. Similar findings in ranitidine and other medications have raised questions about the potential for nitrosamine formation across a wide range of pharmaceutical products. Consequently, pharmaceutical companies and regulatory bodies, such as the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA), have implemented stringent guidelines for the detection, quantification, and mitigation of nitrosamine impurities in pharmaceutical formulations. **Chemical Nature and Formation Mechanism** Nitrosamines typically form when a nitrosating agent, such as sodium nitrite

($NaNO_2$), reacts with an amine, particularly secondary and tertiary amines. The reaction involves the nitrosation of the nitrogen atom in the amine group, leading to the formation of a nitrosamine. While nitrosamines can form under various conditions, the presence of nitrous acid or nitrite in the manufacturing process is a crucial factor in their formation. This is especially relevant in the pharmaceutical industry, where raw materials, excipients, or even process-related contaminants may serve as sources of nitrosating agents. In many cases, the formation of nitrosamines may occur unintentionally during chemical reactions in the synthesis of active pharmaceutical ingredients (APIs), particularly when there is inadvertent exposure to nitrites. Nitrosation reactions can also happen during the storage of certain raw materials, excipients, or final drug formulations, especially under conditions of heat, moisture, or acidity. For example, the use of certain stabilizers, such as sodium nitrite, during API synthesis or the use of contaminated raw materials can unintentionally contribute to nitrosamine formation. Additionally, drugs in tablet, capsule, or injectable forms may accumulate nitrosamine impurities over time if stored improperly or exposed to unfavorable conditions. **Health Implications and Regulatory Response** The carcinogenic potential of nitrosamines is well-documented, with extensive research indicating their ability to cause DNA damage, which leads to an increased risk of cancer in humans. Nitrosamines are known to be highly potent carcinogens, especially when administered in high doses over extended periods. Their impact is particularly concerning because they may be present in very low concentrations in pharmaceuticals, yet still pose significant health risks due to their toxicological properties. Given the potential hazards associated with nitrosamine impurities, regulatory agencies worldwide have taken decisive actions to address the issue. In 2018, the USFDA acknowledged the detection of nitrosamines in several widely used drugs, including those containing valsartan and ranitidine. Following this discovery, the FDA and other international regulatory agencies, such as the EMA and the World Health Organization (WHO), established strict limits for acceptable levels of nitrosamine contamination in pharmaceutical products. The threshold for acceptable nitrosamine impurity levels is typically set at 96 ng/day for a given drug, based on the acceptable daily intake of carcinogenic substances. The details of chemical structure, formula and molecular weights of the drug ad impurity were presented below. Chemical structures are shown in the Table:1

Table.1: Chemical structures of Impurity and Drug substance

Chemical Name	Molecular formula	Molecular weight	Chemical Structure
1-(2-Methoxyphenyl)-N-methylpropan-2-amine. HCl	$C_{11}H_{18}NOCl$	215.72	
N-Nitroso methoxyphenamine	$C_{11}H_{16}N_2O_2$	208.26	

Materials, Instruments and Method:

Materials:

N-Nitroso methoxyphenamine impurity is procured from Synzeal, high pure acetonitrile and formic acid were obtained from Honeywell, in-house water and 1-(2-Methoxyphenyl)-N-methylpropan-2-amine. HCl drug substance were used. Waters make Acquity HSS T3 (100X2.1mm, 1.8µm) column was used for separations.

Analytical instruments:

Waters make LC-MS/MS was used for chromatographic
separations and quantifications, analytical balance of Sartorius make and Shimadzu make were used for all weighing measurements. Micropipette of Brand make was used All the chemicals used were of high purity AR grade. Solvents used were of chromatography grade and analytical instruments used shown in Table:2 and Method conditions[7-12] shown in the Table:3

Table.2: Instruments used in the Experiment

S. No.	Equipment / Instrument Name	Make
1	LC-MS	Waters
2	LC-MS	Agilent
3	Analytical balance	Sartorius
4	Micropipette	Brand
5	Acquity UPLC HSST3 1.8 µm, (2.1 x100mm) column	Waters

Table.3: Chromatographic and mass spectrometric conditions

Liquid Chromatograph (LC) Conditions					
Column	Acquity UPLC HSST3 1.8 µm, (2.1 x100mm)				
Injection Volume	5 µL				
Mobile Phase-A	0.1% Formic acid in water				
Mobile Phase-B	0.1% Formic acid in methanol				
Diluent	Methanol: Water (1:1)				
Purge solution	Methanol: Water (1:1)				
Wash Solution	Methanol: Water (1:1)				
Seal wash	Methanol: Water (1:9)				
Column Oven temperature	45 °C				
Sample manager temperature	10°C				
Gradient Program	Time	Flow	%A	%B	Curve
	Initial	0.30	55	45	Initial
	15.0	0.30	55	45	6
Mass Spectrometer Conditions					
Source of Ionization	ESI (Polarity: Positive)				
Capillary	3.0 kV				
Cone	30 V				
Source Temperature	150 °C				
Desalvation Temperature	500 °C				
Desalvation Gas Flow	1000				
Cone gas flow	100 L/Hr				

PREPARATION OF SOLUTIONS:

Preparation of Mobile Phase-A:

Transfer 1000mL of water into 1litre mobile phase bottle and 1mL of Formic acid. Shake the bottle for 2 minutes.

Preparation of Mobile Phase-B:

Transfer 1000mL of Methanol into 1litre mobile phase bottle and 1mL of Formic acid. Shake the bottle for 2 minutes.

Preparation of diluent:

Transfer 500 mL of water and 500 mL of Methanol into 1litre mobile phase bottle and. Shake the bottle for 2 minutes.

Standard Stock solution:

Weigh and transfer 10mg of impurity standard into 10mL volumetric flask and make-up to the 10mL with methanol.

Preparation of 10 µg/mL standard solution:

Transfer 0.1($) ml of standard stock solution into 10 mL volumetric flask and make up to 10 mL with Diluent.

Note: ($) Volume correction shall be done based on the weight of the standard used in the preparation of standard stock solution.

Preparation of 100ng/mL standard solution:

Transfer 0.2mL of 10 µg/mL standard solution into 20 mL volumetric flask and make up to 20 mL with diluent.

Preparation of standard solution (5ng/mL, 2.5 ppm against test concentration of 2 mg/mL):

Transfer 5.0mL of 100 ng/mL standard solution into 100 mL volumetric flask and make up to 100 mL with diluent.

Sample preparation:

Weigh and transfer 20 mg of sample into 15mL centrifuge tube and add 10mL of diluent. And then vortex for 2.0 minutes and Transfer sample solution into HPLC vial for analysis.

RESULTS AND DISCUSSION:

Analytical method validation data:

System suitability:

By adopting the method developed standard solution of the impurity is injected into the LC/MS-MS system. Following table depicts the results of the same. System suitability results are shown in Table:4

Table.4: Area response of N-Nitroso Methoxyphenamine

Injection	N-Nitroso Methoxyphenamine area response	Acceptance criteria
1	104044	% RSD of analyte peak area response from replicate injections shall not be more than 15.0%
2	104475	
3	105269	
4	103901	
5	103112	
6	104290	
Average	104182	
Standard Deviation	710.209	
%RSD	0.7	
Cumulative % RSD		
7	102736	
Average	103975	
Standard Deviation	847.917	
%RSD	0.8	

Inference: System suitability complied with the acceptance criteria.

Specificity: Following the test protocol, a solution of the drug substance contaminated with the impurity was prepared and injected into the LC-MS/MS apparatus. The findings are shown in the table below specificity details are shown in Table:5 and Specificity chromatogram shown in Figure:1

Table.5: Specifity Retention times

Component	Retention time in standard injection solution	Retention time in spiked test solution
N-Nitroso Methoxyphenamine	8.81	8.80

Inference: The elution order and retention times of impurity peak obtained from standard solutions and spiked test solution are comparable.

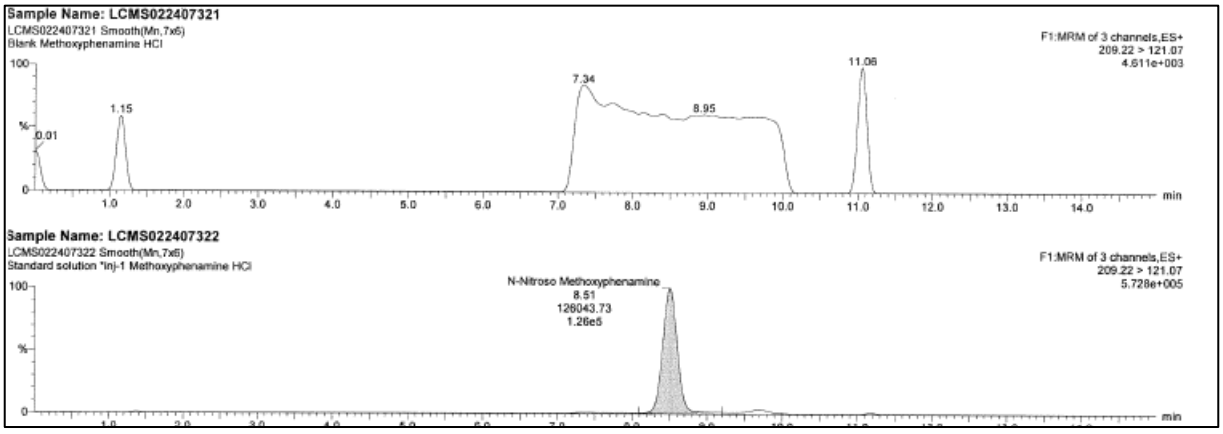


Figure-1: Typical chromatogram depicting the specificity

Limit of Detection and Limit of Quantification: Precision at Limit of Detection and Limit of Quantification were established by creating the solution at the required concentration and injecting it into the LC-MS/MS in replicates. Limit of quantification and Limit of detection area responses are shown in Table:6 and Table:7

Table.6: LOQ area responses and S/N Ratio

Injection	N-Nitroso Methoxyphenamine	S/N Ratio
1	126044	131.48
2	124000	105.22
3	124365	113.96
4	123954	263.13
5	124183	92.92
6	124586	117.02
Average	124522	
Standard Deviation	781.6979	
%RSD	0.6	
Cumulative % RSD		
7	126365	
Average	124785	
Standard Deviation	997.2188	
%RSD	0.8	

Table:7: S/N Ratios in LOD Level standard solution

Preparation	S/N Ratio of N-Nitroso Methoxyphenamine peak
1	41.33
2	45.32
3	73.03
Average	**53**

Linearity:

Linearity is determined by taking five concentrations i.e. 0.25ppm (LOQ), 1.29 ppm, 2.07 ppm, 2.5 ppm and 3.0 ppm reported the correlation coefficient for component. An analysis of linearity confirms that the sample solutions fall within a concentration range where the analyte response is linearly proportional to the analyte concentration. The method's linearity was assessed using varying impurity concentrations from LOQ to 150% of the specification level.Linearity results shown in Table:8 and Linearity graph shown in Figure:2

Table 8: Linearity results

Concentration Level	Average area response	Correlation co-efficient
0.25ppm (LOQ)	0.25801	0.999944
1.29 ppm	1.29008	
2.07 ppm	2.06413	
2.5 ppm	2.58017	0.999944
3.0 ppm	3.09620	

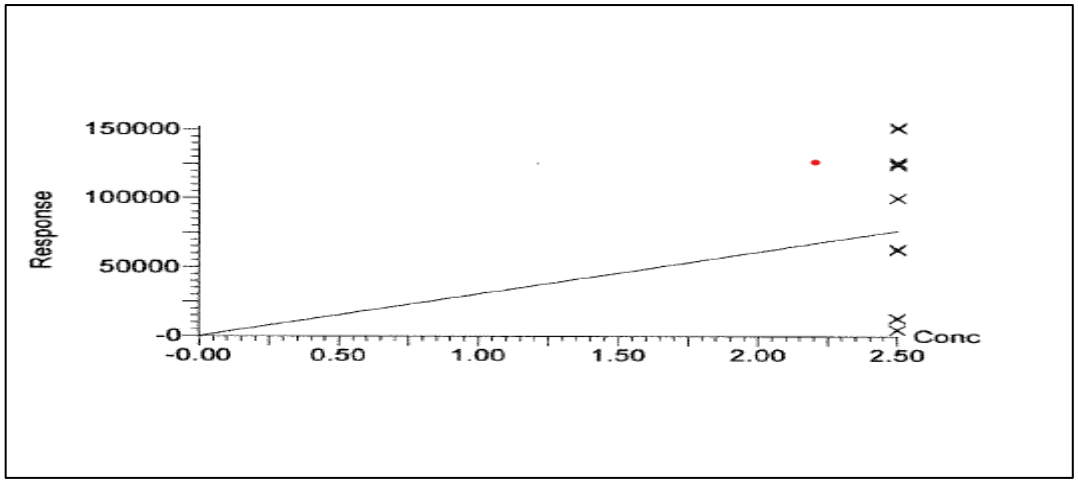


Figure-2: Linearity test result plot between concentration and response

Method Precision and Accuracy:

Following protocol, six replicates of a batch of the drug material polluted with impurity at the specification level were manufactured and injected into the LC-MS/MS.

Acceptance criteria: When utilizing the calibration curve to compute the nominal concentration, the accuracy must be between 80% and 120%, and the % RSD of the analyte area response from the six preparations cannot exceed 20.0%. Method precision results shown in the Table:9

Table 9: Method precision results

Injection	N-Nitroso Methoxyphenamine area response
1	104044
2	104475
3	105269
4	103901
5	103112
6	104290
Average	104182
Standard Deviation	710.209
%RSD	0.7
Cumulative % RSD	
7	102736
Average	103975
Standard Deviation	847.917
%RSD	0.8

Test solution area response:

Injection	N-Nitroso Methoxyphenamine content
Test solution preparation	Not Detected

Spiked test solution area response:

Preparation	N-Nitroso Methoxyphenamine area response
1	102264
2	102321
3	102481
4	103188
5	102881
6	103208

Content of test solution (in ppm):

Injection	N-Nitroso Methoxyphenamine content
Test solution preparation	Not Detected

Content of spiked test preparations (in ppm):

Preparation	N-Nitroso Methoxyphenamine content in ppm
1	2.383
2	2.455
3	2.433
4	2.448
5	2.401
6	2.467
Average	2.431
Standard Deviation	0.0328
%RSD	1.4

Inference: % RSD (Precision) complied within the acceptance criteria.
Recovery: Three replicates of each are used to compare the impurity spikes at 80%, 100%, and 120% levels in diluent without the drug injected into them and the impurity spiked at 80%, 100%, and 120% levels in the presence of the drug. Recovery results are shown in Table:10

Table 10: Recovery results

Level	Preparation	Amount spiked to test sample (in ppm)	Content in test sample (in ppm)	Content of Spiked sample (in ppm)	% Recovery
LOQ	1	0.250	ND	0.245	98.00
	2			0.249	99.60
	3			0.246	98.40
				Average	**98.7**
80%	1	2.001	ND	2.009	100.39
	2			1.973	98.60
	3			2.023	101.09
				Average	**100.0**
100%	1	2.501	ND	2.493	99.68
	2			2.471	98.80
	3			2.445	97.76
				Average	**98.8**
120%	1	3.001	ND	3.007	100.19
	2			2.952	98.36
	3			2.991	99.66
				Average	**99.4**

Solution stability:
The standard and sample solutions spiked with the impurity at the designated level were created and assessed for 24 hours at 25°C ±2°C and 5°C ±3°C after the test process. Solution stability details are shown in the Table:11

Table 11: % RSD for area response in standard solution:

Injection	N-Nitroso Methoxyphenamine (initial)	N-Nitroso Methoxyphenamine (after 24 hours)
1	17561	9857
2	17610	9290
3	16976	9897
4	17581	10037
5	18363	9899
6	17264	10281
Average	17559	9877
Standard Deviation	463.9868	327.0073
%RSD	2.6	3.3
Cumulative % RSD		
7	18064	9931
Average	17631	9885
Standard Deviation	464.5549	299.2167
%RSD	2.6	3.0

Spiked test solution Area response:

Injection	N-Nitroso Methoxyphenamine area response	
Standard solution	17561	9857
Spiked test solution	17363	9481

Content of Spiked test solution (in ppm):

Injection	N-Nitroso Methoxyphenamine area response	
Standard solution	**2.547**	**2.541**
Spiked test solution	**2.484**	**2.412**

Acceptance criteria: Variation between initial results of Initial and 24th Hours spiked test solution & Standard solution stability (individually) should be within ±30% from initial results.

Discussion:

A chromatographic method involves demonstrating specificity, which is the ability of the method to accurately measure the impurity response in the presence of all potential sample components. The chromatographic and mass spectroscopy parameters were fixed and LC-MS/MS system was studied for suitability of residual analysis. The developed method was validated for Linearity, Precision, Accuracy, Specificity, LOD and LOQ.

CONCLUSION:

Based on the above validation results, it is concluded that the test method used for the determination of N-Nitroso Methoxyphenamine impurity in Methoxyphenamine HCl is found to specific, accurate, precise and linear in the range of LOQ to 120% of specification limit. In robustness parameter increased column temperature method is sensitive. Standard and Test solution stability was established up to 24hrs. Results of N-Nitroso Methoxyphenamine for the batch samples analyzed under the scope of the study are found to be below detection limit. Hence method validation is concluded successfully and this test method can be used for routine analysis.

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Conflict of Interest:
The authors declare that there is no conflict of interests regarding the publication of this article.

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