TN-1364

Identification and Sensitive Quantitation of N-nitroso Betahistine Impurity in Betahistine API

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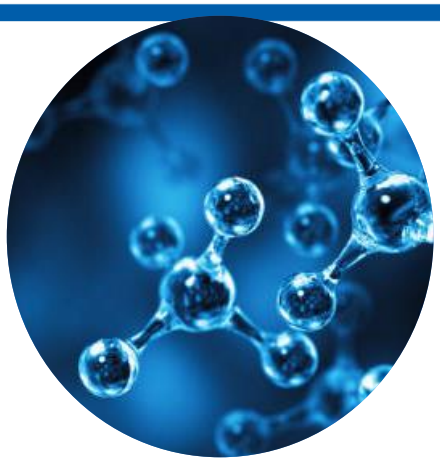


Introduction

Nitrosamines are highly potent carcinogens classified into various risk categories (class 1-5). Categorization was performed using the Carcinogenic Potency Categorization Approach (CPCA), where the severity was determined based on the acceptable intake, activating, or deactivating features defined structurally. N-nitroso Betahistine (NNBH) can be formed in Betahistine (BH) because of the presence of the secondary amine, placing it under a class 1 category as per the CPCA approach.

Due to concerns raised about the potential carcinogenic risk posed by nitrosamines in therapeutics, the EU has set a regulation limit of 18 ng/day. Considering the daily dosage and the regulation limit, N-nitroso Betahistine should be analyzed below the 0.375 ng/mg limit.

This technical note demonstrates a sensitive method for the identification (**Figure 1**) and sensitive quantitation of N-nitroso Betahistine impurity in Betahistine API using triple quadrupole linear ion trap mass spectrometry. A limit of quantitation (LOQ) of 0.05 ng/mL was achieved with baseline separation of N-nitroso Betahistine and Betahistidine API (**Figure 4**).

Sample Preparation

Standards: Calibration curve dilutions of N-nitroso Betahistine were prepared across a range of concentrations in 0.1 % Formic Acid in Water (0.05, 0.1, 0.15, 0.3, 1, 5, 10, 50, and 100 ng/mL).

Samples: 2 mg of Betahistine API was weighed into a suitable vessel, a 4 mL aliquot of 0.1 % Formic Acid in Water was added and vortexed thoroughly to yield a 0.5 mg/mL concentration. A 3 ng/mL solution of N-nitroso Betahistine was spiked in 0.5 mg/mL of Betahistine API solution to achieve a final concentration of 0.15 ng/mL.

LC Conditions

Column: Kinetex™ 2.6 µm Biphenyl
Dimensions: 100 x 3.0 mm
Part No.: 00D-4622-Y0
Mobile Phase: A: 0.1 % Formic Acid in Water
B: 0.1 % Formic Acid in Methanol

Gradient:

Time (min)	% B
0	5
1	5
7	95
7.5	95
8.5	5
12	5

Flow Rate: 0.6 mL/min
Injection Volume: 3 µL
Temperature: 45 °C
LC System: SCIEX® ExionLC™
Detection: MS/MS
Detector: SCIEX QTRAP® 4500
NOTE: The LC flow was diverted to waste for the first 2 min to prevent Betahistadine API from entering the mass spectrometer and after 5 min during column wash.

MS/MS Conditions

Ionization Mode: APCI
Polarity: Positive
Source Temperature: 350 °C
GS1: 35 psi
CUR: 35 psi
CAD: 9
Nebulizer Current: 3 µA
Scan Rate: 10000 Da/s
CE ± CE Spread: 30 ± 15 V
Fixed Fill Time: 100 ms
MS/MS Scan Range: 50-170 Da

Table 1. MRM Transitions.

Analyte	Precursor Ion (m/z)	Fragment Ion (m/z)	CE (V)	CXP (V)	DP (V)
N-nitroso Betahistine-01	166.07	93.0	20	8	20
N-nitroso Betahistine-02	166.07	136.09	10	6	20

Results and Discussion

Baseline chromatographic separation was achieved between Betahistidine and the N-nitroso Betahistidine using the Kinetex™ Biphenyl column. The N-nitroso Betahistidine was retained on the column with a retention time of 2.9 min, while the Betahistidine API was eluted at a retention time of 1.2 min (**Figure 2**). This illustrates one of the primary selectivity benefits for the Biphenyl phase, as the separation of NDSRI from the parent API can be challenging to achieve.

N-nitroso Betahistidine was analyzed across the concentration range of 0.05 to 100 ng/mL. To evaluate reproducibility, each calibration standard was analyzed in triplicate. Linearity was achieved across concentrations ranging from 0.05 to 100 ng/mL with a correlation of determination (R^2) of >0.999 for both quantifier and qualifier ions (**Figure 3**). An LDR of 3.3 orders of magnitude was achieved. No interference in the diluent blank was observed in the calibration curve samples (**Figure 4**).

The specification limit (0.375 ng/mg) was calculated based on the maximum daily dose of 48 mg/day. The N-nitroso Betahistidine was analyzed at 0.150 ng/0.5 mg of API, which is below the calculated specification limit of 0.375 ng/mg. Recovery was calculated against the neat solution, where the peak area from N-nitroso Betahistidine in the control Betahistidine API solution was subtracted from the peak area of the spiked N-nitroso Betahistidine in the Betahistidine API solution. The average recovery was 100.2 % with a %CV of 2.2, evaluated in triplicate (**Table 2**).

Analytical performance was evaluated based on the criteria that the accuracy of the calculated mean should be between 80 % and 120 % at the LOQ and between 85 % and 115 % at the higher concentrations. In addition, the %CV of the calculated mean of the concentration should be <20 % at the LOQ and <15 % at all higher concentrations. The assay accuracy was within ±11 % of the actual concentration and the %CV was <13 %. Calculated percent accuracy and %CV values were within the acceptance criteria at each concentration level (**Figure 5**).

In the Betahistidine API sample, a peak was observed at the retention time of N-nitroso Betahistidine, around 2.87 min (**Figure 1**). The unknown impurity was identified by comparing its MS/MS spectra with an N-nitroso Betahistidine standard. Identification was performed using full scan MS/MS experiments and library searching using SCIEX® OS software. Data acquisition was performed using the linear ion trap feature of the QTRAP® 4500 system through an MRM > Enhanced Product Ion (EPI) experiment. In this case, the selected MRM transitions for N-nitroso Betahistidine were used to create an EPI survey scan in an Information Dependent Acquisition (IDA) experiment setting. Here, the MS/MS spectra from the unknown impurity were compared to standard N-nitroso Betahistidine sample spectra. MS/MS spectra matching identified the impurity as N-nitroso Betahistidine, but it was below the calculated specification limit of 0.375 ng/mg (**Figure 1**).

Figure 1. Detect and Verify Nitrosamine Impurity Using the MRM > EPI Approach on the QTRAP 4500 System.

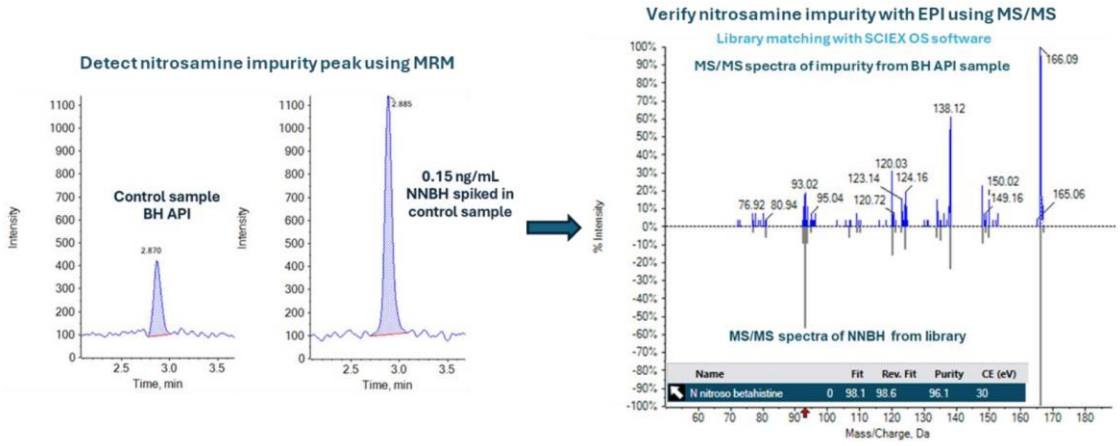


Figure 2 . Good Chromatographic Separation was Achieved Between N-nitroso Betahistidine and Betahistidine API.

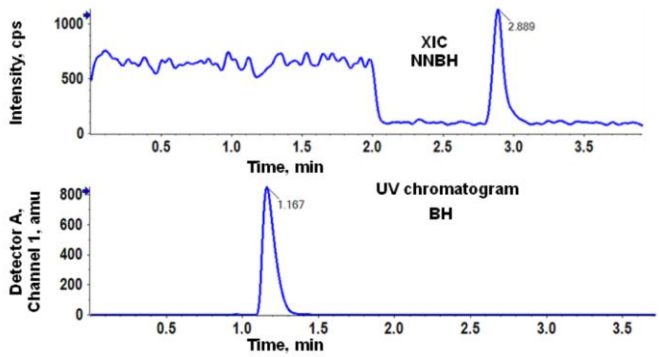
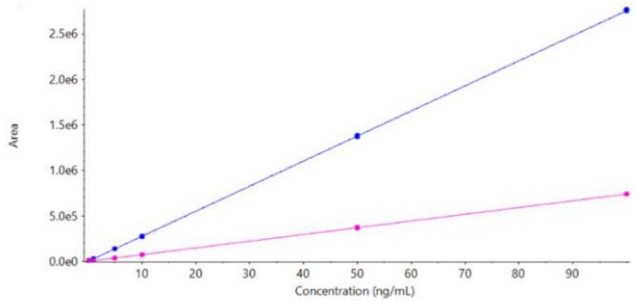


Figure 3 . Calibration Curve for Quantitation of N-nitroso Betahistidine Quantifier Ion (166.07 → 93) and Qualifier Ion (166.07 → 136.09). A Weighing Factor of 1/x was Applied for both Calibration Curves.



Have questions or want more details on implementing this method? We would love to help!
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Figure 4 . Representative Extracted Ion Chromatograms of the Diluent (Left) and LOQ, 0.05 ng/mL (Right).

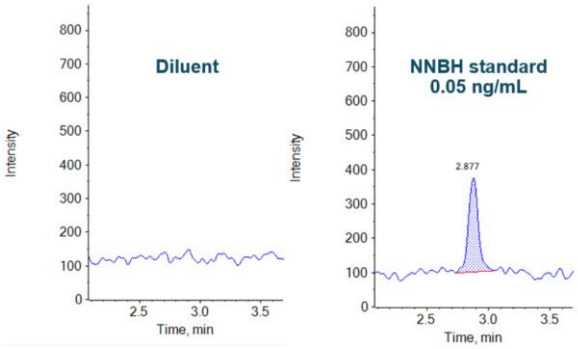

Table 2 . Recovery and Precision Calculation.

ID	Peak Area of NNBH at 0.15 ng/mL Neat Standard	Peak Area of NNBH in BH Control Sample	Peak Area of Spiked 0.15 ng/mL of NNBH in 0.5 mg of BH API Solution
Sample_01	4388	1662	5621
Sample_02	3863	1423	5536
Sample_03	4329	1338	5869
Mean	4193	1474	5675
SD	234.7	118.5	122.3
%CV	5.6	8.0	2.2
Peak Area Response at 0.15 ng/mL after Control Correction			4201
Recovery (%)			100.2

Figure 5 . Quantitative Performance of N-nitroso Betahistidine Quantifier Ion (166.07 → 93.0) and Qualifier Ion (166.07 → 136.09).

NNBH-01(166.07>93.0)

Row	Component Name	Actual Concentration	Num. Values	Mean	Standard Deviation	Percent CV	Average Accuracy across Replicates
1	NNBH_01	0.050	3 of 3	0.055	0.002	3.35	111.
2	NNBH_01	0.100	3 of 3	0.093	0.003	2.92	93.2
3	NNBH_01	0.150	3 of 3	0.152	0.010	6.87	101.
4	NNBH_01	0.300	3 of 3	0.289	0.015	5.07	96.5
5	NNBH_01	1.000	3 of 3	0.987	0.022	2.21	98.7
6	NNBH_01	5.000	3 of 3	4.995	0.059	1.19	99.9
7	NNBH_01	10.000	3 of 3	9.969	0.021	0.209	99.7
8	NNBH_01	50.000	3 of 3	49.879	0.238	0.477	99.8
9	NNBH_01	100.000	3 of 3	100.179	0.316	0.315	100.

NNBH-01(166.07>136.09)

Row	Component Name	Actual Concentration	Num. Values	Mean	Standard Deviation	Percent CV	Average Accuracy across Replicates
1	NNBH_02	0.050	3 of 3	0.053	0.003	6.53	106.
2	NNBH_02	0.100	3 of 3	0.098	0.008	8.69	97.8
3	NNBH_02	0.150	3 of 3	0.150	0.019	12.5	100.
4	NNBH_02	0.300	3 of 3	0.291	0.027	9.35	97.0
5	NNBH_02	1.000	3 of 3	1.006	0.020	2.03	101.
6	NNBH_02	5.000	3 of 3	4.946	0.049	0.987	98.9
7	NNBH_02	10.000	3 of 3	9.944	0.044	0.440	99.4
8	NNBH_02	50.000	3 of 3	49.920	0.392	0.786	99.8
9	NNBH_02	100.000	3 of 3	100.192	0.111	0.111	100.

Conclusions

An LOQ of 0.05 ng/mL was achieved for the quantitation of N-nitroso Betahistidine. Linearity was achieved at concentrations ranging from 0.05 ng/mL to 100 ng/mL with an R^2 >0.999 for both quantifier and qualifier ions covering LDR of 3.3 orders of magnitude. An impurity in Betahistidine API was identified as N-nitroso Betahistidine by comparing the impurity MS/MS spectra with the N-nitroso Betahistidine standard MS/MS spectra using the library matching feature in SCIEX® OS software. Good quantitative performance was demonstrated with accurate and highly reproducible (%CV <13) results using the QTRAP® 4500 system. The method demonstrated the quantitation of N-nitroso Betahistidine impurity below the calculated specification limit (0.375 ng/mg) in the Betahistidine API.

Kinetex™ Ordering Information

2.6 µm Midbore™ Columns (mm)						SecurityGuard™ ULTRA Cartridges (mm)‡
Phases	**30 x 3.0**	**50 x 3.0**	**75 x 3.0**	**100 x 3.0**	**150 x 3.0**	**3/pk**
EVO C18	00A-4725-Y0	00B-4725-Y0	—	00D-4725-Y0	00F-4725-Y0	AJ0-9297
PS C18	00A-4780-Y0	00B-4780-Y0	—	00D-4780-Y0	00F-4780-Y0	AJ0-8950
Polar C18	—	00B-4759-Y0	—	00D-4759-Y0	00F-4759-Y0	AJ0-9531
Biphenyl	—	00B-4622-Y0	—	00D-4622-Y0	00F-4622-Y0	AJ0-9208
XB-C18	00A-4496-Y0	00B-4496-Y0	00C-4496-Y0	00D-4496-Y0	00F-4496-Y0	AJ0-8775
C18	00A-4462-Y0	00B-4462-Y0	00C-4462-Y0	00D-4462-Y0	00F-4462-Y0	AJ0-8775
C8	00A-4497-Y0	00B-4497-Y0	00C-4497-Y0	00D-4497-Y0	00F-4497-Y0	AJ0-8777
HILIC	00A-4461-Y0	—	—	00D-4461-Y0	00F-4461-Y0	AJ0-8779
Phenyl-Hexyl	—	00B-4495-Y0	—	00D-4495-Y0	00F-4495-Y0	AJ0-8781
F5	—	00B-4723-Y0	—	00D-4723-Y0	00F-4723-Y0	AJ0-9321

for 3.0 mm ID

‡SecurityGuard ULTRA Cartridges require holder, Part No.: AJ0-9000

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