TN-1365

Identification and Sensitive Quantitation of N-nitroso N-desmethyl Orphenadrine Impurity in Orphenadrine Citrate 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 N-desmethyl Orphenadrine (NNDO) can be formed in Orphenadrine Citrate (OC) because of the presence of the secondary amine, placing it under the class 1 category as per the CPCA approach.

Due to increased concerns posed by the presence of nitrosamines in therapeutics, the EU has set a regulation limit of 18 ng/day. Considering the daily dosage and the regulation limit, N-nitroso N-desmethyl Orphenadrine should be analyzed below the 90 ng/mg limit.

This technical note demonstrates a sensitive method for the identification (**Figure 1**) and sensitive quantitation of N-nitroso N-desmethyl Orphenadrine impurity in Orphenadrine Citrate API using the QTRAP version of the SCIEX 7500 system. A limit of quantitation (LOQ) of 2.5 pg/mL was achieved with baseline separation of N-nitroso N-desmethyl Orphenadrine and Orphenadrine Citrate API (**Figure 2**).

Sample Preparation

Standards: Calibration curve dilutions of N-nitroso N-desmethyl Orphenadrine were prepared across a range of concentrations using a Acetonitrile / Water (50:50, v/v) diluent (2.5, 5, 10, 50, 100, 500, 1000, 5000, and 10000 pg/mL).

Samples: A 2 mg of Orphenadrine Citrate API was weighed into a suitable vessel. A 20 mL aliquot of diluent (Acetonitrile / Water (50:50, v/v)) was added and vortexed thoroughly to yield a 0.1 mg/mL concentration. A 100 pg/mL solution of N-nitroso N-desmethyl Orphenadrine was spiked in 0.1 mg/mL of Orphenadrine Citrate API solution to achieve a final concentration of 5 pg/mL.

LC Conditions

Column: Kinetex™ 2.6 µm C8
Dimensions: 100 x 2.1 mm
Part No.: 00D-4497-AN
Mobile Phase: A: 5 mM Ammonium Formate + 0.1 % Formic Acid in Water
B: Acetonitrile

Gradient:

Time (min)	% B
0	40
2	40
5	98
8	98
8.1	40
12	40

Flow Rate: 0.5 mL/min
Injection Volume: 3 µL
Temperature: 40 °C
LC System: SCIEX® ExionLC™
Detection: MS/MS
Detector: SCIEX QTRAP® 7500
NOTE: The LC flow was diverted to waste for the first 2 min to prevent Orpenadrine Citrate API from entering the mass spectrometer and after 5 min during column wash.

MS/MS Conditions

Ionization Mode: ESI
Polarity: Positive
Source Temperature: 300 °C
GS1: 45 psi
CUR: 70 psi
CAD: 8
IS: 1700 V
Scan Rate: 10000 Da/s
CE ± CE Spread: 30 ± 15 V
Fixed Fill Time: 50 ms
MS/MS Scan Range: 50-290 Da

Table 1. MRM Transitions.

Analyte	Precursor Ion (m/z)	Fragment Ion (m/z)	CE (V)	CXP (V)	DP (V)
N-nitroso N-desmethyl Orphenadrine-01	285.0	181.09	20	6	35
N-nitroso N-desmethyl Orphenadrine-02	285.0	166.11	45	10	35

Results and Discussion

Baseline chromatographic separation was achieved between Orphenadrine Citrate and the N-nitroso N-desmethyl Orphenadrine. The N-nitroso N-desmethyl Orphenadrine retained on the Kinetex™ C8 column much longer with a retention time of 3.8 min, while the Orphenadrine Citrate API eluted at a retention time of 1.9 min (**Figure 2**).

N-nitroso N-desmethyl Orphenadrine was analyzed across the concentration range of 2.5 to 10000 pg/mL. To evaluate reproducibility, each calibration standard was analyzed in triplicate. Linearity was achieved across concentrations ranging from 2.5 to 10000 pg/mL with a correlation of determination (R^2) of >0.999 for both quantifier and qualifier ions (**Figure 3**). An LDR of 3.6 orders of magnitude was achieved. No interference in the diluent blank was observed in the calibration curve samples (**Figure 4**).

The specification limit (90 pg/mg) was calculated based on the maximum daily dose of 200 mg/day. The N-nitroso N-desmethyl Orphenadrine was analyzed at 5 pg/0.1 mg of API, which is below the calculated specification limit of 90 pg/mg. Recovery was calculated against the neat solution, where the peak area from N-nitroso N-desmethyl Orphenadrine in the control Orpehnadrine Citrate API solution was subtracted from the peak area from spiked N-nitroso N-desmethyl Orphenadrine in Orphenadrine Citrate. The average recovery was 90 % with a %CV of 3.8, 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 ±9 % of the actual concentration and the %CV was <9. Calculated percent accuracy and %CV values were within the acceptance criteria at each concentration level (**Figure 5**).

In the Orphenadrine Citrate API sample, a peak was observed at the retention time of N-nitroso N-desmethyl Orphenadrine, around 3.8 min (**Figure 1**). The unknown impurity was identified by comparing its MS/MS spectra with the standard N-nitroso N-desmethyl Orphenadrine standard. Identification was performed using full scan MS/MS experiments with library searching. Data acquisition was performed using the linear ion trap feature of the QTRAP® 7500 system using an MRM to Enhanced Product Ion (EPI) experiment. In this case, the selected MRM transitions for N-nitroso N-desmethyl Orphenadrine 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 was compared to standard N-nitroso N-desmethyl Orphenadrine sample spectra. MS/MS spectra matching identified the impurity as N-nitroso N-desmethyl Orphenadrine (**Figure 1**), but it was below the calculated specification limit of 90 pg/mg.

Figure 1. Detect and Verify Nitrosamine Impurity Using the MRM > EPI Approach on the QTRAP 7500 System.

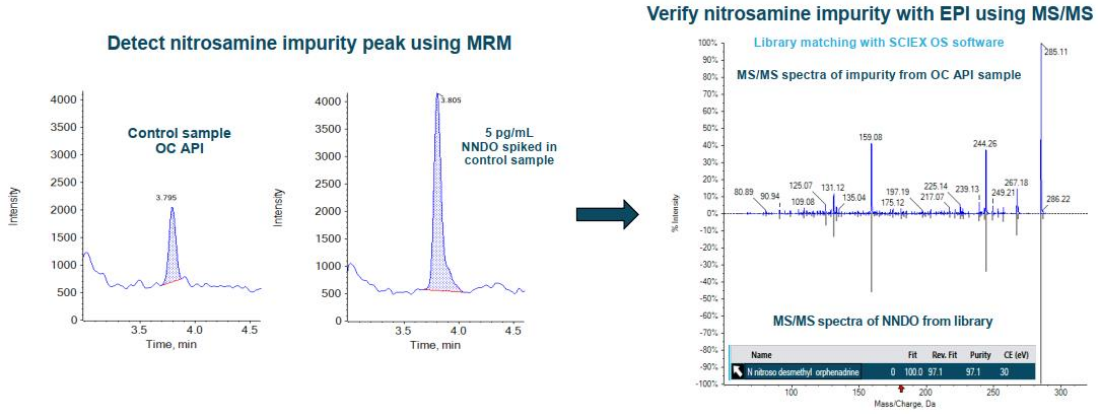


Figure 2 . Good Chromatographic Separation was Achieved Between N-nitroso N-desmethyl Orphenadrine and Orphenadrine Citrate API.

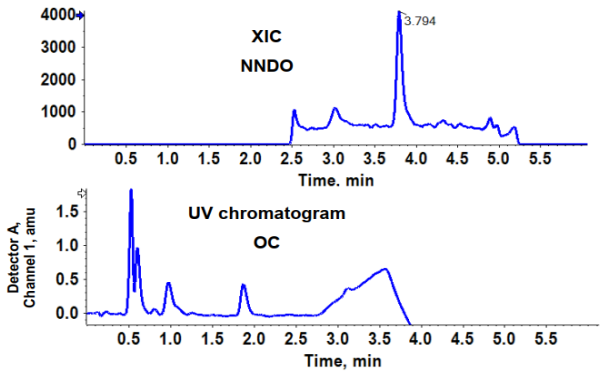
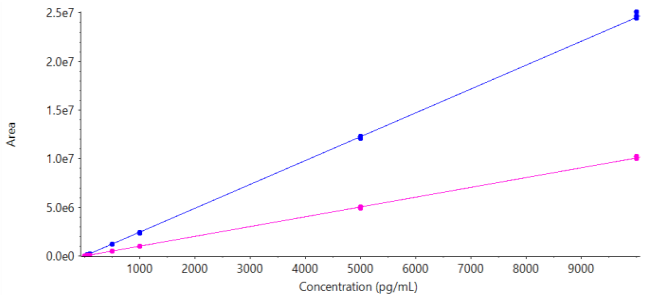


Figure 3 . Calibration Curve for Quantitation of N-nitroso N-desmethyl Orphenadrine Quantifier Ion (285 → 181.09) and Qualifier Ion (285 → 166.11). A Weighing Factor of 1/x was Applied for both Calibration Curves.



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, 2.5 pg/mL (Right).

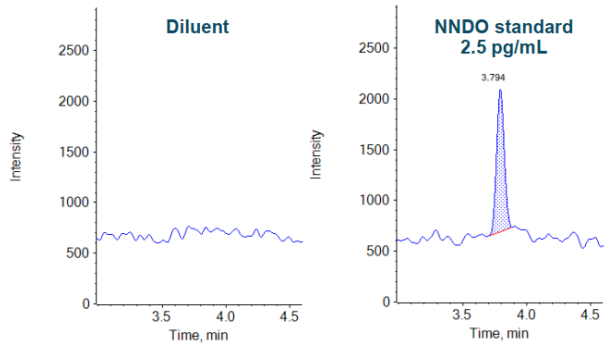

Table 2 . Recovery and Precision Calculation.

ID	Peak Area of NNDO at 5 pg/mL Neat Standard	Peak Area of NNDO in OC Control Sample	Peak Area of Spiked 5 pg/mL of NNDO in 0.1 mg of OC API Solution
Sample_01	13405	6548	18164
Sample_02	13349	5760	18439
Sample_03	12807	5824	18999
Mean	13187	6289	18154
SD	269.7	582.4	696
%CV	2.0	9.3	3.8
Peak Area Response at 0.15 ng/mL after Control Correction			11865
Recovery (%)			100.2

Figure 5 . Quantitative Performance of N-nitroso N-desmethyl Orphenadrine Quantifier Ion (285 → 181.09) and Qualifier Ion (285 → 166.11).

Row	Component Name	Actual Concentration	Num. Values	Mean	Standard Deviation	Percent CV	Average Accuracy across Replicates
1	NNDO-01	2.50	3 of 3	2.362	0.037	1.57	94.5
2	NNDO-01	5.00	3 of 3	5.407	0.135	2.49	108.
3	NNDO-01	10.00	3 of 3	10.676	0.452	4.23	107.
4	NNDO-01	50.00	3 of 3	47.976	0.969	2.02	96.0
5	NNDO-01	100.00	3 of 3	98.815	2.410	2.44	98.8
6	NNDO-01	500.00	3 of 3	486.642	3.627	0.745	97.3
7	NNDO-01	1000.00	3 of 3	980.718	11.817	1.20	98.1
8	NNDO-01	5000.00	3 of 3	4968.405	51.023	1.03	99.4
9	NNDO-01	10000.00	3 of 3	10107....	138.167	1.37	101.

Row	Component Name	Actual Concentration	Num. Values	Mean	Standard Deviation	Percent CV	Average Accuracy across Replicates
1	NNDO-02	2.50	3 of 3	2.529	0.045	1.79	101.
2	NNDO-02	5.00	3 of 3	4.736	0.421	8.89	94.7
3	NNDO-02	10.00	3 of 3	10.606	0.433	4.09	106.
4	NNDO-02	50.00	3 of 3	49.755	1.413	2.84	99.5
5	NNDO-02	100.00	3 of 3	99.131	2.717	2.74	99.1
6	NNDO-02	500.00	3 of 3	494.118	3.562	0.721	98.8
7	NNDO-02	1000.00	3 of 3	992.681	10.993	1.11	99.3
8	NNDO-02	5000.00	3 of 3	4974.377	86.185	1.73	99.5
9	NNDO-02	10000.00	3 of 3	10182....	107.332	1.05	102.

Conclusions

An LOQ of 2.5 pg/mL was achieved for N-nitroso N-desmethyl Orphenadrine in Orphenadrine Citrate API. Linearity was achieved at concentrations ranging from 2.5 pg/mL to 10000 pg/mL with an R^2 >0.999 for both quantifier and qualifier ions covering a linear dynamic range of 3.6 orders of magnitude. An impurity in Orphenadrine Citrate API was identified as N-nitroso N-desmethyl Orphenadrine by comparing the impurity MS/MS spectra with the N-nitroso N-desmethyl Orphenadrine standard MS/MS spectra. Good quantitative performance was demonstrated with accurate and highly reproducible (%CV <9) results on the QTRAP® 7500 system. The method demonstrated the quantitation of N-nitroso N-desmethyl Orphenadrine impurity below the calculated specification limit (90 pg/mg) in the Orphenadrine Citrate API.

Kinetex™ Ordering Information

2.6 µm Minibore Columns (mm)						SecurityGuard™ ULTRA Cartridges (mm)‡
Phases	**30 x 2.1**	**50 x 2.1**	**75 x 2.1**	**100 x 2.1**	**150 x 2.1**	**3/pk**
EVO C18	00A-4725-AN	00B-4725-AN	—	00D-4725-AN	00F-4725-AN	AJ0-9298
PS C18	00A-4780-AN	00B-4780-AN	—	00D-4780-AN	00F-4780-AN	AJ0-8951
Polar C18	00A-4759-AN	00B-4759-AN	—	00D-4759-AN	00F-4759-AN	AJ0-9532
Biphenyl	00A-4622-AN	00B-4622-AN	—	00D-4622-AN	00F-4622-AN	AJ0-9209
XB-C18	00A-4496-AN	00B-4496-AN	00C-4496-AN	00D-4496-AN	00F-4496-AN	AJ0-8782
C18	00A-4462-AN	00B-4462-AN	00C-4462-AN	00D-4462-AN	00F-4462-AN	AJ0-8782
C8	00A-4497-AN	00B-4497-AN	00C-4497-AN	00D-4497-AN	00F-4497-AN	AJ0-8784
HILIC	00A-4461-AN	00B-4461-AN	00C-4461-AN	00D-4461-AN	00F-4461-AN	AJ0-8786
Phenyl-Hexyl	00A-4495-AN	00B-4495-AN	00C-4495-AN	00D-4495-AN	00F-4495-AN	AJ0-8788
F5	00A-4723-AN	00B-4723-AN	—	00D-4723-AN	00F-4723-AN	AJ0-9322

for 2.1 mm ID

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

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