TN-1335

Low-level Quantification of 10 Mutagenic Nitrosamine Impurities in Acyclovir

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Introduction

Nitrosamines are a class of probable carcinogenic and mutagenic compounds that have caused the recall of more than 100 compounds since 2018. This includes the recent recall of Acyclovir, a pharmaceutical product used to treat viral infections such as shingles and chickenpox. The US FDA has amended the maximum permissible daily intake limits for nitrosamine compounds, which for most drug products is now 30 ng/g. This is equivalent to a maximum daily dose of less than 880 mg/day. Given that acyclovir has a maximum daily dose of 800 mg, a nitrosamine limit of 30 ng/g can be implemented.

In this technical note, we demonstrate a highly selective and sensitive method for quantifying 10 nitrosamines in Acyclovir. Baseline chromatographic separation was achieved between the 10 nitrosamines and the Acyclovir active pharmaceutical ingredient (API) using a Luna Omega Polar C18 HPLC column (**Figure 1**). Low-level quantification was achieved in spiked API samples. Accurate and highly reproducible quantitative performance was reached, meeting critical requirements for nitrosamine analysis using the SCIEX QTRAP 6500+ system (**Figure 2**). Linearity was reached across a wide linear range covering 0.025-20 ng/mL.

Sample Preparation

Standard preparation: Each Nitrosamine was mixed at 100 µg/mL and diluted in water to generate a 1000 ng/mL mixture.

Sample preparation: A 100 mg (±5 mg) sample of the Acyclovir API was weighed into a suitable vessel. A 2.5 mL aliquot of water was added, shaken to mix and then vortexed for 30 seconds. The solution was sonicated for 15 minutes and centrifuged at 3901 RCF for 5 minutes. The supernatant was removed and filtered through a 0.2 µm PTFE filter and transferred to an HPLC vial for analysis. The resulting sample concentration was 40 mg/mL.

Spiked sample preparation: A 100 mg (±5 mg) sample of the API was weighed into a suitable vessel. A 2.5 mL aliquot of a 1 ng/mL Nitrosamine mix standard solution was added before being shaken by hand. The sample was vortexed for 30 seconds and centrifuged at 4500 rpm for 5 minutes. The supernatant was removed and filtered using a 0.22 µm PVDF syringe filter and transferred to an HPLC vial for analysis. The final concentration was 40 mg/mL with a spike concentration of 1 ng/mL of the Nitrosamine mix. Each Nitrosamine was present at a spike concentration of 25 ng/g relative to the sample.

LC Conditions

Column: Luna™ Omega 3.0 µm Polar C18
Dimensions: 150 x 3.0 mm
Part No.: 00F-4760-Y0
Mobile Phase: A: 0.1 % Formic Acid in Water
B: 0.1 % Formic Acid in Methanol

Gradient:

Time (min)	%B
0.00	2
1.00	2
20.0	98
22.0	98
22.1	2
24.0	2

Flow Rate: 0.45 mL/min
Injection Volume: 20 µL
Temperature: 35 °C
LC System: SCIEX® ExionLC™
Detection: MRM
Detector: SCIEX QTRAP® 6500+

MRM Conditions

Mode: Positive (APCI ionization)
Source Temperature: 350 °C
GS1: 45 psi
CUR: 35 psi
CAD: Medium
Nebulizing Current: 3 µA

MRM Transitions and Parameters

Analyte	Q1 (m/z)	Q3 (m/z)	DP (V)	CE (V)
N-Nitrosodimethylamine (NDMA)	75.0	43.1	55	21
N-Nitrosodi-n-propylamine (NDIPA)	131.1	89.0	65	12
N-Nitrosomethylethylamine (NMEA)	89.0	61.0	20	16
N-Nitrosodiethylamine (NDEA)	103.0	75.0	70	14
1-Nitrosopyrrolidine (NPYR)	101.1	55.1	40	21
1-Nitrosopiperidine (NPIP)	115.1	69.0	40	28
N-Nitrosomethylphenylamine (NMPA)	137.1	66.0	70	21
Ethyl(nitroso)(propan-2-yl)amine (NEIPA)	117.0	75.0	60	14
4-[Methyl(nitroso)amino]butanoic Acid (NMBA)	147.0	117.0	60	9
N-Nitrosodiisopropylamine (NDPA)	131.1	43.0	65	12

Results and Discussion

The calibration curves for 10 Nitrosamines were plotted across a concentration range of 0.025-20 ng/mL (**Figure 2**). Strong linearity was achieved with correlation coefficients >0.99 for all compounds analyzed (**Table 1**). The linear dynamic range was greater than 3 orders of magnitude for all Nitrosamines. Each LOQ level was evaluated in 6 replicates. The chromatograms at the LOQ and the matrix blank for all 10 Nitrosamines are highlighted in **Figure 3**. Interferences were not observed in the matrix blanks for any of the Nitrosamines analyzed (**Figure 3**). The observed accuracy and precision levels met the specified regulatory recommendations for Nitrosamine analysis. The overall coefficient of variation (CV) was <20 % and accuracy was within ±20 % of the nominal concentration at the LOQ level. The quantitative performance for each nitrosamine is summarized in **Table 1**.

Accuracy and precision metrics were evaluated in standard solutions and spiked samples. A 1 ng/mL concentration in spiked solution (equivalent to 25 ng/g in sample concentration) was used for the assessment. **Figure 4** shows the representative chromatograms of the spiked sample equivalent to a 25 ng/g concentration. The acceptable criteria for accuracy and precision at this concentration level are ±30 % of the nominal concentration and ≤25 % for %CV. The overall accuracy was within ±20 % of the nominal concentration and the %CV was <20 %, meeting the specified requirements. **Table 2** summarizes the accuracy and %CV for each Nitrosamine in spiked samples. The spike concentration was below the recommended limit (30 ng/g), indicating that the assay meets the specified requirements for Nitrosamine analysis. Accuracy was within ±19.5 % of the nominal concentration with %CV <10.4 %.

Figure 1. Good Chromatographic Separation was Achieved Between the 10 Nitrosamines and the Acyclovir API.

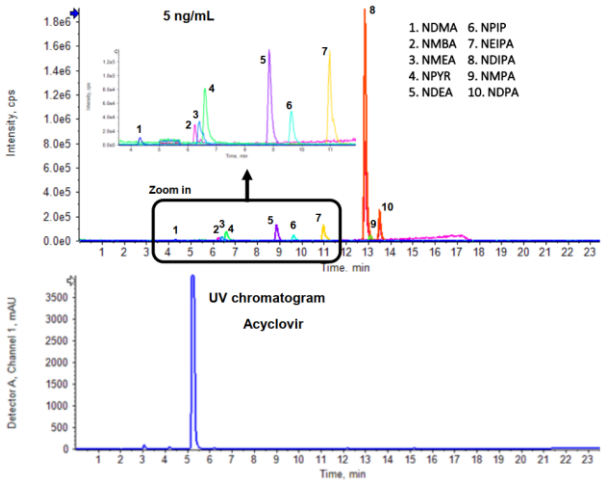

Figure 2. Calibration Curves Showing the Quantitative Responses of 10 Nitrosamines.

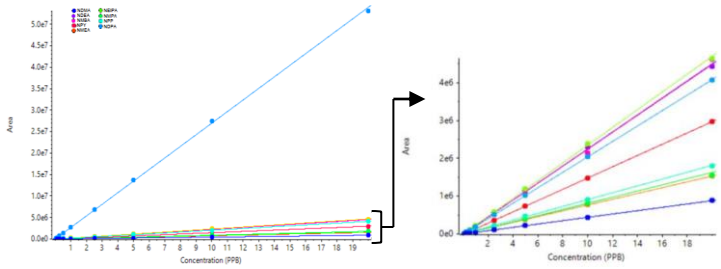

Table 1. Linearity, LOQ, %CV, and Average Accuracy for the Analysis of 10 Nitrosamines.

Compound	LOQ (ng/mL)	CV (%)	Average Accuracy (%)	Linearity Range (ng/mL)	Correlation Coefficient (r^2)
NDMA	0.100	12.3	103	0.100 – 20	0.999
NDIPA	0.025	3.90	92.3	0.025 – 20	0.999
NMEA	0.050	12.2	99.2	0.050 – 20	0.999
NDEA	0.100	5.30	80.0	0.100 – 20	0.999
NPYR	0.100	18.7	106	0.100 – 20	0.999
NPIP	0.250	11.2	82.4	0.250 – 20	0.999
NMPA	0.250	8.30	90.0	0.250 – 20	0.999
NEIPA	0.050	19.6	104	0.050 – 20	0.999
NMBA	0.050	17.2	113	0.050 – 20	0.999
NDPA	0.025	13.0	108	0.025 – 20	0.999

Figure 3. Chromatograms of 10 Nitrosamines at the Limit of Quantitation (LOQ).

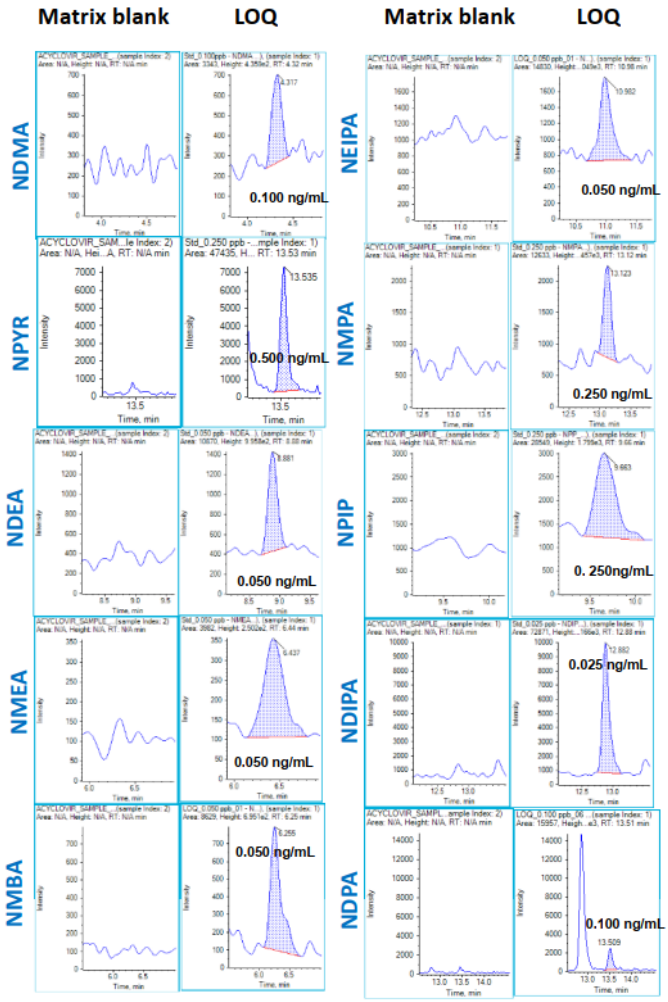

Figure 4. Chromatograms of 10 Nitrosamines Spiked in a 40 mg/mL Solution of the Acyclovir API.

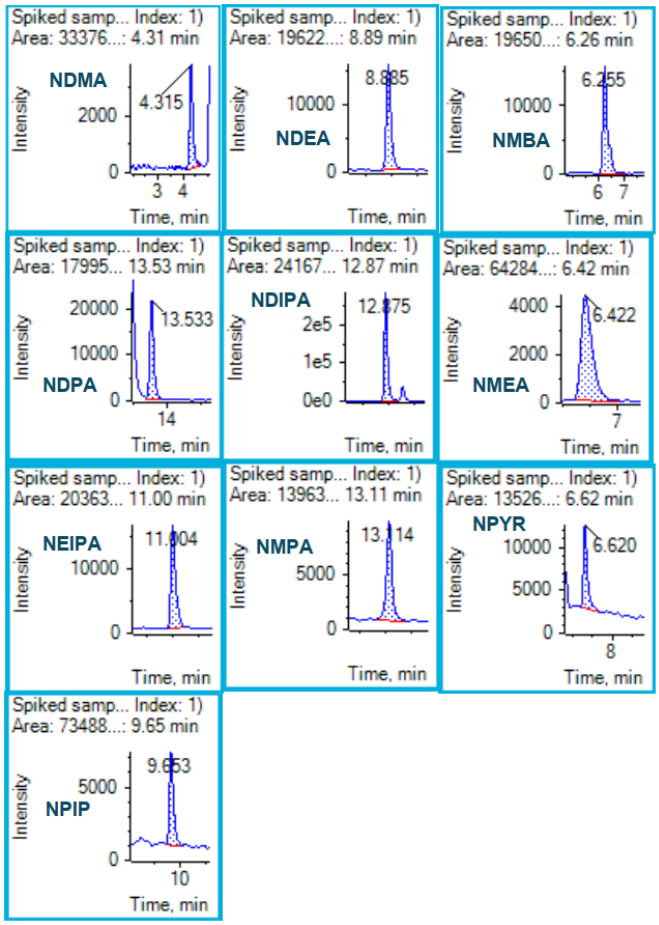

Table 2. Accuracy and %CV Values in Spiked Samples.

Compound	Accuracy (%)	CV (%)
NDMA	81.5	4.10
NDIPA	87.5	1.90
NMEA	80.5	3.90
NDEA	84.3	3.30
NPYR	82.1	10.4
NPIP	83.9	2.30
NMPA	84.5	7.90
NEIPA	85.6	1.90
NMBA	86.0	4.30
NDPA	82.7	4.10

Conclusions

Low-level quantification for 10 Nitrosamines using a Luna™ Omega 3.0 µm Polar C18 HPLC column in conjunction with the SCIEX® QTRAP® 6500+ system was achieved. Baseline chromatographic separation was achieved between the 10 Nitrosamines and the Acyclovir API. Accurate and highly reproducible quantitative performance was demonstrated for all 10 Nitrosamines with strong linearity, meeting the specified acceptance criteria. The method demonstrated the quantification of Nitrosamine impurities below the current recommended limit (30 ng/g) in the Acyclovir drug product. Using a straightforward sample preparation, multiple preparations of the un-spiked sample generated consistent results.

Luna Omega Ordering Information

3 µm MidBore™ Columns (mm)				SecurityGuard™ Cartridges (mm)
Phases	**50 x 3.0**	**100 x 3.0**	**150 x 3.0**	**4 x 2.0*/10pk**
Polar C18	00B-4760-Y0	00D-4760-Y0	00F-4760-Y0	AJ0-7600
PS C18	00B-4758-Y0	00D-4758-Y0	00F-4758-Y0	AJ0-7605
C18	00B-4784-Y0	00D-4784-Y0	00F-4784-Y0	AJ0-7611
SUGAR	—	—	00F-4775-Y0	AJ0-4496

for ID: 2.0 – 3.0 mm

*SecurityGuard Analytical Cartridges require holder, Part No.: KJ0-4282

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