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Liquid Chromatograph Mass Spectrometer LCMS-8060NX

Application News

Quantitation of 7 N-nitrosamines in Monoclonal Antibody (mAb) Formulations Using LC-MS/MS

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User Benefits

- An LC-MS/MS method for the low-level quantitation of N-nitrosamines in mAb formulations
- A quick, easy and reliable method for complex biological formulation such as mAb

■ Introduction

N-nitrosamine (Figure 1) impurities have long been a concern for pharmaceutical manufacturers, and their testing has become increasingly critical. Some of these compounds are listed as Class 1 mutagens in ICH M7 and have been monitored extensively in since 2018. However, following the conclusion of the review under Article 5(3), the Committee for Medicinal Products for Human Use (CHMP) of European Medicines Agency (EMA) considered that there is also a risk of presence of N-nitrosamines in biological medicinal products as well. This is for the biological medicines with the following risk factors namely biologicals containing chemically synthesized fragments, nitrosating reagents in processes and contamination from packaging, storage and such due to nitrocellulose.

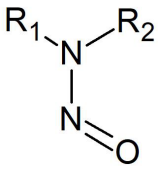

Figure 1: Chemical structure of N-nitrosamine

Monoclonal antibodies (mAbs) are large, complex biomolecules that can interfere with LC-MS/MS analysis by clogging the chromatography column and contaminating the MS system. To address this, a quick, reliable, and robust sample preparation method was used for low-level quantitation. This application news presents a validated LC-MS/MS procedure for quantifying seven N-nitrosamines in mAb formulations using the Nexera™ X3 UHPLC coupled with the LCMS-8060NX Triple Quadrupole Mass Spectrometer. (Figure 2) The seven N-nitrosamines and four labelled internal standards (IS) include N-nitroso-N-methyl-4-aminobutyric acid (NMBA); N-nitroso-dimethylamine (NDMA); N-nitroso-diethylamine (NDEA); N-nitroso-ethyl-isopropylamine (NEIPA), N-nitroso-diisopropylamine (NDIPA); N-nitroso-dipropylamine (NDPA), N-nitroso-dibutylamine (NDBA); N-Nitroso-N-methyl-4-aminobutyric Acid-d3 (NMBA-d3), N-Nitrosodimethylamine-13C2-d6 (NDMA-13C2 d6), N-Nitrosodiethylamine-d10 (NDEA-d10) and N-Nitroso-di-n-butylamine-d18 (NDBA-d18). The above listed compounds cover the scope of regulatory methods from USP, USFDA and EDQM for N-nitrosamines testing.

Figure 2: Nexera™ X3 UHPLC coupled with an LCMS™-8060NX

■ Experimental

Locally procured individual N-nitrosamine standards were analyzed in scan mode. An LC method (Table 1) was developed with an aim to separate all N-nitrosamines under study (Figure 2) which was achieved using Shimadzu Shim-pack Scepter C8, 150 mm x 4.6 mm I.D. and 5 µm LC column. Further, steps such as precursor ion selection, Multiple Reaction Monitoring (MRM) optimization at different Collision Energies (CE) and voltage optimization were performed using LabSolutions auto MRM optimization function to obtain MRMs and their optimum CEs (Table 2). The mAb blank formulation mixture was processed using an optimized sample preparation protocol (Figure 3). This blank sample matrix was then used to prepare an eight-point calibration curve for the seven N-nitrosamines under study, ranging from 50 to 2000 ppb, spiked with four different internal standards. The limit of quantitation (LOQ) was found to be 50 ppb. The S/N and % RSD at LOQ are shown in Table 3.
(All concentrations mentioned above are relative to sample.)

Method

Table 1: Analytical conditions

HPLC System	: Nexera™ X3
Column	: Shim-pack Scepter™, C8-120 (150 mm × 4.6 mm I.D., 5 µm, P/N: 227-31041-05)
Column Oven	: 40 °C
Mobile Phases	: MP-A: 0.1 % Formic acid in LC-MS grade water ; MP-B: LC-MS grade methanol
Flow Rate	: 0.5 mL/min
Gradient Program (B%)	: 40 % (0-1.0 min) → 100 % (12.0-15.0 min) → 40 % (15.5-20.0 min)
Injection Volume	: 30 µL
LC-MS System	: LCMS™-8060NX
Ionization Source	: APCI
LC-MS Temperatures	: Interface: 270 °C Desolvation Line: 220 °C
LC-MS Gas Flows	: Nebulizing Gas: 3 L/min Drying Gas: 3 L/min

Table 2: MRM transitions for 7 N-nitrosamines and 4 ISTD

Compound	Sample Type	IS ID	Precursor *m/z*	Product *m/z*	CE
NMBA	Target	IS-1	146.90	44.10	-14
NDMA	Target	IS-2	74.90	58.10	-18
NDEA	Target	IS-3	103.00	29.05	-16
NEIPA	Target	*	117.10	75.10	-12
NDIPA	Target	*	131.10	89.15	-12
NDPA	Target	*	131.10	89.15	-12
NDBA	Target	IS-4	159.10	40.95	-22
NMBA-d3	IS-1	NA	150.00	120.00	-11
NDMA-13C2 d6	IS-2	NA	83.00	47.00	-11
NDEA-d10	IS-3	NA	113.20	34.10	-17
NDBA-d18	IS-4	NA	177.00	46.00	-15

* For these compounds, an external standard calibration method is used

Sample Analysis

Incubate the mAb sample/spiked sample at 70°C for 30 mins in water bath

Centrifuge the sample/spiked sample at 12000 rpm for 5 mins at 5°C

Collect the supernatant and inject in LC-MS/MS

Figure 3: mAb sample preparation protocol

Table 3: Coefficient of determination for calibration curves, repeatability of area ratios for LOQ solution and S/N ratio for LOQ solution (Conc. expressed are relative to sample)

Compound	r^2	CC Range (ppb)	LOQ		
			Conc. (ppb)	% RSD (n=6)	S/N
NMBA	0.996			12.6	18
NDMA	0.997			16.0	97
NDEA	0.999			1.5	102
NEIPA	0.999	50-2000	50	2.0	100
NDIPA	0.999			1.8	103
NDPA	0.998			3.9	102
NDBA	0.999			3.1	106

Sample results summary for all 6 mAb is tabulated in table 4.

Table 4: Sample summary

Samples	Concentration in ppb						
	NMBA	NDMA	NDEA	NEIPA	NDIPA	NDPA	NDBA
mAb-1	Below LOQ						
mAb-2							
mAb-3							
mAb-4							
mAb-5							
mAb-6							

■ Results and Discussion

Figure 4 depicts the calibration curve and 50 ppb matrix matched standard (Representative chromatograms of 4 compounds).

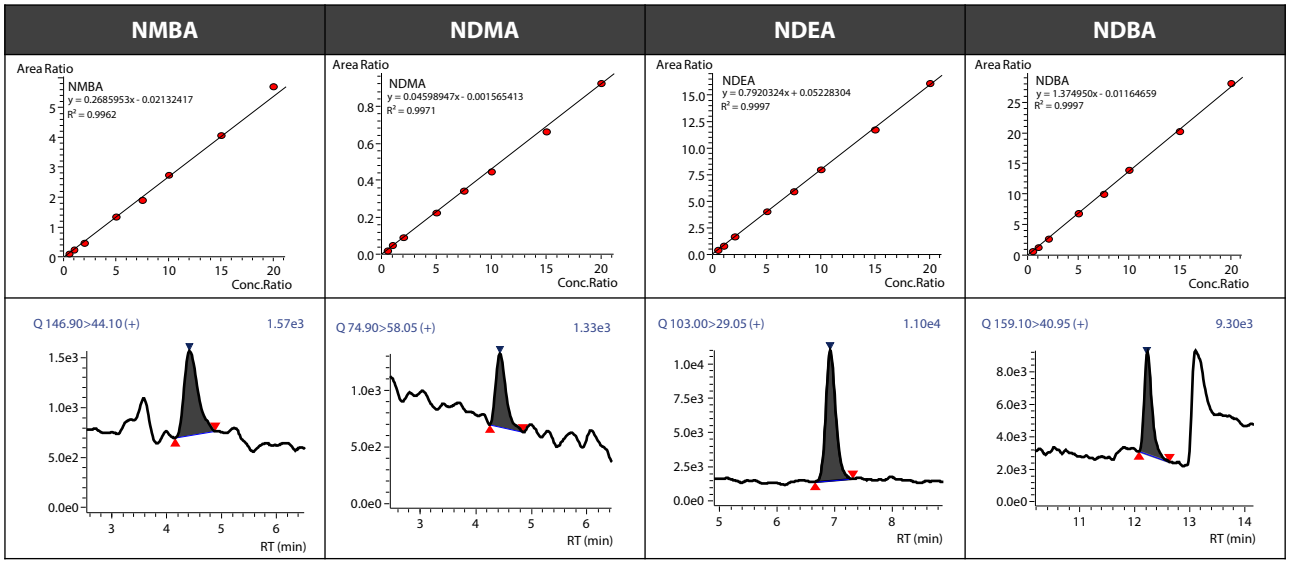

Figure 4: Calibration curve and chromatogram of LOQ solution for NMBA, NDMA, NDEA & NDBA as representative compounds

Figure 5 depicts the typical chromatogram for all 7 N-nitrosamines.

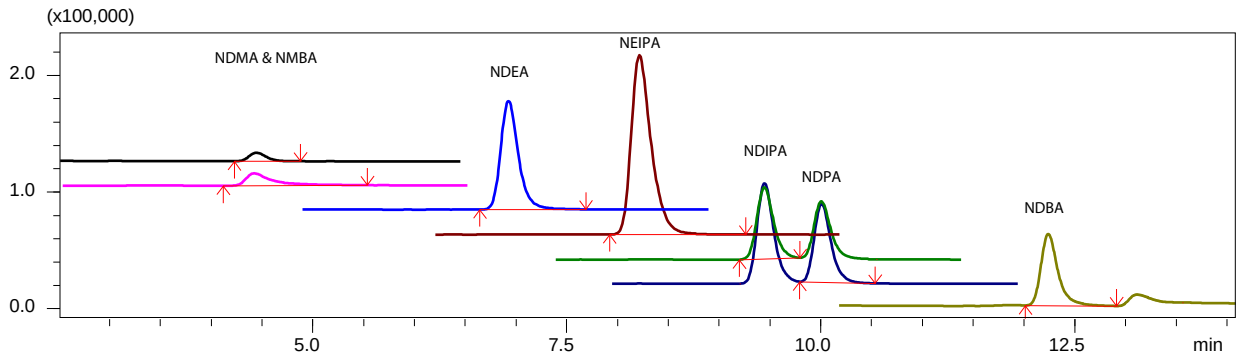

Figure 5: Typical chromatogram for separation of all 7 N-nitrosamines

The amount in sample, amount obtained, amount spiked and % recovery are shown in Table 5.

Table 5: Sample spiked study for mAb sample at 200 ppb (Results expressed are relative to sample concentration)

% Recoveries of N-nitrosamines in mAb-1 sample				
Comp.	**Amt. in sample (ppb)**	**Amt. obtained (ppb)**	**Amt. spiked (ppb)**	**% Recovery**
NMBA		227	200	114
NDMA		244	200	122
NDEA		182	200	91
NEIPA	Below LOQ	184	200	92
NDIPA		183	200	92
NDPA		139	200	70
NDBA		231	200	116

■ Conclusion

- Quantitation of 7 N-nitrosamines in 6 mAb formulation samples was successfully demonstrated on Shimadzu LCMS-8060NX.
- Repeatability for all N-nitrosamines were found to be less than 20.0 %.
- Recoveries for all N-nitrosamines were found to be between 70-130 %.
- The newly developed, patented lens system (UF-Qarray II and UF-Lense) of LCMS-8060NX improves system robustness by efficiently introducing only ions into the mass spectrometer and removing unwanted neutral particles and contaminants.

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