SHIMADZU

WP 484

Quantitation of 7 N-Nitrosamines in Monoclonal antibody (mAb) formulations using LC-MS/MS

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1. Overview

N-nitrosamine impurity, are organic compounds of the chemical structure $R_2N{-}N{=}O$, where R is usually an alkyl group. These compounds are listed as Class 1 mutagens in ICH M7[1]. N-nitrosamines have been monitored extensively in pharmaceuticals 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 & contamination from packaging, storage such due to nitrocellulose[2].

2. Introduction

Biopharmaceuticals are medical drugs produced using biotechnological tools which includes nucleic acids and proteins such as antibodies. Monoclonal antibodies (mAbs) studied in this poster are one such type of biomolecules which are large and complex in nature, this complexity interferes during LC-MS/MS analysis by clogging the chromatography column and contaminating the MS ionization source as well as lens system. To counter this a quick, easy, reliable & robust sample preparation was employed resulting in low level detection. This poster describes a validated LC-MS/MS procedure for quantitation of 7 N-nitrosamines in mAb formulation samples was performed using an Ultra High Performance Liquid Chromatograph (UHPLC) Nexera™ X3 coupled with an LCMS-8060NX, a Triple Quadrupole Mass Spectrometer from Shimadzu Corporation, Japan (Figure 1). The seven N-nitrosamines and four labelled internal standards (IS) includes 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-C13-d6 (NDMA-C13 d6), N-Nitrosodiethylamine-d10 (NDEA-d10) and N-Nitroso-di-n-butylamine-d18 (NDBA-d18) The above listed compounds covers the scope of regulatory methods from USP, USFDA & EDQM for N-nitrosamines testing.

3. Methods

3-1. LC-MS/MS analysis

Individual Certified Reference Standard (CRS) for all 7 N-nitrosamines and 4 internal standards from United States Pharmacopeia (USP) were procured. Stock solutions for individual N-nitrosamines and IS were prepared and analysed in scan mode. Further, steps such as precursor ion selection, Multiple Reaction Monitoring (MRM) optimization at different Collision Energies (CE) and voltage optimization were performed using Shimadzu's LabSolutions auto MRM optimization feature to obtain MRMs and their optimum CEs (Table 2). An LC method (Table 1) was developed with an aim to separate 7 N-nitrosamines under study (Figure 2) which was achieved using Shimadzu make Shim-pack Scepter C8, 150 mm x 4.6 mm I.D. and 5 µm LC column. For quantitation, eight-point linearity of 7 N-nitrosamines ranging from 50.0-2000.0 ppb were prepared in formulation buffer spiked with 4 different internal standards. The limit of quantitation (LOQ) was found to be 50.0 ppb. The S/N & % RSD at LOQ are shown in Table 3.

Figure 1: Shimadzu Nexera™ X3 UHPLC coupled with an LCMS-8060NX Triple quadrupole

3-2. Analytical conditions

Table 1. Instrument parameters for LC-MS/MS

HPLC System	: Nexera™ X3
Column	: Shim-pack Scepter C8-120, 5 µm 4.6 x 150 mm (P/N :227-31041-05)
Column Oven	: 40 ° C
Mobile Phases	: A-0.1% Formic acid in LC-MS grade water B-LC-MS grade methanol
Flow Rate	: 0.5 mL/min
Gradient program (B%)	: 0-1 min → 40 (%); 1-12 min → 40-100 (%); 12-15 min → 100 (%); 15-15.5 min → 100-40 (%); 20 min → STOP.
Injection Volume	: 30 µL
LC-MS System	: LCMS™-8060NX
Ionization Source	: APCI
LC-MS Temperatures	: Interface: 270° C Desolvation Line: 220° C Heater Block: 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	Type	Precursor m/z	Product m/z	CE
NMBA	Target	146.9	44.1	-14
NMBA-d3	IS	150	120	-11
NDMA	Target	74.9	58.1	-18
NDMA-C13d6	IS	83	47	-15
NDEA	Target	103	29.05	-16
NDEA-d10	IS	113.2	34.1	-17
NEIPA	Target	117.1	75.1	-12
NDIPA	Target	131.1	89.15	-12
NDPA	Target	131.1	89.15	-12
NDBA	Target	159.1	40.95	-22
NDBA-d18	IS	177	46	-15

3-3. Sample preparation

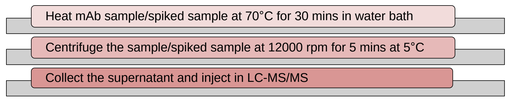

Figure 2 depicts the chromatographic separation of all 7 N-nitrosamines

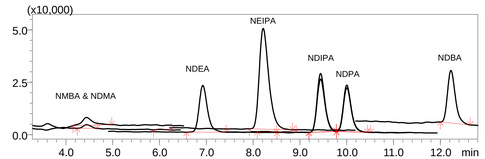

Figure 2: Chromatogram for separation of all 7 N-nitrosamines

4. Results and Discussion

Figure 3 depicts the calibration curve and 50.0 ppb matrix matched standard (Representative chromatograms of 3 compounds)

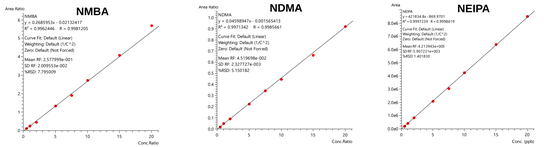

Figure 4: Calibration curves for NMBA, NDMA & NEIPA as representative compounds

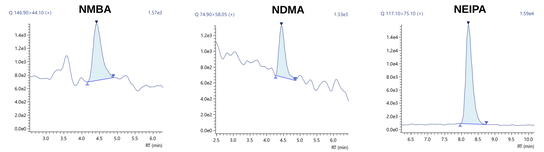

Figure 5: Chromatograms of 0.5 ppb for NMBA, NDMA & NEIPA as representative compounds

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)

Sr. No.	Abbr.	r^2	CC Range (ppb)	LOQ		
				Conc. (ppb)	% RSD (n=6)	S/N
1	NMBA	0.996	50.0-2000.0	50.0	12.6	18
2	NDMA	0.997			16.0	97
3	NDEA	0.999			1.5	102
4	NEIPA	0.999			2.0	100
5	NDIPA	0.999			1.8	103
6	NDPA	0.998			3.9	102
7	NDBA	0.999			3.1	106

Summary of all 7 N-nitrosamines detected are included in Table 4.

Table 4: Sample summary

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

The amount in sample, amount obtained, amount spiked & % 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	Below LOQ	227	200	114
NDMA	Below LOQ	244	200	122
NDEA	Below LOQ	182	200	91
NEIPA	Below LOQ	184	200	92
NDIPA	Below LOQ	183	200	92
NDPA	Below LOQ	139	200	70
NDBA	Below LOQ	231	200	116

5. 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 IonFocus ion source unit and patented lens system (UF-Qarray II) of LCMS-8060NX improves system robustness by efficiently introducing only ions into the mass spectrometer and removing unwanted neutral particles and contaminants.

6. References

[1] WHO Information Note; Update on Nitrosamine impurities, 20 Nov. 2019.
[2] Questions and answers for marketing authorization holders/applicants on the CHMP Opinion for the Article 5(3) of Regulation (EC) No 726/2004 referral on nitrosamine impurities in human medicinal products, 30 March 2023; EMA/409815/2020 Rev.15