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HRAM LC-MS method for the determination of nitrosamine impurities in drugs

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Application benefits

- Detection and quantification of nine nitrosamines with a single liquid chromatography-high resolution accurate mass (HRAM) mass spectrometry method
- Quantitation of nitrosamine impurities in ranitidine drug substance and product below the daily acceptable intake level, that meets the requirements of FDA regulatory guidelines
- Use of Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) software for both data collection and processing in a 21 CFR 11 compliant environment with full data integrity and security capabilities for cGMP facilities

Goal

To demonstrate fast, highly sensitive quantitation of nine nitrosamines with a Thermo Scientific™ Orbitrap Exploris™ 120 mass spectrometer, and the use of the LC-MS method to measure nitrosamine impurities in commercially available ranitidine drug substances and products

Introduction

What are nitrosamines?

Nitrosamines are small molecular weight chemical substances that are probable human carcinogens, and their presence in medicines is considered unacceptable by regulators. Since 2018, the United States Food and Drug Administration (US FDA) has announced a series of voluntary recalls of drug products following the detection of genotoxic nitrosamine impurities. To ensure the health and safety of the patients taking these medications, international medicine regulatory agencies have set acceptable daily intake limits for nitrosamines.[1,2] Given these low action limits, pharmaceutical manufacturers and contract testing organizations are developing methods to quantify nitrosamines in excipients, active pharmaceutical ingredients (APIs), and drug products to ensure batches do not exceed regulatory acceptance levels and enable control of impurities within their supply chains.

Reliable detection and quantitation of nitrosamines in drug products are often challenging due to the presence of complex formulation matrix and the trace level of these impurities relative to the high concentration of API. Orbitrap-based mass spectrometry is capable of selectively determining analytes at low concentrations with high confidence, even in the presence of high background.

Several validated methods from the US FDA have demonstrated high selectivity, sensitivity, and quantitation of nitrosamine impurities in several drug products.[3,4,5] Given numerous recalled drug products, and the importance to control and limit the origin of these impurities in API and drug products, the US FDA has recently published guidance for the pharmaceutical industry to mandate risk assessment and implement control strategies to limit the formation of these impurities in all human drugs.[6] New guidelines also suggest to expand the nitrosamine panels to seven impurities, as well as establishing a new limit in which if more than one nitrosamine was detected in the drug products, the total nitrosamine contents cannot exceed 30 ppb, or more than 26.5 ng/day.

Here we describe a newly developed and validated LC-MS method using the Thermo Scientific™ Vanquish™ Horizon UHPLC system coupled to a Orbitrap Exploris 120 mass spectrometer to detect and quantify nine nitrosamines in a single analytical run. We also explore the use of an excipient formulation as an alternative matrix to a neat solution for the construction of the calibration plot and its use for measuring the nitrosamine impurities.

The method is performed using atmospheric pressure chemical ionization (APCI) and can comfortably quantify impurities below the newly recommended limit in both neat and excipient solution. Data acquisition and processing were carried out in compliance-ready Chromeleon CDS software to meet the regulatory requirements for user access, audit trails, data integrity, and data security. Lastly, this method was applied to detect and quantify nitrosamines in commercially available over-the-counter ranitidine drug products. This method is also applicable for the determination of nitrosamines in other drug products.

Experimental

Reagents and consumables

- Water, UHPLC-MS grade, Thermo Scientific (P/N W81)
- Methanol (MeOH), UHPLC-MS grade, Thermo Scientific (P/N A4581)
- Formic acid, Fisher Chemical™ Optima™ LC/MS solvent (P/N A117-10X1AMP)
- Ranitidine hydrochloride, Sigma-Aldrich (R101-5G)
- Nitrosamine reference standards (See Table 1)
- N,N-dimethylformamide (DMF), HPLC grade, Sigma-Aldrich (P/N 270547)
- Over-the-counter ranitidine 300 mg tablet

Sample preparation

Excipient formulation preparation: A formulation matrix was prepared to mimic ranitidine drug formulation ingredient by mixing 750 mg of microcrystalline cellulose, 30 mg of sodium croscarmellose, 30 mg of magnesium stearate, 2000 mg of hypromellose E15, and 1000 mg of lactose. All ingredients were provided by our partner at Patheon in Bend, Oregon.

Neat and excipient standards: Pooled neat standards ranging from 10 to 5000 ng/mL were prepared by diluting the 1 mg/mL stock solutions with pure methanol. A working solution of pooled internal standards at 500 ng/mL was prepared by diluting the 1 mg/mL stock solution with pure methanol. Neat standards ranging from 0.1 to 50 ng/mL were prepared by mixing 10 µL of working standards with 10 µL of working internal standards, followed by adding 980 µL of pure methanol.

Table 1. Nitrosamine reference standards

Standards	CAS	Vendor	P/N
N-Nitrosodiethylamine (NDEA)	55-18-5		
N-Nitrosodimethylamine (NDMA)	62-75-9		
N-Nitrosodi-n-butylamine (NDBA)	924-16-3		
N-Nitroso-di-n-propylamine (NDPA)	621-64-7	Restek	31898
N-Nitrosomethylethylamine (NMEA)	10595-95-6		
N-Nitrosopiperidine (NPIP)	100-75-4		
N-Nitrosopyrrolidine (NPYR)	930-55-2		
N-Ethyl-N-nitroso-2-propanamine (NEIPA)	16339-04-1	Enamine	EN300-1296534
N-Nitroso-di-isopropylamine (NDIPA)	601-77-4	Enamine	EN300-7456222
NDEA-D_{10}	55-18-5	Cambridge Isotope Laboratories	DLM-7982-S
NDMA-D_6	62-75-9	Restek	33910
NDBA-D_{18}	924-16-3	Cambridge Isotope Laboratories	D-6711-0.05g
NDPA-D_{14}	621-64-7	Cambridge Isotopes	DLM-2131-S
NMEA-D_3	10595-95-6	CDN Isotopes	D-6874-0.01g
NPIP-D_{10}	100-75-4	CDN Isotopes	D-4139-0.05g
NPYR-D_8	930-55-2	Cambridge Isotopes	DLM-8252-1.2
NEIPA-D_5	16339-04-1	Toronto Research Chemicals	E932796
NDIPA-D_{14}	601-77-4	Toronto Research Chemicals	N525602

Excipient standards ranging from 0.1 to 50 ng/mL were prepared by mixing 10 µL of working solution of pooled standards and 10 µL of working solution of pooled internal standards with 30 mg of excipient mixture, allowing it to dry at room temperature for 30 min, and resolubilizing it with 1 mL of methanol. The mixture was shaken for 40 min with a mechanical shaker, followed by centrifugation for 15 min at 4000 RPM. 750 µL of supernatant solutions were decanted and then filtered using a 0.2 µm PVDF syringe filter.

A 1 ppm DMF reference standard was prepared by performing: 1:1,000,000 dilution of the HPLC grade DMF with pure methanol. 5 µL of the reference standard was injected to verify the presence of DMF in ranitidine drug substance and product by matching retention time and monitored isotopic ions.

Ranitidine substance and drug product preparation:
Ranitidine substance extraction was carried out by resolubilizing 30 mg of ranitidine hydrochloride with 1 mL of methanol to make a final concentration of 30 mg/mL. 10 µL of 500 ng/mL internal standard was added to give a final concentration of 5 ng/mL.

300 mg of ranitidine tablet extract was prepared by mixing 10 mL of methanol with ground tablet to make a final concentration of 30 mg/mL. 100 µL of 500 ng/mL internal standard was added to give a final concentration of 5 ng/mL. The mixture was shaken for 40 min on a mechanical shaker, followed by centrifugation for 15 min at 4000 RPM. 750 µL of supernatant solutions were decanted and then filtered using a 0.2 µm PVDF syringe filter.

Sample recovery and extraction reproducibility evaluation: For sample recovery evaluation, the extracted excipient samples were prepared by spiking neat standards at 2 ng/mL and 5 ng/mL levels into blank excipient before the extraction process. For spiked excipient samples, the same neat standards were spiked into blank excipient after the extraction process. In both cases, internal standards were added after the extraction process. The peak area ratio (standard over internal standard) of the extracted excipient was compared with the peak area ratio of the spiked excipient for % recovery calculation.

LC-MS method

A single targeted LC-MS method was developed using a Vanquish Horizon UHPLC system coupled to an Orbitrap Exploris 120 mass spectrometer. 5 µL of samples were injected onto a Thermo Scientific™ Acclaim™ Polar Advantage II column using the LC gradient and conditions outlined in Table 2. All nitrosamines were analyzed using an APCI probe with optimized source parameters and scan settings as outlined in Tables 3 and 4, respectively.

Table 2. LC and autosampler conditions

Parameters	Value		
HPLC column	Acclaim Polar Advantage II, 100 × 2.1 mm, 2.2 µm (P/N 068990)		
Column temperature	40 °C, still air		
Flow rate	0.5 mL/min		
Mobile phase A	Water + 0.1% formic acid		
Mobile phase B	Methanol + 0.1% formic acid		
Gradient	*Time (min)*	*% Mobile phase A*	*% Mobile phase B*
	0.0	95	5
	0.5	95	5
	8.0	5	95
	9.0	5	95
	9.1	95	5
	12.0	95	5
Injection volume	5 µL		
Needle wash solution	80% Methanol with 0.1% formic acid		
Seal rinse solution	10% Methanol with 0.1% formic acid		
Autosampler temperature	4 °C		
Needle wash option	Before and after injection		
Wash speed and time	30 µL/s for 10s		

Table 3. MS ion source parameters

Parameter	Value
Ionization	APCI
Polarity	Positive
Spray current	2 µA
Sheath gas	45
Auxiliary gas	10
Sweep gas	0.5
Ion transfer tube temperature	200 °C
Vaporizer temperature	300 °C

Table 4. Scan settings

Parameter	Value	
Scan mode	tSIM	tMS^2
Polarity	Positive	Positive
Resolution	120,000	120,000
AGC target	1e5	1e5
Maximum IT	Auto	Auto
Isolation window	*m/z* 2.0	*m/z* 2.0
Scan range	Auto	Auto
RF lens %	100	100

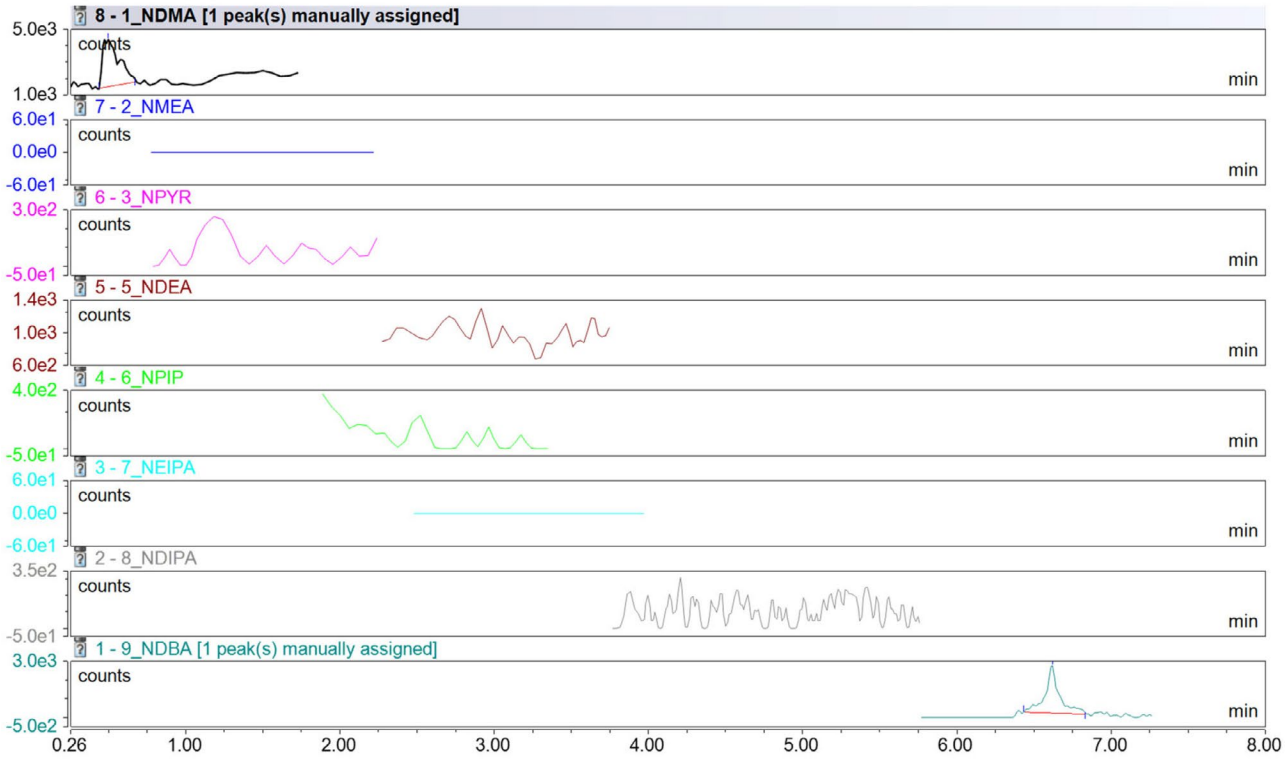
Compliance-ready Chromeleon 7.2.10 CDS software was used for both data acquisition and analysis to meet regulatory requirements including US FDA 21 CFR Part 11 and European Commission (EU) Annex 11.

Results and discussion

Selective determination of nitrosamine impurities

The method was operated in targeted selective ion monitoring (tSIM) and targeted tandem MS (tMS^2) mode (previously PRM) with fast scan speed (3 Hz at 120,000 resolution setting) for optimal sensitivity and selectivity of target nitrosamines (Table 5). Figure 1 shows a comparison of the extracted ion chromatogram (XIC) of each nitrosamine impurity in blank excipient with a 0.5 ng/mL check standard at a mass tolerance setting of 3 ppm. Other than NDBA, no other endogenous interferences in blank excipient extract were observed. Although using the current gradient conditions, the method could not baseline resolve the interferent from NDBA, the amount of interferent to NDBA was <20% at 0.1 ng/mL, lowest calibrator, and insignificant at 0.5 ng/mL.

a)



b)

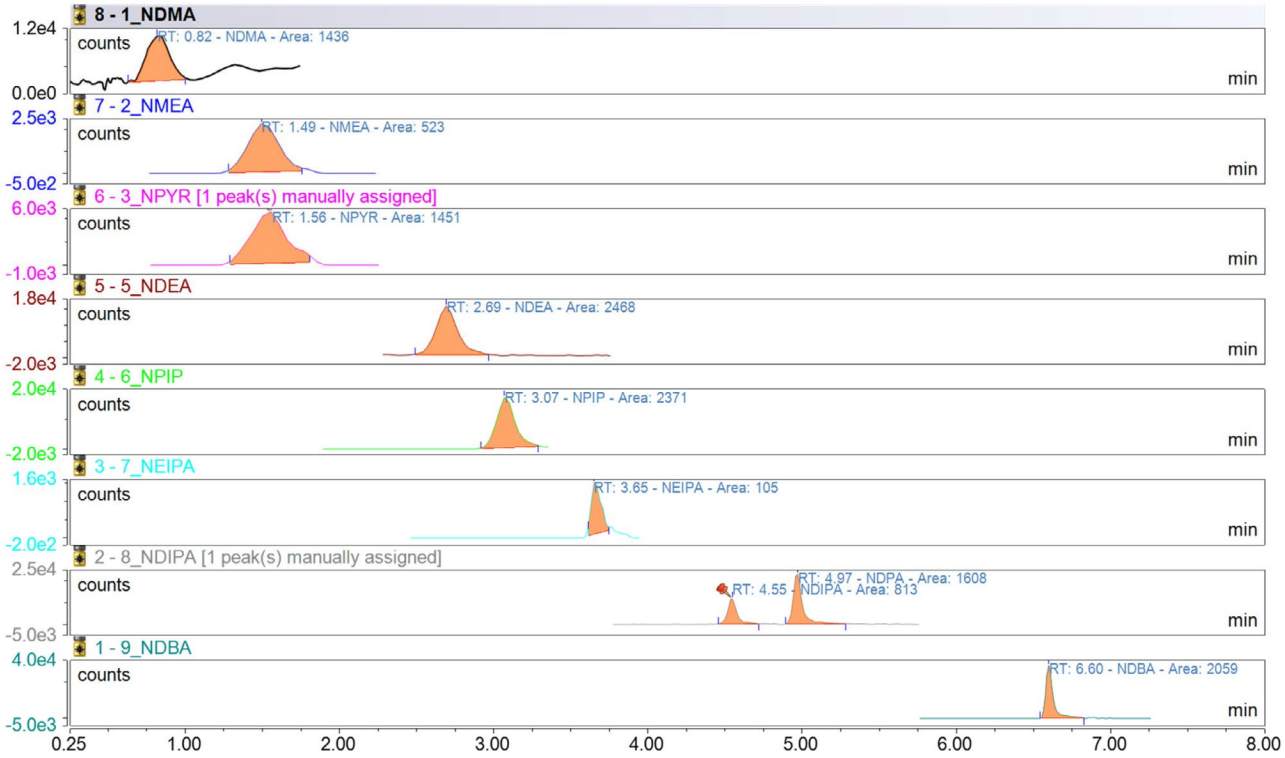


Figure 1. XIC of quantitation ion of nitrosamine impurities in a) blank excipient, and b) 0.5 ng/mL check standard at a mass tolerance setting of 3 ppm

Table 5. Optimized MS condition for target nitrosamines

	Scan type	Scan Start – End (min)	Polarity	*m/z* of Quan. ion	*m/z* of Qual. Ion	Normalized CE (%)
NDMA	tMS^2	0.25–1.75	Positive	75.0552	43.0290	60
NDMA-D_6	tMS^2	0.25–1.75	Positive	81.0928	46.0480	60
NMEA	tMS^2	0.75–2.25	Positive	61.0397	89.0708	15
NMEA-D_3	tMS^2	0.75–2.25	Positive	64.0585	92.0898	30
NPYR	tMS^2	0.75–2.25	Positive	101.0709	55.0540	60
NPYR-D_8	tMS^2	0.75–2.25	Positive	109.1212	62.0980	30
NDEA	tMS^2	1.95–3.45	Positive	103.0866	75.0550	45
NDEA-D_{10}	tMS^2	1.95–3.45	Positive	113.1493	81.0930	45
NPIP	tMS^2	2.35–3.85	Positive	115.0866	69.0699	60
NPIP-D_{10}	tMS^2	2.35–3.85	Positive	125.1494	78.1260	60
NEIPA	tMS^2	2.85–4.35	Positive	75.0553	117.1022	15
NEIPA-D_5	tMS^2	2.85–4.35	Positive	80.0866	122.1336	15
NDIPA	tSIM	3.75–5.25	Positive	131.1179	-	-
NDIPA-D_{14}	tSIM	3.75–5.25	Positive	145.2058	-	-
NDPA	tSIM	4.25–5.75	Positive	131.1179	-	-
NDPA-D_{14}	tSIM	4.25–5.75	Positive	145.2058	-	-
NDBA	tMS^2	5.75–7.25	Positive	159.1492	103.0866	15
NDBA-D_{18}	tMS^2	5.75–7.25	Positive	177.2622	66.1264	15

Sample extraction and recovery for the analysis of nitrosamines

A critical challenge for reliably detecting and quantifying trace levels of nitrosamine impurities in complex drug formulation matrices is the need to develop a reproducible extraction protocol to maximize sample extraction efficiency. In response to this challenge, sample recovery and reproducibility of the extraction process were evaluated by spiking the nitrosamine neat standards at 2 and 5 ng/mL levels into the blank excipient matrix before and after the extraction process. Table 6 shows typical recovery and reproducibility values for the extracted nitrosamines with five replicate injections. The recovery for all nitrosamines during the extraction process was between 95 and 105%, and the reproducibility of the replicate injections was within 10% RSD.

Table 6. Results of sample recovery and reproducibility analysis (n=5)

	2 ng/mL		5 ng/mL	
	% Recovery	% RSD	% Recovery	% RSD
NDMA	95	5.5	99	3.2
NMEA	96	6.3	98	1.2
NPYR	99	7.4	100	3.9
NDEA	97	2.4	98	3.2
NPIP	96	2.6	99	5.5
NEIPA	99	8.4	98	2.2
NDIPA	99	2.8	98	2.2
NDPA	95	4.4	96	8.4
NDBA	99	2.9	101	1.5

Achieving regulatory performance limits for the analysis of nitrosamines in drug products

With the newly recommended detection limits from the US FDA (less than 30 ppb for a total of 7 nitrosamines[6]), it is imperative for new methods to accurately and reproducibly detect and quantify nitrosamine impurities in drug products below that threshold level.

With the fast scanning speeds available on the Orbitrap Exploris 120 mass spectrometer, even at the highest resolution setting, all target nitrosamines at 17 ppb (0.5 ng/mL) could be detected and quantified. Table 7 shows a typical accuracy and precision of 17 ppb check standard in excipient.

Calibration curves were constructed by plotting the peak area ratios of standard over internal standard against the concentrations of standard, with a 1/× weighting. Figure 2 shows the calibration curve for all compounds, and Table 8 lists the instrument limits of detection (LOD), lowest limits of quantitation limits (LLOQ), and linearity values for all nitrosamines in excipient. In addition to excipient standards, LOD and LLOQ were also evaluated for all nitrosamines in a neat solution. The data are not shown here, but the regression coefficient (R^2) for all plots was above 0.99 and the resultant LOD and LLOQ for all compounds were almost identical between the two matrices, suggesting the method is resilient to any matrix effects for the tested formulation.

Table 7. Accuracy and precision of 0.5 ng/mL (17 ppb) check standard in excipient (n=5)

	%Accuracy	%RSD
NDMA	92	10.9
NMEA	95	4.2
NPYR	92	2.7
NDEA	95	3.4
NPIP	98	5.1
NEIPA	93	7.8
NDIPA	97	2.9
NDPA	96	3.0
NDBA	97	4.1

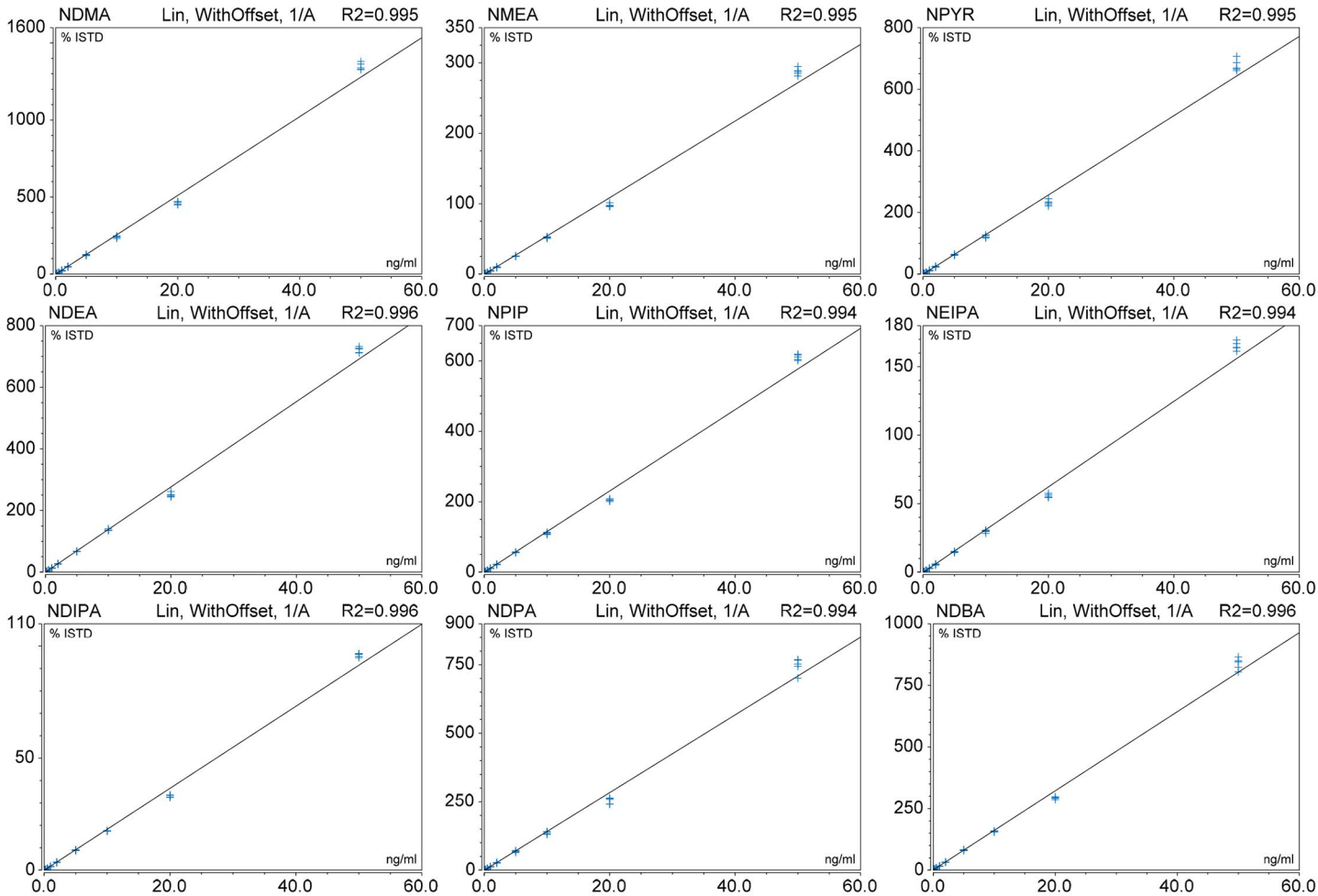


Figure 2. Calibration curves for all compounds in excipient

Table 8. Instrument LOD, LLOQ, and linearity for all nitrosamines in excipient containing samples

	LOD		LLOQ		
	ng/mL	PPB	ng/mL	PPB	Linearity
NDMA	0.2	6.8	0.2	6.8	
NMEA	0.2	6.8	0.2	6.8	
NPYR	0.2	6.8	0.2	6.8	
NDEA	0.1	3.4	0.1	3.4	
NPIP	0.2	6.8	0.2	6.8	LLOQ – 50
NEIPA	0.5	17.0	0.5	17.0	
NDIPA	0.1	3.4	0.1	3.4	
NDPA	0.1	3.4	0.1	3.4	
NDBA	0.1	3.4	0.5	3.4	

LOD defined as within 20% accuracy, and 15% RSD.

LOQ defined as within 15% accuracy, and 15% RSD.

PPB is calculated based on 30 mg/mL of drug substance and product extract.

Nitrosamine impurity levels in ranitidine drug product

Nitrosamines were quantified in commercially available ranitidine drug substances and tablets. The measured amount of NDMA in 30 mg/mL ranitidine drug substance exceeded the upper limit of calibration and was estimated to be more than 7 ppm (Figure 3a), whereas the measured amount of NDMA in 300 mg ranitidine tablet was 82 ppb (Figure 3b), both of which exceeded the acceptable regulatory limit. In both cases, the data was processed with a mass tolerance setting of 3 ppm. The quantitation was unaffected when reducing the mass tolerance setting to 1 ppm.

However, if data were processed with a mass tolerance setting >20 ppm (a typical mass tolerance achievable with time-of-flight mass spectrometers), this would result in a significant overestimation of NDMA concentrations due to co-elution with DMF. As shown in Figure 4a, the mass spectrum of NDMA at the apex contained two interfering ions, *m/z* of 75.05707 and 75.06340, matching the theoretical *m/z* of DMF ^{15}N isotope (75.05700) and ^{13}C isotope (75.06340) with sub-ppm mass accuracy. The presence of DMF was further confirmed by monitoring the monoisotopic mass of the molecular ion (*m/z* 74.0600) in addition to the mass of the two isotopes with an injection of the DMF reference standard. The resultant retention time was almost identical to the retention time of NDMA under current LC condition (data not shown). The mass difference between NDMA and DMF ^{15}N isotope is only 21 ppm, and DMF in pharmaceuticals is allowed to be up to 880 ppm as per the ICH Q3C(R6) guideline. A minimum resolution setting of 45,000 and a maximum mass tolerance of 15 ppm are required to prevent overestimation of NDMA when quantifying NDMA using the monoisotopic ion. As a hypothetical illustration shown in Figure 4b, the determined NDMA was 13% higher when processing the quantitation using a 25 ppm mass tolerance setting as supposed to 3 ppm (Figure 3b). This finding is consistent with the recently published US FDA article in The American Association of Pharmaceutical Scientists Journal, reporting several false-positive results for NDMA levels in tested metformin drug products due to the presence of co-eluting DMF, insufficient instrument mass accuracy and resolution, and inadequate mass tolerance setting for data processing.[7]

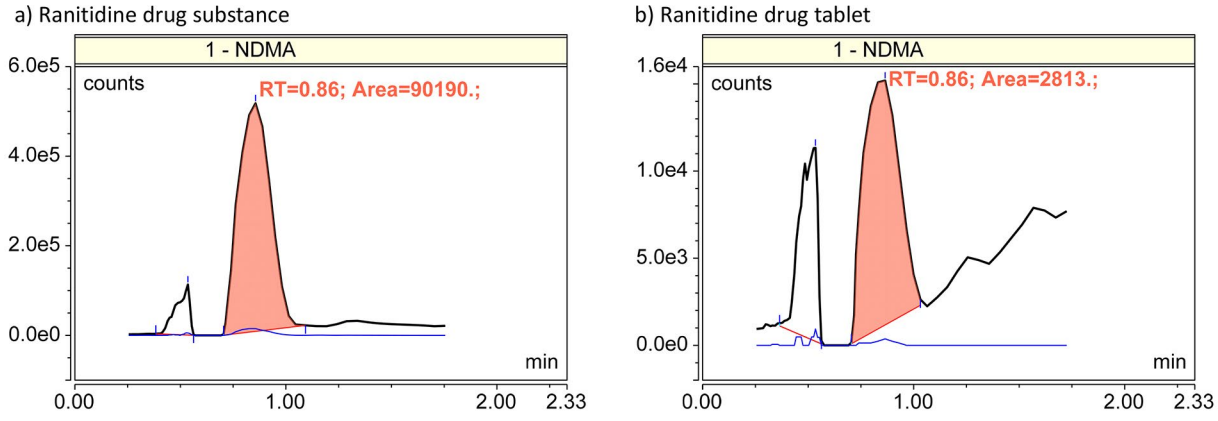


Figure 3. NDMA in ranitidine drug substance and tablet. Data processed with a mass tolerance setting of 3 ppm.

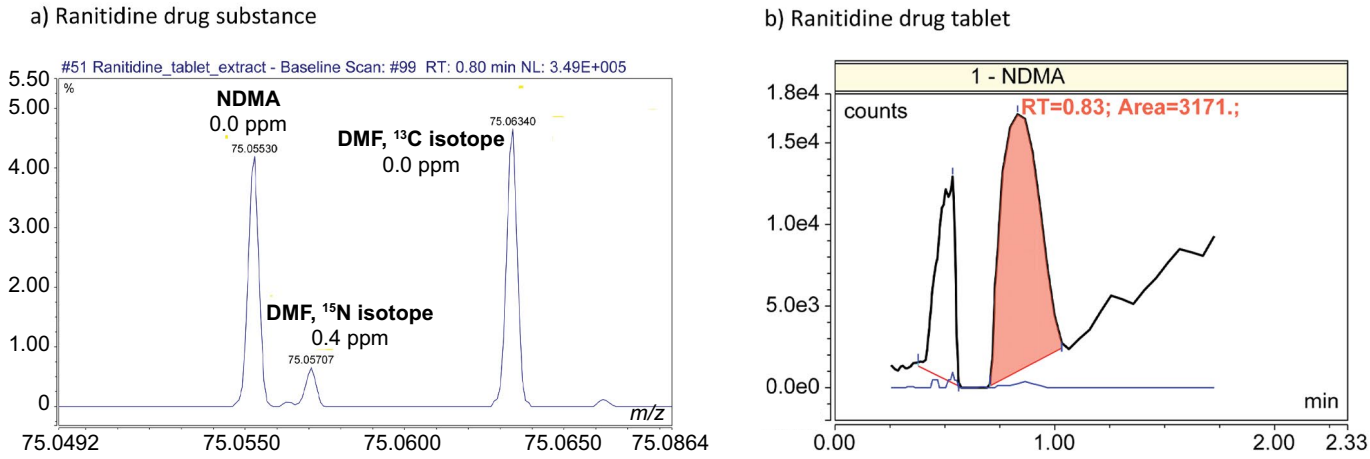


Figure 4. Mass spectrum of ranitidine drug tablet showing co-elution of NDMA and DMF, which could cause overestimation of NDMA when inadequate mass tolerance setting was used for data processing. a) Spectrum of ranitidine drug tablet sample containing NDMA and DMF with a resolution setting of 120,000; b) overestimation of NDMA when processing the data with mass tolerance set at 25 ppm; the resultant quantitation is 13% higher as compared with results obtained at 3 ppm (Figure 3b).

Conclusion

A rapid, highly selective, and sensitive method was developed using the Acclaim Polar Advantage II column, Vanquish Horizon UHPLC system, Orbitrap Exploris 120 mass spectrometer, and Chromeleon CDS software for detection and quantitation of nine nitrosamines in commercially available ranitidine drug products. By combining the robust and reproducible chromatography with the 120,000 mass resolving power, fast scanning speed, and sub-ppm mass accuracy of the Orbitrap Exploris 120 system, the resultant method can provide reliable and confident quantitation of nine nitrosamine impurities to meet the September 2020 US FDA regulatory acceptance limits.

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