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Pharma

Highly sensitive and robust LC-HRAM-MS method for simultaneous quantitation of sixteen nitrosamines in multiple drug products

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Keywords

Nitrosamine quantitation, drug products, high resolution, Orbitrap, single method, sartans, metformin, impurity analysis, genotoxic impurities (GTIs)

Goal

The aim of this study was to evaluate and report on the quantitative capabilities for the maximum number of genotoxic nitrosamine impurities using a single method employing a Thermo Scientific™ Vanquish™ Flex Quaternary UHPLC system coupled with a Thermo Scientific™ Q Exactive™ Plus Hybrid Orbitrap™ mass spectrometer in sartan and metformin drug samples (APIs and tablets).

Introduction

The sartan group of drugs is widely used for the treatment of high blood pressure, heart failure, kidney failure in diabetes, and chronic kidney diseases. The main drug products are Losartan, Valsartan, Irbesartan, Azilsartan, and Olmesartan.[1]

Metformin is a biguanide drug product and the first-line medication for the treatment of type 2 diabetes. Controlling high blood sugar helps prevent kidney damage, blindness, nerve problems, and loss of limbs.[2]

Nitrosamines are common in water and foods, including cured and grilled meats, dairy products, and vegetables. Everyone is exposed to some level of nitrosamines, however due to their classification as probable carcinogens (i.e., potential genotoxic impurities), the presence of nitrosamines in drug products has led to regulatory authorities issuing

guidance to ensure that the levels are kept within acceptable limits. Nitrosamine impurities are formed during production of metformin and sartans that contain a specific ring structure known as a tetrazole ring under certain conditions including the usage of certain solvents, reagents, and other raw materials (Figures 1 and 2). These impurities are classified as probable carcinogens (i.e., potential genotoxic impurities).

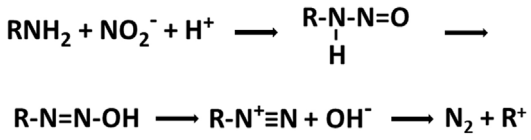

Figure 1. Nitrosamine formation from primary amines

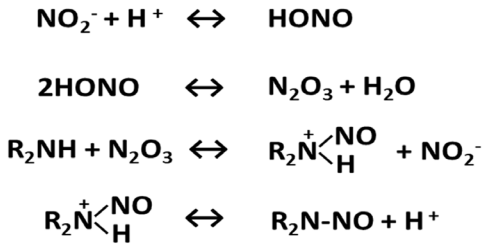

Figure 2. Nitrosamine formation from secondary amines

A dominant Valsartan API supplier in China reported the detection of *N*-nitroso dimethylamine (NDMA) in their product in July 2018. Investigations carried out by the European Medicines Agency and the U.S. FDA, showed that NDMA may cause cancer, and thus recall procedures for Valsartan drugs were started. Furthermore, the U.S. FDA found an additional unexpected genotoxic impurity, *N*-nitroso diethylamine (NDEA), in three batches of the recalled Valsartan drugs on September 13, 2018. The recall affected more than half of the United States' supply of the drug. Since then, additional nitrosamine impurities were detected in other drugs belonging to the sartans family. In September 2019, the U.S. FDA announced that preliminary tests also found low levels of NDMA in Ranitidine products, and large pharmaceutical companies announced recalls of the respective generic Ranitidine products.

The U.S. FDA imposes a maximum daily exposure to nitrosamines in different drugs in the range of low-sub ppm levels in the final product, but those levels are expected to decrease to non-detectable levels as soon as the manufacturing process is modified to avoid any nitrosamine formation.

The U.S. FDA has developed and published different methodology to detect these nitrosamine impurities in Ranitidine, Sartans, and Metformin recently using gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS), and liquid chromatography-high resolution mass spectrometry (LC-HRMS).[3-6] The method was published on quantitation of eight different impurities in Metformin using a single LC-HRMS/MS method.[2] However, following that method there were multiple demands and guidelines highlighting different lists of nitrosamine impurities.

In this regard, we document robust and highly sensitive LC-HRAM, parallel reaction monitoring (PRM), and targeted single ion monitoring (t-SIM) methods for the simultaneous quantitation of 16 different *N*-nitrosamine impurities for two different drug samples. The 16 impurities employed in the current study are *N*-nitroso dimethylamine (NDMA), *N*-nitroso-diethylamine (NDEA), *N*-ethyl-*N*-nitroso-2-propanamine (NEIPA), *N*-nitroso-diisopropylamine (NDIPA), *N*-nitroso-di-*n*-propylamine (NDPA), *N*-nitroso-methylphenylamine (NMPA), *N*-nitroso-di-*n*-butylamine (NDBA), *N*-nitroso-*N*-methyl-4-aminobutyric acid (NMBA), *N*-Nitroso-*N*-methylethylamine (NMEA), *N*-nitrosopiperidine (NPIP), *N*-nitroso diamyl amine (NDA), *N*-nitroso morpholine (NMOR), *N*-nitroso methyl isopropyl amine (NIPMA), *N*-nitrosodiphenylamine (NDPhA), *N*-nitrosopyrrolidine (NPYR), and *N*-nitroso-*N*-ethylaniline (NNEA).

Experimental

Reagents

- Water (H_2O), Fisher Chemical™ Optima™ LC/MS solvent (CAS: 7732-18-5) (P/N AAB-W6-4)
- Methanol (MeOH), Fisher Chemical™ Optima™ LC/MS solvent (CAS: 67-56-1) (P/N AAB-A456-4)
- Acetonitrile (MeCN), Fisher Chemical™ Optima™ LC/MS solvent (CAS: 75-05-8) (P/N A9554)
- Formic acid, Fisher Chemical™ Optima™ LC/MS solvent (CAS: 64-18-6) (P/N A117-50)
- Metformin tablets/API marketed product
- Valsartan tablets/API marketed product
- Nitrosamine Impurities Standard from Clean Chem (Details provided in Table 1)

Table 1. Nitrosamine impurity information

Abbreviation	Chemical name	Chemical formula	Monoisotopic mass
NDMA	*N*-nitroso-dimethylamine	$C_2H_6N_2O$	74.048012
NMEA	*N*-nitroso-methyl ethylamine	$C_3H_8N_2O$	88.06311
NMBA	*N*-nitroso-*N*-methyl-4-aminobutyric acid	$C_5H_{12}N_2O$	116.094963
NDEA	*N*-nitroso-diethylamine	$C_4H_{10}N_2O$	102.079315
NEIPA	*N*-ethyl-*n*-nitroso-2-propanamine	$C_5H_{12}N_2O$	116.094963
NDIPA	*N*-nitroso-diisopropylamine	$C_6H_{14}N_2O$	130.110611
NDPA	*N*-nitroso-di-*n*-propylamine	$C_6H_{14}N_2O$	130.110611
NMPA	*N*-nitroso-methylphenylamine	$C_7H_8N_2O$	136.06311
NPIP	*N*-Nitrosopiperidine	$C_5H_{10}N_2O$	115.08659
NIPMA	*N*-Nitroso methyl isopropyl amine	$C_4H_{10}N_2O$	103.08659
NNEA	*N*-nitroso-*N*-ethylaniline	$C_8H_{10}N_2O$	150.08659
NDPhA	*N*-Nitrosodiphenylamine	$C_{12}H_{10}N_2O$	199.08659
NMOR	*N*-Nitroso Morpholine	$C_4H_8N_2O_2$	
NPYR	*N*-nitrosopyrrolidine	$C_4H_8N_2O$	101.07094
NDBA	*N*-nitroso-di-*n*-butylamine	$C_8H_{18}N_2O$	159.14919
NDA	*N*-Nitroso diamyl amine	$C_{10}H_{22}N_2O$	187.18049

Equipment

- Analytical balance, Sartorius, model no: BSA2245-CBJ
- Vortex mixer, Tarsons, SPINIX™ Vortex Shaker
- 15 mL centrifuge tubes, Axygen™, SCT-15ML25-S
- Mechanical shaker or vortex mixer Tarsons, SPINIX™ Vortex Shaker
- Thermo Scientific™ Choice™ PVDF (hydrophilic) syringe filters, 0.2 µm pore size, 25 mm diameter, 100 pk (P/N CH2225-PV)
- Refrigerated centrifuge, Thermo Scientific™ Heraeus™ Megafuge™ 40R centrifuge
- Thermo Scientific™ SureSTART™ HPLC vials

Sample preparation

Each nitrosamine impurity's certified reference material standard was used to prepare the stock solution by dissolving 10 mg of the respective standard in 10 mL of methanol (LC-MS grade). Furthermore, a 100 ng/mL intermediate working standard solution was prepared in methanol through a serial dilution approach. The intermediate working solution (100 ng/mL) was further diluted to prepare the linearity solution(s) in the range of 0.3 to 50 ng/mL. Solutions for recovery studies were prepared using a similar approach.

The required number of tablets were powdered using a mortar and pestle, reconstituted in LC-MS grade 100% methanol to obtain a target concentration of 100 mg/mL of API. Samples were subjected to mechanical shaking for about 45 minutes. Extracted sample solutions were centrifuged for 15 minutes at 4,500 rpm and filtered through 0.22 µm PVDF syringe membrane filter. These filtered sample solutions were injected into the LC-HRMS for further analysis.

LC-HRMS conditions

A Vanquish Flex Quaternary UHPLC system coupled to a Q Exactive Plus Orbitrap mass spectrometer was used for the data acquisition. UHPLC configurations and parameters are listed in Table 2, and the mass spectrometer instrument and acquisition parameters for the nitrosamine impurities are listed in Tables 3 and 4.

Data analysis

All nitrosamine standards and drug samples were analyzed using the LC-HRMS method described in Tables 1 and 2. Data analysis was performed using Thermo Scientific™ Chromeleon™ 7.2.10 Chromatography Data System (CDS) software, which is a globally accepted gold standard for acquiring, analyzing, and reporting LC-MS and LC-HRMS datasets as per compliance requirements. The extraction parameters are given in Table 5.

Table 2. Liquid chromatography configuration and parameters

Parameter	Value
UHPLC	Thermo Scientific™ Vanquish™ Flex Quaternary UHPLC system with 25 µL sample loop Vanquish Quaternary Pump F (P/N VF-P20-A), Mixer volume: 350 µL Vanquish Split Sampler FT (P/N VF-A10-A) Vanquish Column Compartment H (P/N VH-C10-A) Vanquish Diode Array Detector HL (P/N VH-D10-A)
Analytical column	Thermo Scientific™ Hypersil GOLD™ 5 µm, 250 × 4.6 mm column (P/N 25005-254630)
Isolator column	Thermo Scientific™ Hypercarb™ 5 µm, 100 × 4.6 mm column (P/N 35005-104630)
Mobile phase	A: 0.1% Formic acid in water (degassed) B: 0.1% Formic acid in methanol (degassed)
Flow rate	0.400 mL/min
Gradient	Time (min) %A %B 00.0 86 14 05.0 86 14 07.0 50 50 11.0 33 67 16.0 33 67 19.0 20 80 30.0 20 80 30.1 86 14 35.0 86 14
Diluent	100% Methanol, with 99.9 purity
Autosampler (°C)	5
Column temperature (°C)	40 (Forced-air mode)
Needle wash	95:5 Acetonitrile:water (v:v)
Injection volume (µL)	10.0
Run time (min)	35

Table 3. Q Exactive Plus mass spectrometer instrument HESI source parameters

Ion source parameter	Value
Ion source type	HESI-II (ESI source)
Spray voltage (V)	3,500 (Pos) and 2,500 (Neg)
Sheath gas (arb)	45
Auxiliary gas (arb)	15
Sweep gas (arb)	0
Ion transfer tube temp. (°C)	250
Vaporizer temp. (°C)	400
S-lens (V)	55

Table 4a. MS acquisition parameters (impurities 1–8)

	Impurity number							
	1	2	3	4	5	6	7	8
Impurity name	NDMA	NMBA	NMEA	NDEA	NEIPA	NDIPA	NDPA	NMPA
Scan type	PRM	t-SIM	PRM	PRM	PRM	t-SIM	t-SIM	t-SIM
Polarity	Positive	Negative	Positive	Positive	Positive	Positive	Positive	Positive
m/z isolated	75.055	145.062	89.07	103.087	117.102	131.118	131.118	137.071
NCE	80	NA	15	60	10	NA	NA	NA
Isolation Width (*m/z*)	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Micro scans	3	3	3	3	3	3	3	3
Resolution	35,000	70,000	35,000	35,000	35,000	70,000	70,000	70,000
AGC target	2.0E+05	1.0E+06	2.0E+05	1.0E+06	2.0E+05	1.0E+06	1.0E+06	1.0E+06
Max. IT (ms)	100	100	100	100	100	100	100	100

Table 4b. MS acquisition parameters (impurities 9–16)

	Impurity number							
	9	10	11	12	13	14	15	16
Impurity name	NDBA	NPIP	NIPMA	NPYR	NMOR	NDA	NNEA	NDPhA
Scan type	PRM	PRM	PRM	PRM	PRM	PRM	t-SIM	PRM
Polarity	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
m/z isolated	159.149	115.087	103.087	101.071	117.066	187.181	151.087	199.087
NCE	50	60	45	95	10	50	NA	45
Isolation Width (*m/z*)	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Micro scans	3	3	3	3	3	3	3	3
Resolution	35,000	35,000	35,000	35,000	35,000	35,000	70,000	35,000
AGC target	2.0E+05	2.0E+05	1.0E+06	2.0E+05	1.0E+06	2.0E+05	1.0E+06	1.0E+06
Max. IT (ms)	100	100	100	100	100	100	100	100

Table 5a. Extraction parameters (impurities 1–8)

	Impurity number							
	1	2	3	4	5	6	7	8
Impurity name	NDMA	NMBA	NMEA	NDEA	NEIPA	NDIPA	NDPA	NMPA
Scan type	PRM	t-SIM	PRM	PRM	PRM	t-SIM	t-SIM	t-SIM
m/z to extract	75.055	145.062	61.039	103.086	75.055	131.118	131.118	137.071
RT (min)	8.9	12.1	14.06	18.36	19.81	21.16	21.83	21.46

Tolerance used for *m/z* extraction is 15 ppm (as per U.S. FDA method)[1]

Table 5b. Extraction parameters (impurities 9–16)

	Impurity number							
	9	10	11	12	13	14	15	16
Impurity name	NDBA	NPIP	NIPMA	NPYR	NMOR	NDA	NNEA	NDPhA
Scan type	PRM	PRM	PRM	PRM	PRM	PRM	t-SIM	PRM
m/z to extract	103.087 159.149	69.069	61.039	55.054	117.066	55.397 71.085	151.087	169.088
RT (min)	27.05	18.88	18.17	14.17	11.03	31.44	22.96	27.71

Tolerance used for *m/z* extraction is 15 ppm (as per U.S. FDA method)[1]

Results and discussion

Representative chromatograms of nitrosamine impurities and drug matrices are shown in Figures 3–8.

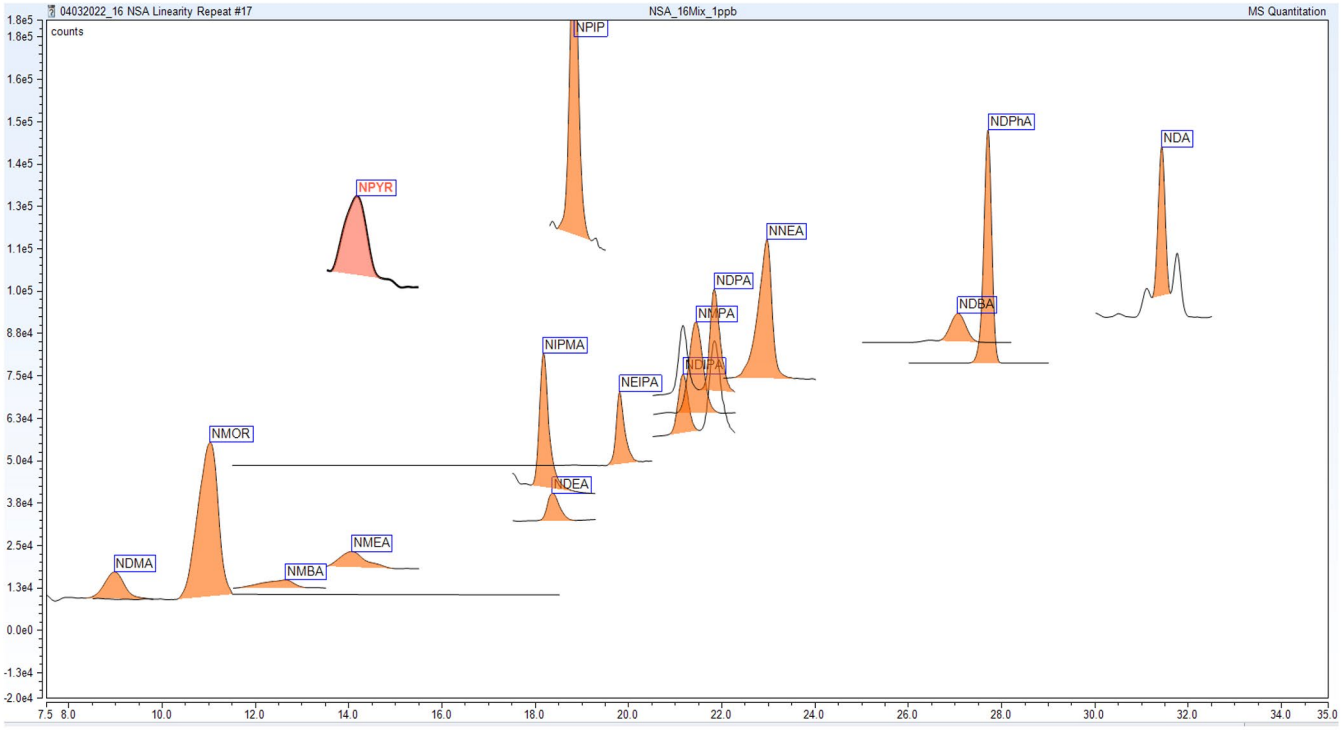

Figure 3. Extracted ion chromatograms for all 16 impurities in a single window

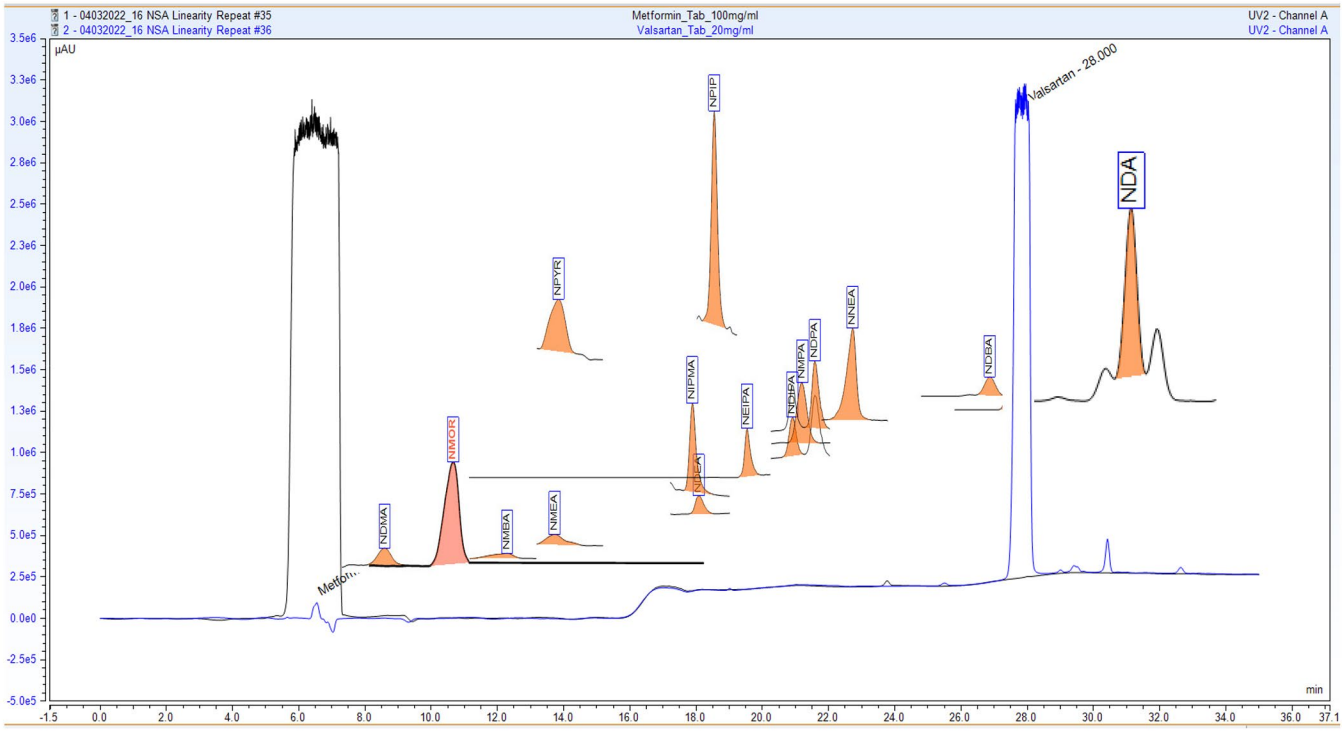

Figure 4. Overlay view of MS and UV chromatograms of nitrosamine impurities with Sartan and Metformin

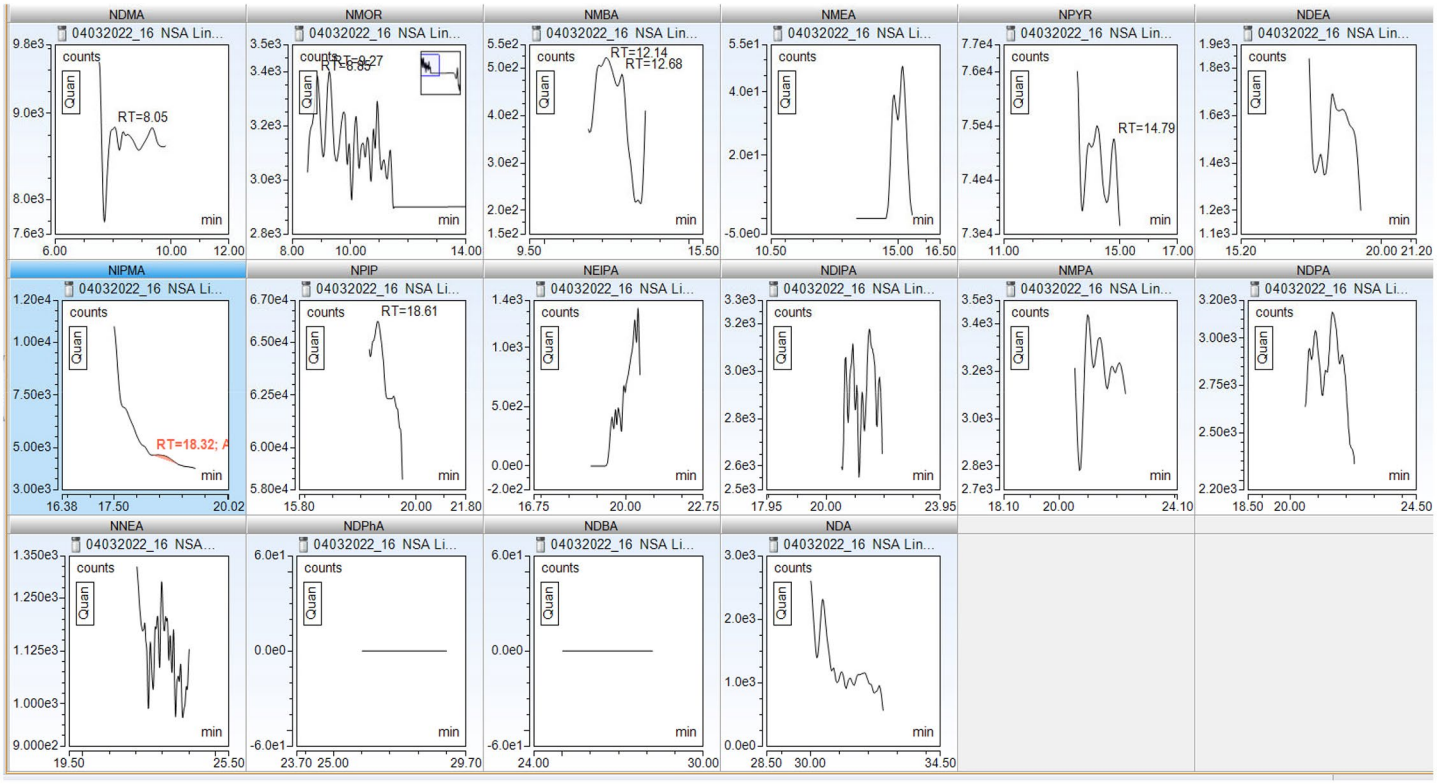

Figure 5. EICs of solvent blank injections

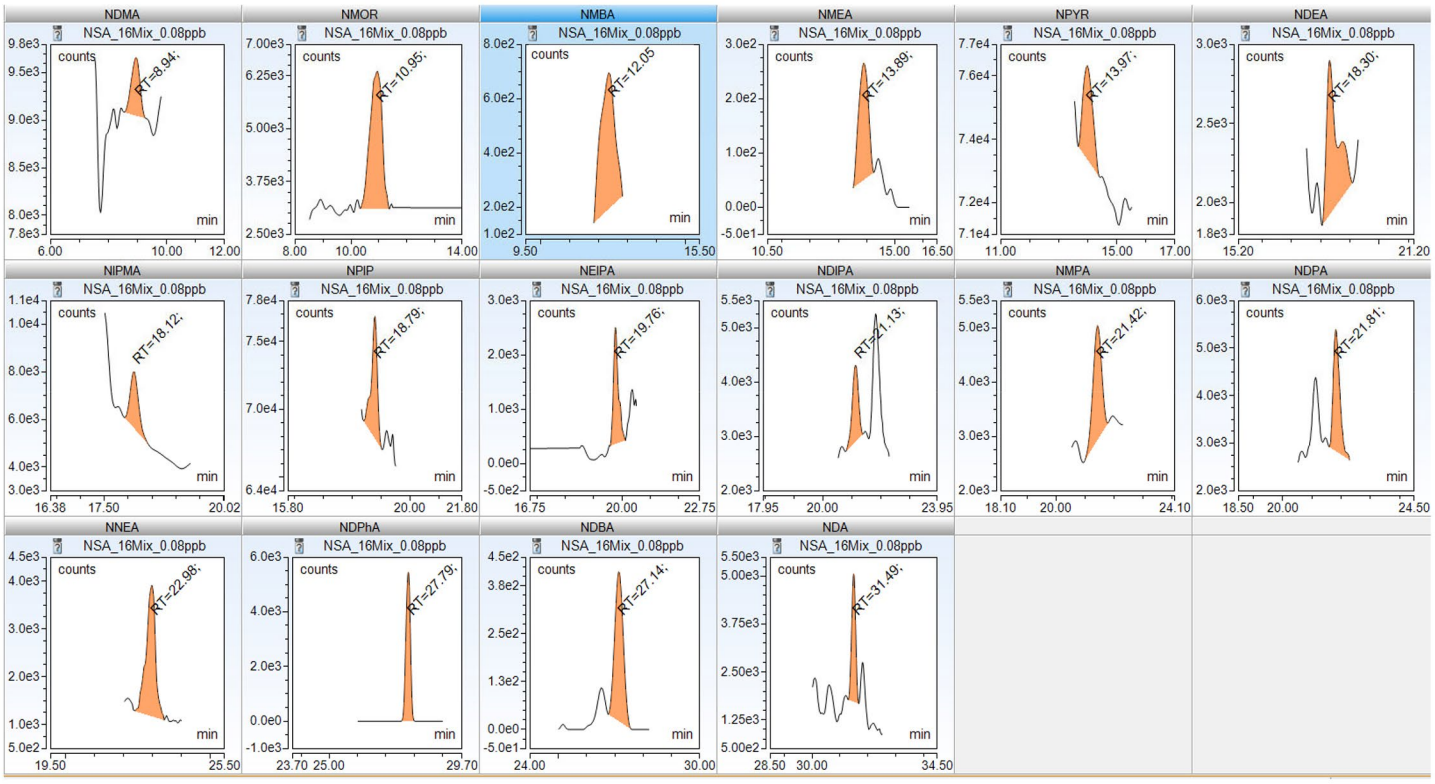

Figure 6. EICs of nitrosamine standard injection at respective LOD levels (0.08 ppb)

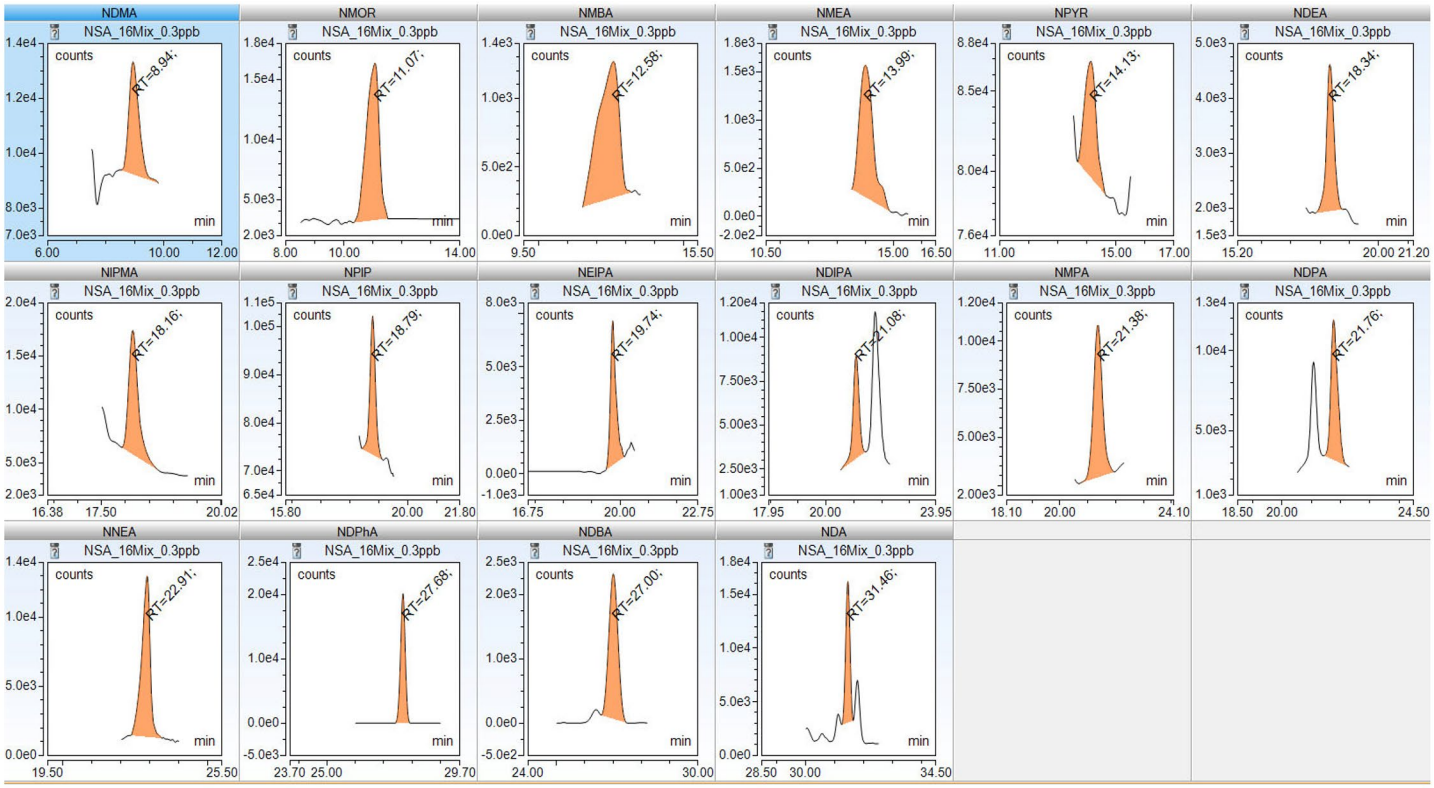

Figure 7. EICs of nitrosamine standard injection at respective LOQ levels (0.3 ppb)

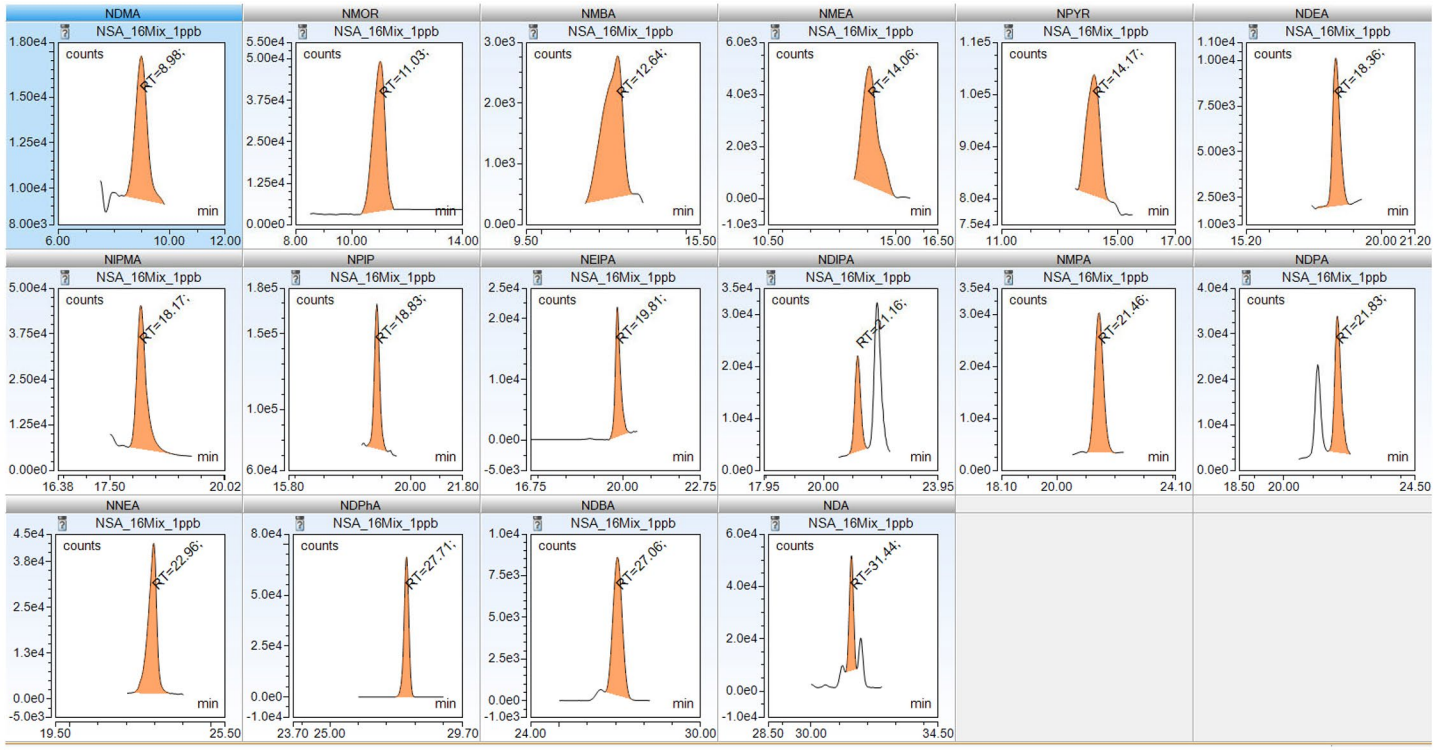

Figure 8. EICs for nitrosamine standard injections at 1 ng/mL (1ppb)

Linearity

The linearity plot employing neat impurity standard concentrations of 0.3, 0.5, 1, 3.15, 6.25, 12.5, 25, and 50 ng/mL is shown below (Figure 9). R^2 values were greater than 0.999 for all the impurity standards, displaying linear responses throughout the concentration ranges.

The chromatographic performance for all impurities was attained using the high-resolution Q Exactive Plus Hybrid Orbitrap MS instrument. In this study, we have demonstrated the specificity and sensitivity of the instrument to detect ultralow levels of nitrosamine contents with very good signal to noise (S/N) and peak shape.

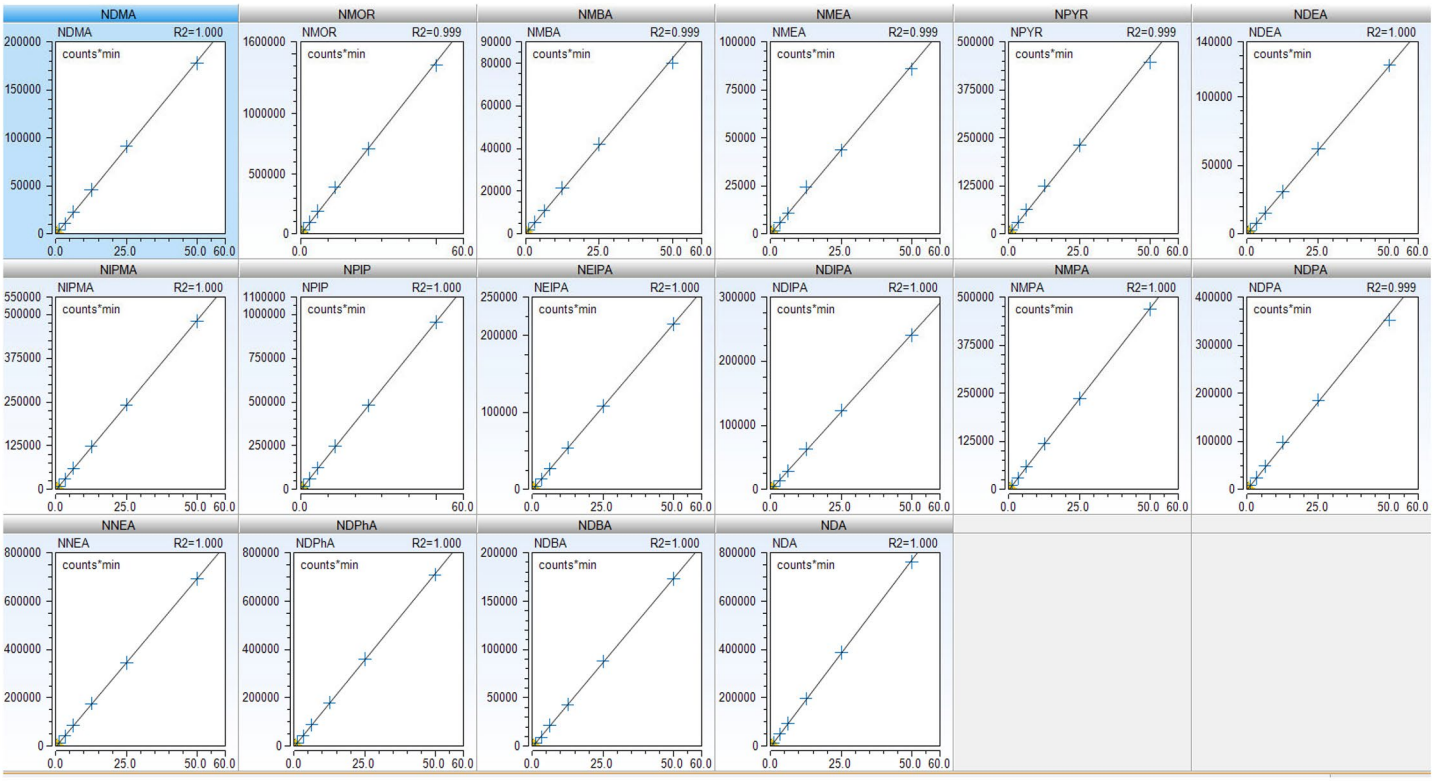

Figure 9A. Linearity curve plot of nitrosamine impurity standards (0.3–50 ng/mL)

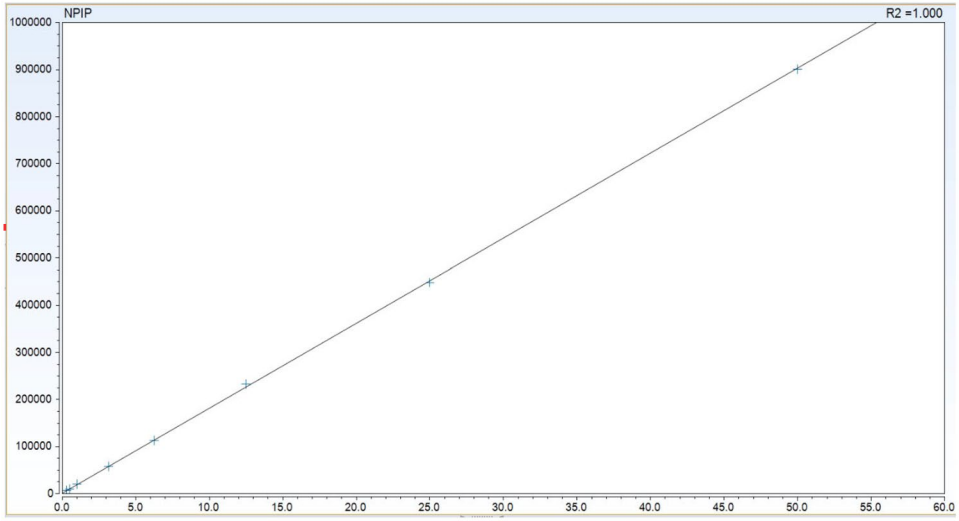

Figure 9B. Zoom in of linearity curve plot of NPIP standard (0.3–50 ng/mL)

Table 6. Quantitative data analysis output of the 16 nitrosamine impurities

Compound	LOD		LOD	LOQ		LOQ	R^2	Weighting factor	Linearity range	
	ng/mL	ppm	S/N	ng/mL	ppm	S/N			ng/mL	ppm
NDMA	0.08	0.008	20	0.3	0.003	46	0.999	1/X	0.3–50	0.003–0.5
NMEA	0.08	0.008	25	0.3	0.003	49	0.999			
NMBA	0.08	0.008	21	0.3	0.003	33	0.999			
NDEA	0.08	0.008	19	0.3	0.003	65	1.000			
NEIPA	0.08	0.008	46	0.3	0.003	172	1.000			
NDIPA	0.08	0.008	67	0.3	0.003	137	1.000			
NDPA	0.08	0.008	37	0.3	0.003	287	0.999			
NMPA	0.08	0.008	39	0.3	0.003	62	1.000			
NDBA	0.08	0.008	44	0.3	0.003	247	1.000			
NPIP	0.08	0.008	18	0.3	0.003	56	1.000			
NIPMA	0.08	0.008	121	0.3	0.003	192	1.000			
NPYR	0.08	0.008	46	0.3	0.003	104	0.999			
NMOR	0.08	0.008	48	0.3	0.003	137	0.999			
NDA	0.08	0.008	44	0.3	0.003	102	1.000			
NNEA	0.08	0.008	28	0.3	0.003	172	1.000			
NDPhA	0.08	0.008	34	0.3	0.003	85	1.000			

Discussion and summary

Key outputs attained in the current study are as below:

- In this study using a Vanquish Flex Quaternary UHPLC coupled with a Q Exactive Plus Orbitrap MS, we were able to confidently measure the contents of 16 nitrosamine impurities for two different matrices (Valsartan and Metformin) in a single method. In the described method, Valsartan was observed to elute after 24.5 min and Metformin before 6.8 min.
- This method has been optimized and evaluated to quantify the possible lowest levels of the mentioned nitrosamines.
- The % RSD of LOQ and standard concentration levels of each nitrosamine's impurities were found to be less than 10% (Table 7).
- Recovery has been performed at three different levels including the 1.0 ng/mL (standard) level. The results were within permissible limit (80–120%) (Tables 8 and 9).

Table 7a. Reproducibility of the method attained using six replicate injections of nitrosamine impurity mix at LOQ and standard levels. Compounds 1–8.

#	Reproducibility data							
	NDMA	NMOR	NMEA	NMBA	NPYR	NIPMA	NPIP	NDPA
Standard %RSD	4.8	2.6	7.4	6.1	8.4	1.9	3.9	2.8
LOQ %RSD	7.5	2.7	3.7	6.4	8.3	5.1	2.5	3.9

Table 7b. Reproducibility of the method attained using six replicate injections of nitrosamine impurity mix at LOQ and standard levels. Compounds 9–16.

#	Reproducibility data							
	NNEA	NDPhA	NMPA	NDBA	NDEA	NEIPA	NDIPA	NDA
Standard %RSD	1.7	1.0	2.4	1.3	4.2	1.8	3.4	3.7
LOQ %RSD	2.5	2.8	5.2	3.4	7.0	3.2	0.9	3.3

Table 8a. Recovery results for the 16 nitrosamine impurities in Metformin drug sample. Compounds 1–8.

Levels	Recovery results for Metformin drug matrix							
	NDMA	NMOR	NMEA	NMBA	NPYR	NIPMA	NPIP	NDPA
%RSD Low level (0.5 ng/mL)	81.2	88.5	102.3	104.3	120.0	112.0	118.0	119.3
%RSD Mid level (1 ng/mL)	87.2	91.2	103.8	92.3	101.6	100.7	102.6	107.5
% RSD High level (2 ng/mL)	89.7	96.0	101.2	91.7	107.9	103.5	98.6	110.9

Table 8b. Recovery results for the 16 nitrosamine impurities in Metformin drug sample. Compounds 9–16.

Levels	Recovery results for Metformin drug matrix							
	NNEA	NDPhA	NMPA	NDBA	NDEA	NEIPA	NDIPA	NDA
%RSD Low level (0.5 ng/mL)	116.0	115.2	110.2	119.1	102.8	92.3	101.6	115.6
%RSD Mid level (1 ng/mL)	106.0	103.2	100.4	108.4	93.2	85.9	102.0	112.3
% RSD High level (2 ng/mL)	106.7	102.3	106.3	112.7	94.5	92.2	106.6	115.5

Table 9a. Recovery results for the 16 nitrosamine impurities in Valsartan drug sample. Compounds 1–8.

Levels	Recovery results for Valsartan drug matrix							
	NDMA	NMOR	NMEA	NMBA	NPYR	NIPMA	NPIP	NDPA
%RSD Low level (0.5 ng/mL)	111.8	101.0	115.1	96.2	93.6	118.9	116.6	112.7
%RSD Mid level (1 ng/mL)	89.3	105.9	97.5	97.1	110.5	107.0	101.8	101.8
% RSD High level (2 ng/mL)	85.5	98.6	91.5	87.7	107.2	104.2	97.7	96.7

Table 9b. Recovery results for the 16 nitrosamine impurities in Valsartan drug sample. Compounds 9–16.

Levels	Recovery results for Valsartan drug matrix							
	NNEA	NDPhA	NMPA	NDBA	NDEA	NEIPA	NDIPA	NDA
%RSD Low level (0.5 ng/mL)	120.9	NA	105.5	108.3	112.4	106.5	115.5	114.9
%RSD Mid level (1 ng/mL)	98.8	NA	92.5	94.5	90.2	93.5	110.9	98.6
% RSD High level (2 ng/mL)	92.1	NA	92.8	100.1	89.0	94.1	104.6	81.6

Conclusion

The Q Exactive Plus Orbitrap MS instrument can be employed to simultaneously identify and quantify 16 different nitrosamine impurities at very low concentrations as per regulatory requirements. In view of its capability to deliver high resolution and accurate mass, the Q Exactive Plus Orbitrap MS can be confidently employed to resolve near isobaric interferences from the analytes of interest. Such unique capabilities of this platform highlight its routine utility for small molecule quantitation such as nitrosamines. The method is highly sensitive, robust, and reproducible even at lower concentrations utilizing only 10 µL injection volume.

References

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