Application Note

Pharmaceutical Small Molecules

Simultaneous Determination of Eight Nitrosamine Impurities in Metformin Using the Agilent 6470 Triple Quadrupole LC/MS

Detection of regulated genotoxic impurities from the drug manufacturing process

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Abstract

The determination of nitrosamine impurities in drug substances and drug products is a critical regulatory requirement, with required sensitivity limits posing immediate challenges in developing sensitive analytical methods. The list of APIs and drug products for nitrosamine determination has expanded beyond angiotensin II receptor blocker (ARB) drugs, and is evident from the recent recalls of metformin by various regulatory bodies such as the FDA, EDQM, and Health Sciences Authority (HSA) due to the presence of NDMA. This application note has developed a highly sensitive triple quadrupole-based LC/MS/MS method for the simultaneous determination of eight nitrosamine impurities in metformin drug substance. Metformin is an antihyperglycemic agent of the biguanide class, used for the management of type II diabetes. This application note describes a highly selective and sensitive LC/MS/MS method using the Agilent 6470 triple quadrupole LC/MS for the detection and quantification of N-nitrosodimethylamine (NDMA), N-nitrosodiethylamine (NDEA), N-nitrosoethylisopropylamine (NEIPA), N-nitroso-N-methyl-4-aminobutyric acid (NMBA), N-nitrosodiisopropylamine (NDIPA), N-nitrosomethylphenylamine (NMPA), N-nitrosodi-*n*-propylamine (NDPA), and N-nitrosodibutylamine (NDBA) impurities in metformin drug substance.

Introduction

Nitrosamine impurities (Figure 1) recently became a focus for regulatory agencies such as the U.S. Food & Drug Administration (US FDA) and European Medicines Agency (EMA), when the US FDA announced a recall of ARB drug products such as losartan and valsartan, due to the potential for these products to contain one of the nitrosamine impurities. Metformin was also added to the recall list due to the presence of NDMA, which has started initially with Singapore's Health Sciences Authority, followed by the European Directorate for the Quality of Medicines (EDQM) and US FDA. These nitroso compounds are classified as probable human carcinogens and are believed to have been introduced into finished medicines as trace-level by-products of the manufacturing process.

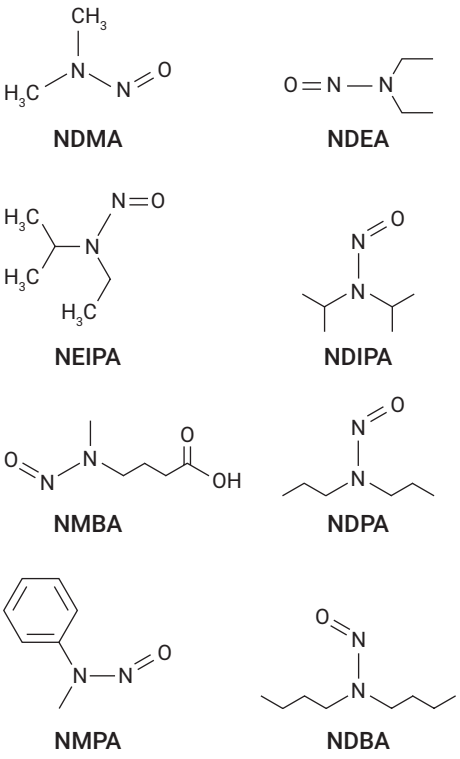

Figure 1. Chemical structures of nitrosamine impurities studied.

Liquid chromatography/tandem mass spectrometry (LC/MS/MS)-based methods are generally very specific and highly sensitive. For this reason, these have served as the basis for development of this method to detect and quantify eight nitrosamine impurities in metformin drug substance. The method described in this application note was carried out on the 6470 LC/TQ, providing a comprehensive analysis of eight nitrosamine impurities at very low detection limits.

Experimental

Chemicals and reagents

All the nitrosamine standards (NDMA, NDEA, NMBA, NEIPA, NDIPA, NMPA, NDPA, and NDBA) used in this study were locally sourced from PS3 Labs LLP (Hyderabad, TS, India). Other LC/MS-grade solvents (e.g., methanol, water) were purchased from Honeywell (Charlotte, NC, USA). Formic acid was purchased from Fluka (now of Honeywell).

Sample preparation

Metformin drug substance: 100 mg of metformin drug substance was weighed and placed in a 15 mL centrifuge tube. Thorough dissolution was done by adding 5 mL of solvent and vortexing until all visible particles had dissolved.

LC configuration and parameters

Table 1. UHPLC configuration and settings.

Parameter	Value
Instruments	Agilent 1290 Infinity II high-speed pump (G7120A) Agilent 1290 Infinity II multisampler (G7167B) Agilent 1290 Infinity II multicolumn thermostat (G7116B) Agilent 1290 Infinity II variable wavelength detector (G7114B)
Needle Wash	Methanol/water (80/20)
Sample Diluent	Water/methanol (95/5)
Multisampler Temperature	10 °C
Injection Volume	20 µL
Analytical Column	Agilent InfinityLab Poroshell HPH-C18, 4.6 × 150 mm, 2.7 µm (p/n 693975-702(T))
Column Temperature	40 °C
Mobile Phase A	0.1 % formic acid in water
Mobile Phase B	0.1 % formic acid in methanol
Flow Rate	0.5 mL/min
Gradient	Time (min) % A % B Flow (mL/min) 0 95 5 0.5 2 95 5 0.5 7 40 60 0.5 10 25 75 0.5 11 10 90 0.5 16.5 10 90 0.5 16.6 95 5 0.5 20.0 95 5 0.5
Stop Time	20 minutes
UV Wavelength	230 nm

Data analysis

Data were acquired and analyzed using the Agilent MassHunter Acquisition software version 10. MRM transitions were obtained and optimized using the Agilent MassHunter Acquisition Optimizer software to determine the optimal precursor and product ions, fragmentor voltages, and collision energies upon injection of a neat solution at a concentration level of 1,000 ng/mL, 1 µL injection volume in flow injection mode.

Results and discussion

Method development was performed using different columns and gradient conditions for the optimization of chromatographic separation between metformin and NDMA. This step is critical to reduce the matrix effects from the API on the targeted compounds. Additionally, separation must be achieved between NDIPA and NDPA because these are positional isomers and have isobaric mass. Instrument MS/MS parameters were optimized to maximize sensitivity. Critical parameters such as specificity, reproducibility, linearity, recovery, LOQ, and LOD were characterized to ensure the method performance.

Triple quadrupole mass spectrometer configuration and parameters

Table 2. Mass spectrometer configuration and source settings.

Parameter	Value
Instrument	Agilent 6470 triple quadrupole LC/MS (G6470A)
Ion Source	Atmospheric pressure chemical ionization (APCI)
MS/MS Mode	MRM
Ion Mode	Positive
Drying Gas Temperature	300 °C
Drying Gas Flow	7 L/min
Nebulizer Pressure	25 psi
APCI Heater	350 °C
APCI Needle Positive	4 µA
Capillary Voltage, Positive	4000 V
MS1/MS2 Resolution	0.7/0.7 (unit/unit)
Dwell Time	50 ms

MS/MS compound information for analytes

Table 3. Detailed MRM settings in MRM mode in 6470 LC/TQ.

Compound	Precursor Ion (*m/z*)	Product Ion (*m/z*)	Dwell Time (ms)	Fragmentor (V)	Collision Energy (V)	CAV (V)	Polarity
NDMA (quantifier)	75	43.1	50	110	16	3	+
NDMA (qualifier)	75	58	50	80	10	2	+
NMBA (quantifier)	147	117	50	60	4	2	+
NMBA (qualifier)	147	44	50	60	12	2	+
NDEA (quantifier)	103	75	50	78	12	4	+
NDEA (qualifier)	103	47	50	78	20	4	+
NEIPA (quantifier)	117	74.9	50	82	8	8	+
NEIPA (qualifier)	117.1	47.1	50	82	15	8	+
NDIPA (quantifier)	131	89.1	50	80	5	4	+
NDIPA (qualifier)	131	43	50	80	20	4	+
NDPA (quantifier)	131	89.1	50	80	5	4	+
NDPA (qualifier)	131	43	50	80	20	4	+
NMPA (quantifier)	137	66	50	70	20	5	+
NMPA (qualifier)	137	107	50	70	10	5	+
NDBA (quantifier)	159.1	57	50	86	12	4	+
NDBA (qualifier)	159.1	41.3	50	86	20	4	+

Table 4. Diverter valve program used to divert metformin peak to waste.

Program Number	Start Time (min)	Scan Type	Diverter Valve
1	0	MRM	Waste
2	4.4	MRM	MS

Reproducibility data including bracketing standards are captured in Table 5. LOQ and LOD limits and signal-to-noise (S/N) values are captured in Tables 6 and 7. The calibration concentrations ranged from 0.1 to 50 ng/mL with specific details mentioned in Table 8. The eight nitrosamine impurities display linear responses throughout the concentration range, with R^2 values greater than 0.99 for all (R^2 >0.99).

Figures 2 and 3 are representative extracted-ion MRM chromatograms from the 6470 LC/TQ showing elution of all the eight nitrosamine impurities in a 0.6 ng/mL standard solution and spiked in metformin (20 mg/mL), respectively. A diverter valve program (Table 4) was used to divert the high concentrations of metformin to waste.

Table 5. Representative data for reproducibility of the method at 0.6 ng/mL including bracketing standards.

	S. No.	NDMA	NMBA	NDEA	NEIPA	NDIPA	NDBA	NDPA	NMPA
Initial Replicates	1	8,798	11,733	6,259	21,342	4,922	21,189	2,991	1,907
	2	9,818	11,268	6,134	20,204	4,370	19,602	2,729	1,868
	3	8,368	11,099	6,107	20,263	4,485	19,795	2,872	1,962
	4	9,134	12,661	5,483	21,795	4,825	21,128	2,868	2,016
	5	9,525	12,285	5,632	21,060	4,623	20,479	3,119	1,962
	6	9,124	12,357	5,832	20,706	4,779	20,599	3,164	1,998
Bracketing Standard	7	8,855	11,921	6,045	20,991	4,472	19,936	2,735	2,051
	Avg.	9,088.9	11,903.4	5,927.4	20,908.7	4,639.4	20,389.7	2,925.4	1,966.3
	Std. Dev.	480.3	578.1	286.5	571.2	207.6	634.5	173.1	63.1
	%RSD	5.3	4.9	4.8	2.7	4.5	3.1	5.9	3.2

Table 6. S/N ratio data for quantitation limit for all eight nitrosamine impurities.

S. No	Name of Impurity	Actual Concentration (ng/mL)	With Respect to Test Concentration 20 mg/mL (ppm)	S/N
1	NDMA	0.2	0.01	23.83
2	NDEA	0.2	0.01	76.24
3	NEIPA	0.2	0.01	126.13
4	NDIPA	0.2	0.01	100.57
5	NDPA	0.1	0.005	31.29
6	NMPA	0.1	0.005	84.25
7	NDBA	0.1	0.005	213.66
8	NMBA	0.1	0.005	153.39

*** S/N was calculated using the RMS algorithm, noise width (0.6 minutes) reference selected as sample using Agilent MassHunter Quantitative Analysis 10 software.**

Table 7. S/N ratio data for detection limit for all eight nitrosamine impurities.

S. No	Name of Impurity	Actual Concentration (ng/mL)	With Respect to Test Concentration 20 mg/mL (ppm)	S/N
1	NDMA	0.04	0.002	5.87
2	NDEA	0.04	0.002	12.61
3	NEIPA	0.04	0.002	24.87
4	NDIPA	0.04	0.002	29.72
5	NDPA	0.02	0.001	7.35
6	NMPA	0.02	0.001	14.55
7	NDBA	0.02	0.001	53.93
8	NMBA	0.02	0.001	30.86

*** S/N was calculated using the RMS algorithm, noise width (0.6 minutes) reference selected as sample using Agilent MassHunter Quantitative Analysis 10 software.**

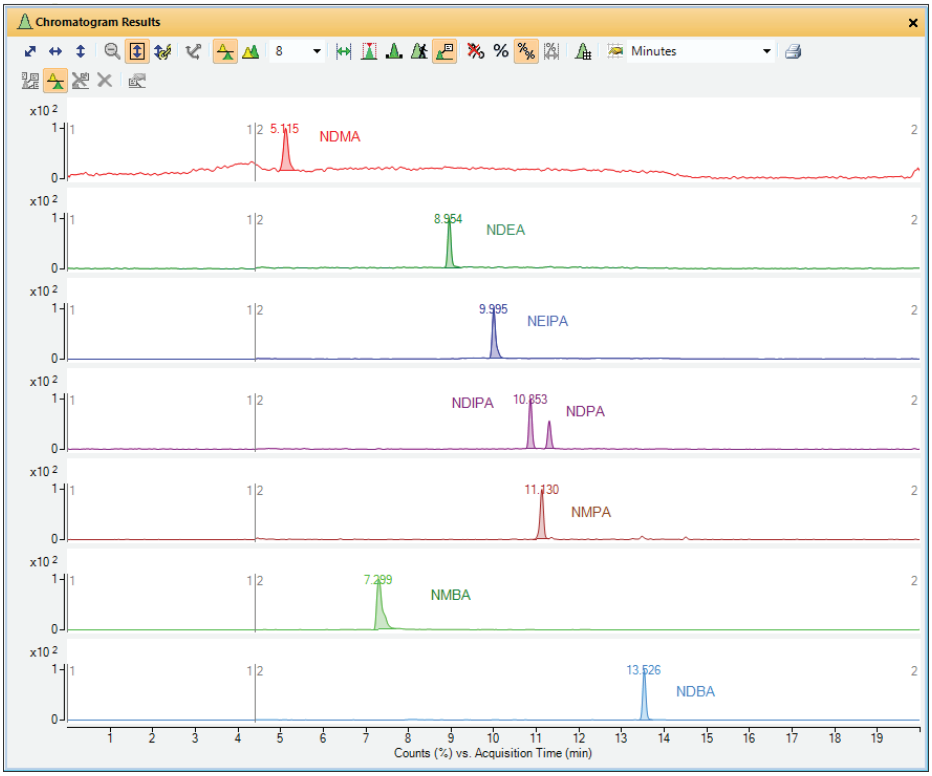

Figure 2. Representative MRM chromatogram of all the nitrosamine impurities (0.6 ng/mL) in a standard solution.

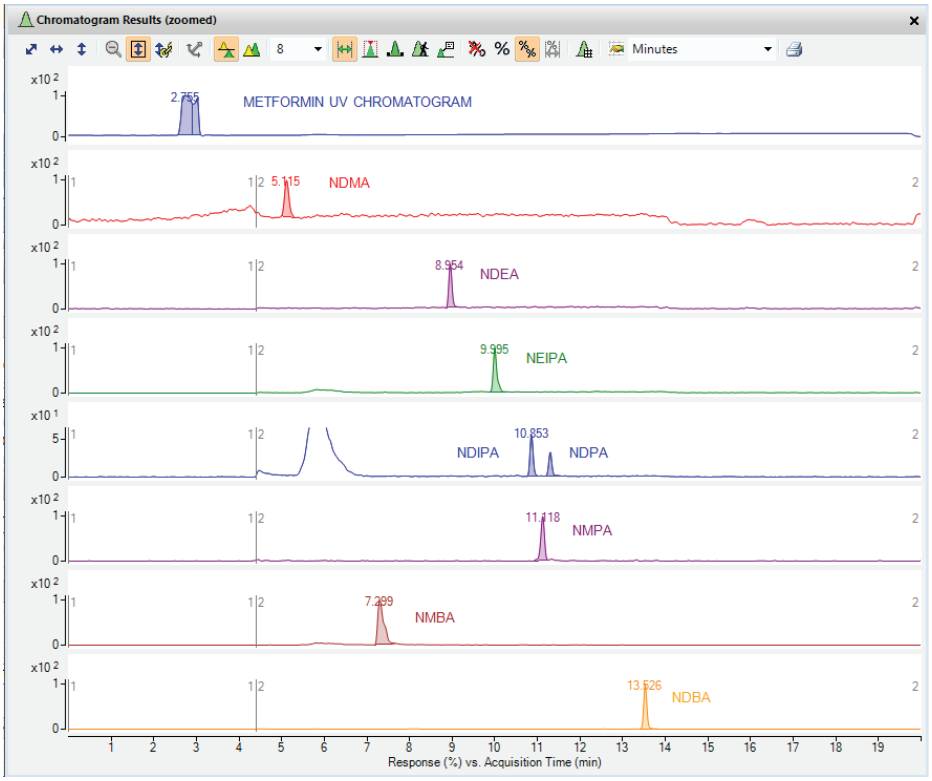

Figure 3. Representative MRM chromatogram of all the nitrosamine impurities (0.6 ng/mL) along with the metformin UV chromatogram.

Accuracy and reproducibility

Calibration curves for all the nitrosamine impurities are shown in Table 8. Each nitrosamine target demonstrated an accuracy rate within 15% of the expected concentration, and reproducibility across all levels exhibited CVs less than 15%. Figure 4 shows the calibration curves generated from a 6470 LC/TQ system.

Table 8. Regression coefficient values for linearity range 0.1 to 50 ng/mL for all eight nitrosamine impurities.

S. No	Name of Impurity	Actual Concentration (ng/mL)	With Respect to Test Concentration 20 mg/mL	R^2 Value
1	NDMA	0.1 to 50	0.005 to 2.5 ppm	0.995
2	NDEA	0.1 to 50	0.005 to 2.5 ppm	0.994
3	NEIPA	0.1 to 50	0.005 to 2.5 ppm	0.998
4	NDIPA	0.1 to 50	0.005 to 2.5 ppm	0.993
5	NDPA	0.1 to 50	0.005 to 2.5 ppm	0.995
6	NMPA	0.1 to 50	0.005 to 2.5 ppm	0.995
7	NDBA	0.1 to 50	0.005 to 2.5 ppm	0.994
8	NMBA	0.1 to 50	0.005 to 2.5 ppm	0.992

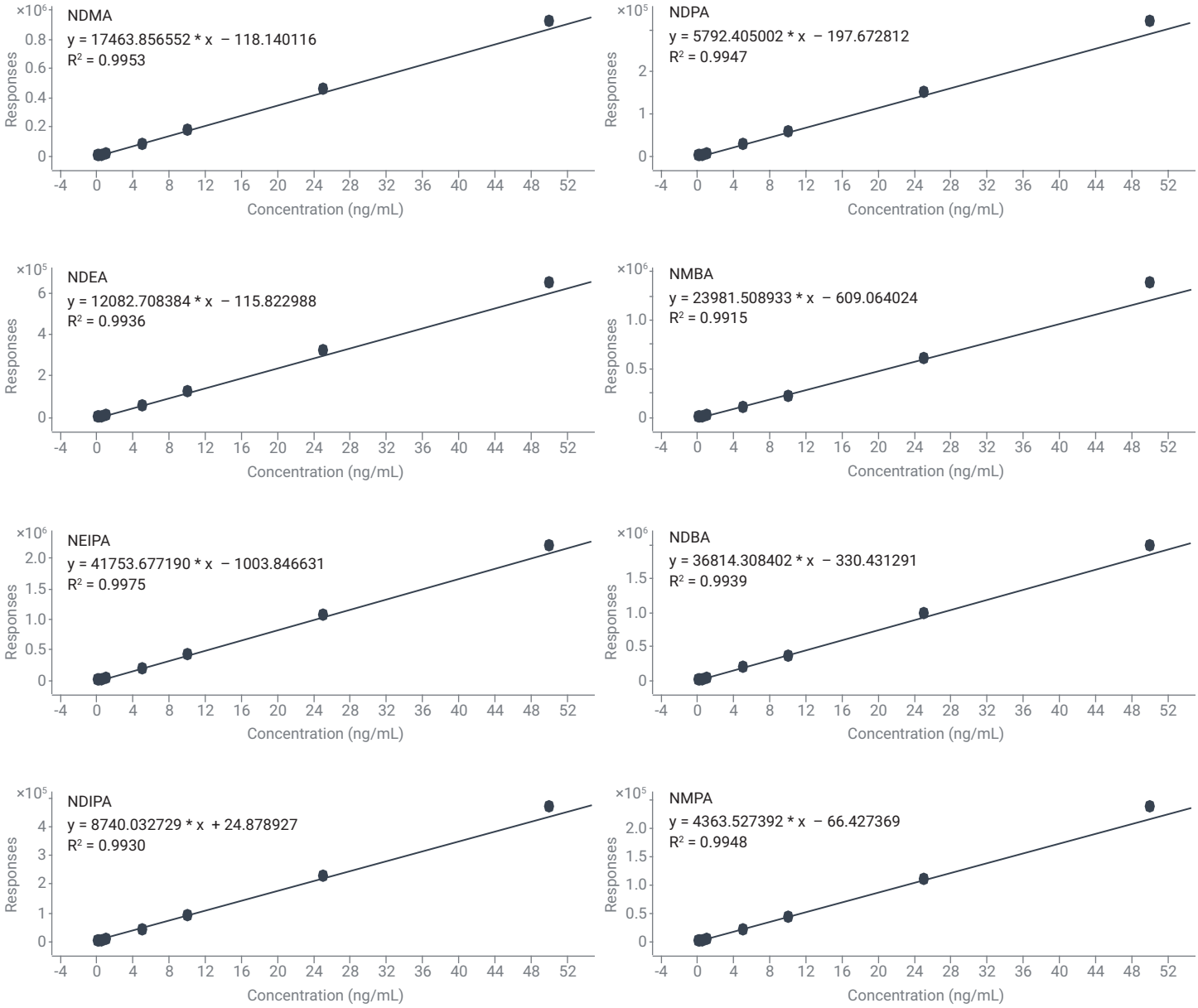

Figure 4. Representative calibration curves from an Agilent 6470 LC/TQ for all the nitrosamine impurities using a $1/x^2$ weighting factor.

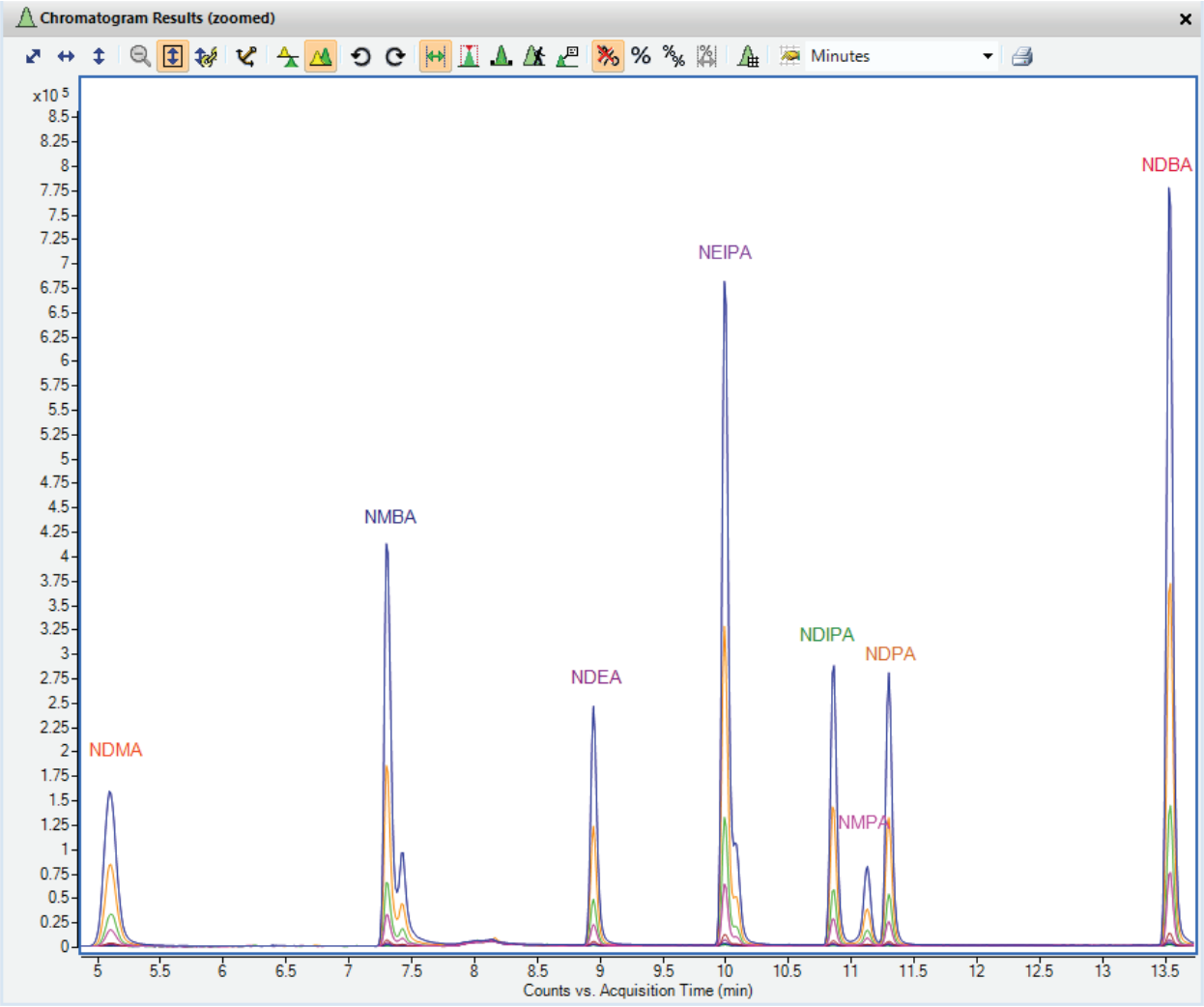

Figure 5. Representative chromatographic overlay of all calibration standards for eight nitrosamine impurities.

Table 9. Summary of recovery experiments in metformin drug substance.

Nitrosamine Impurity	Spiked Concentration (ng/mL) Metformin API (20 mg/mL)**	Concentration wrt to Test (ppm)	Recovery %*
NDMA	0.6	0.03	101.2
NDEA	0.6	0.03	98.4
NEIPA	0.6	0.03	94.9
NDIPA	0.6	0.03	102.6
NDPA	0.6	0.03	95.8
NMPA	0.6	0.03	101.5
NDBA	0.6	0.03	102.0
NMBA	0.6	0.03	97.1

* Recovery experiment performed in triplicate injections

** Since the Agilent 6470 LC/TQ is capable of very low limits of detection, a metformin sample concentration of 20 mg/mL, which is sufficient to reach regulatory limits, has been used. The sensitivity can be further improved by increasing the sample concentration subject to matrix effect and chromatographic separation.

Conclusion

The 6470 LC/TQ can analyze nitrosamine impurities at the very low concentration levels demanded by regulatory requirements. This application note is intended to demonstrate the reproducibility and sensitivity of the 6470 LC/TQ in the detection of eight nitrosamine impurities at low concentration levels in metformin drug substance.

References

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