Application Note

Pharmaceutical Small Molecules

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

Detection of regulated genotoxic impurities from the drug manufacturing process

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Abstract

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. This is evidenced by the recent recalls of metformin by various regulatory bodies like the U.S. Food & Drug Administration (FDA), European Directorate for the Quality of Medicines (EDQM), and Health Sciences Authority (HSA) due to the presence of N-nitroso-dimethylamine (NDMA).

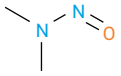
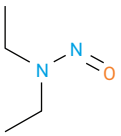
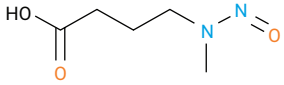
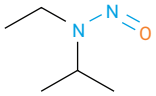
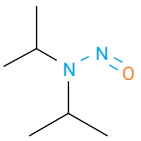
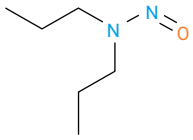
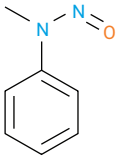
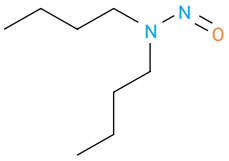
In this application note, we have developed a highly sensitive, triple quadrupole-based, liquid chromatography/tandem mass spectrometry (LC/MS/MS) method for the simultaneous determination of eight nitrosamine impurities in metformin drug substance and drug products.

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 NDMA, N-nitroso-diethylamine (NDEA), N-ethyl-*n*-nitroso-2-propanamine (NEIPA), N-nitroso-N-methyl-4-aminobutyric acid (NMBA), N-nitroso-diisopropylamine (NDIPA), N-nitroso-methylphenylamine (NMPA), N-nitroso-di-*n*-propylamine (NDPA), and N-nitroso-di-butylamine (NDBA) impurities in metformin drug substance and drug products.

Introduction

Nitrosamine impurities recently became a focus for regulatory agencies like the 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; this began with Singapore's Health Sciences Authority, followed by the 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.

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 and drug products. The methods described in this application note were carried out on the 6470 LC/TQ, providing a comprehensive analysis of eight nitrosamine impurities at very low detection limits by using two different sample preparation and column chemistries.

Nitrosamine Compounds	Chemical Structure
N-nitroso-dimethylamine (NDMA)	
N-nitroso-diethylamine (NDEA)	
N-nitroso-N-methyl-4-aminobutyric acid (NMBA)	
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)	

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

Method 1

Drug substance sample preparation

The complete details of this sub-method are already reported in the Agilent application note Simultaneous Determination of Eight Nitrosamine Impurities in Metformin Using the Agilent 6470 Triple Quadrupole LC/MS (publication number 5994-2286EN).

Drug product sample preparation

Crush the appropriate number of tablet(s) and weigh 100 mg equivalent weight of metformin API and transfer into a 15 mL centrifuge tube. Add 250 µL of methanol and sonicate for 15 minutes. Add LC/MS-grade water to bring the total volume to 5 mL, such that the final target concentration is 20 mg/mL. Sonicate again for 15 minutes, followed by 10 minutes shaking using a shaker. After extraction, centrifuge the sample for 15 minutes at 4,500 rpm. Filter the supernatant using a 0.22 µm PVDF syringe filter, discard the first 1 mL, and transfer the filtered sample into an HPLC vial for LC/MS analysis.

Method 2

Drug substance sample preparation

Metformin drug substance: Weigh and place 500 mg of metformin drug substance in a 15 mL centrifuge tube. Dissolve thoroughly by adding 5 mL of methanol, and vortex until all visible particles are dissolved. Filter the solution using a 0.22 µm PVDF syringe filter to eliminate any undissolved particles, discard the first 1 mL, and transfer the filtered sample into an HPLC vial for LC/MS analysis.

Drug product sample preparation

Crush the appropriate number of tablet(s) to obtain a target concentration of 100 mg/mL of API in methanol, and transfer into a 15 mL centrifuge tube. Add the appropriate volume of methanol and mix for about a minute using a vortex mixer. Shake the sample for 40 minutes using a shaker.

After extraction, centrifuge the sample for 15 minutes at 4,500 rpm. Filter the supernatant using a 0.22 µm PVDF syringe filter, discard the first 1 mL, and transfer the filtered sample into an HPLC vial for LC/MS analysis.

LC configuration and parameters

Table 1. UHPLC configuration and settings.

Parameter	Value	
	Method 1	Method 2
Instruments	• Agilent 1290 Infinity II high-speed pump (G7120A) • Agilent 1290 Infinity II multisampler (G7167B) • Agilent 1290 Infinity II multicolumn thermostat (G7116B) • Agilent 1260 Infinity diode array detector (G1315C)	• Agilent 1290 Infinity II high-speed pump (G7120A) • Agilent 1290 Infinity II multisampler (G7167B) • Agilent 1290 Infinity II multicolumn thermostat (G7116B) • Agilent 1260 Infinity diode array detector (G1315C)
Needle Wash	Methanol: water (80:20)	Methanol: water (80:20)
Sample Diluent	Water: methanol (95:5)	Methanol
Multisampler Temperature	10 °C	10 °C
Injection Volume	20 µL	5 µL
Analytical Column	Agilent InfinityLab Poroshell HPH-C18, 4.6 × 150 mm, 2.7 µm (p/n 693975-702(T))	Agilent InfinityLab Poroshell 120 PFP, 3.0 x 150 mm, 2.7 µm (p/n 693975-308)
Column Temperature	40 °C	40 °C
Mobile Phase A	0.1% formic acid in water	0.1% formic acid in water
Mobile Phase B	0.1% formic acid in methanol	0.1% formic acid in methanol
Flow Rate	0.5 mL/min	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	Time (min) / % A / % B / Flow (mL/min) 0 / 95 / 5 / 0.5 3 / 95 / 5 / 0.5 14 / 40 / 60 / 0.5 17 / 10 / 90 / 0.5 19 / 10 / 90 / 0.5 19.1 / 95 / 5 / 0.5 22 / 95 / 5 / 0.5
Stop Time	20 minutes	22 minutes
UV Wavelength	230 nm	230 nm

Triple quadrupole mass spectrometer configuration and parameters

Table 2. Mass spectrometer configuration and source settings.

Parameter	Value	
	Method 1	Method 2
Instrument	Agilent 6470 triple quadrupole LC/MS (G6470A)	Agilent 6470 triple quadrupole LC/MS (G6470A)
Ion Source	Atmospheric pressure chemical ionization (APCI)	Atmospheric pressure chemical ionization (APCI)
MS/MS Mode	MRM	MRM
Ion Mode	Positive	Positive
Drying Gas Temperature	300 °C	300 °C
Drying Gas Flow	7 L/min	7 L/min
Nebulizer Pressure	25 psi	25 psi
Apci Heater	350 °C	350 °C
Apci Needle Positive	4 µA	4 µA
Capillary Voltage, Positive	4,000 V	4,000 V
MS1/MS2 Resolution	0.7/0.7 (unit/unit)	0.7/0.7 (unit/unit)
Dwell Time	50 ms	50 ms
Diverter Valve Diverted to MS	4.4 min	2.3 min

MS/MS compound information for analytes

Table 3. Detailed MRM settings in MRM mode using the Agilent 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	18	3	+
NDMA (qualifier)	75	58	50	80	12	2	+
NMBA (quantifier)	147	117	50	60	4	2	+
NMBA (qualifier)	147	44	50	60	16	2	+
NDEA (quantifier)	103.1	75.1	50	80	11	3	+
NDEA (qualifier)	103.1	47.1	50	80	19	3	+
NEIPA (quantifier)	117.1	75.1	50	75	8	4	+
NEIPA (qualifier)	117.1	47.1	50	75	18	3	+
NDIPA (quantifier)	131	43.1	50	75	12	8	+
NDIPA (qualifier)	131	89.1	50	75	6	4	+
NDPA (quantifier)	131	43	50	80	20	4	+
NDPA (qualifier)	131	89.1	50	80	5	4	+
NMPA (quantifier)	137	66	50	70	20	5	+
NMPA (qualifier)	137	107	50	70	10	5	+
NDBA (quantifier)	159.1	57.2	50	90	12	4	+
NDBA (qualifier)	159.1	41.1	50	90	22	4	+

Data analysis

Data was acquired and analyzed using Agilent MassHunter Acquisition software version 10. MRM transitions were obtained and optimized using 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 1

Method development was performed using different columns and gradient conditions for the optimization of chromatographic separation between metformin and NDMA. This step is very 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 masses. Instrument MS/MS parameters were optimized to maximize sensitivity. Critical parameters like specificity, reproducibility, linearity, recovery, LOQ, and LOD were characterized to ensure the method performance.

LOQ, LOD limits, and S/N values are captured in Table 4. The calibration concentrations ranged from 0.1 to 50 ng/mL or 0.1 to 25 ng/mL with specific details mentioned in Table 4. 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). All eight calibration curves are shown in Figure 3. Reproducibility data for eight replicates, including bracketing standards, is captured in Table 5. This data shows excellent results at 0.5 ng/mL concentration level. The recovery data at three different spiked concentration levels of 0.2 ng/mL (0.01 ppm), 0.5 ng/mL (0.025 ppm), and 1 ng/mL (0.05 ppm) show excellent results and are captured in Table 6.

Figures 1 and 2 are representative extracted-ion MRM chromatograms from the 6470 LC/TQ showing elution of all the eight nitrosamine impurities in a 0.5 ng/mL (0.025 ppm) standard solution and spiked in metformin (20 mg/mL), respectively.

Accuracy and reproducibility

Calibration curves details for all the nitrosamine impurities are shown in Table 4. Each nitrosamine target demonstrated an accuracy rate within 15% of the expected concentration, and reproducibility across all levels exhibited CVs less than 15%. Figure 3 shows the calibration curves generated using Method 1 from a 6470 LC/TQ system.

Table 4. Result summary of Method 1. Data includes signal-to-noise (S/N), calculated LOD/ LOQ, coefficient of regression, and calibration curve fit. All standards used 1/x weighted calibration curve.

Compound	LOD		LOD S/N)	LOQ		LOQ (S/N)	R^2	Linearity Range	
	ng/mL	ppm**		ng/mL	ppm			ng/mL	ppm
NDMA	0.05	0.0025	10	0.15	0.0075	26.9	0.998	0.15 to 50	0.0075 to 2.5
NDEA	0.025	0.00125	9.55	0.1	0.005	28.15	0.997	0.1 to 50	0.005 to 2.5
NMBA	0.025	0.00125	87.98	0.1	0.005	536.2	0.996	0.1 to 50	0.005 to 2.5
NEIPA	0.025	0.00125	114	0.1	0.005	203.97	0.997	0.1 to 50	0.005 to 2.5
NDIPA	0.05	0.0025	30.68	0.1	0.005	45.98	0.997	0.1 to 25	0.005 to 1.25
NDPA	0.05	0.0025	48.6	0.1	0.005	86.01	0.997	0.1 to 25	0.005 to 1.25
NMPA	0.05	0.0025	27	0.1	0.005	41.97	0.998	0.1 to 50	0.005 to 2.5
NDBA	0.05	0.0025	81.84	0.1	0.005	149.95	0.997	0.1 to 25	0.005 to 1.25

*S/N was calculated using the Auto-RMS algorithm, using Agilent MassHunter Quantitative Analysis 10 software.
**ppm concentration is the actual concentration with respect to test concentration 20 mg/mL.

Table 5. Representative data for reproducibility of the method at 0.5 ng/mL including bracketing standard using Method 1.

	S. No.	NDMA	NDEA	NMBA	NEIPA	NDIPA	NDPA	NMPA	NDBA
Initial Replicates	1	22747	9015	8761	41071	24618	31651	1386	13678
	2	24529	8529	8456	40838	22460	30466	1431	13544
	3	23021	9080	9120	39335	23176	31453	1388	13716
	4	24649	9235	9112	41309	23525	30648	1382	13609
	5	23306	8855	9010	40980	21834	30608	1408	13399
	6	23607	8855	8629	41056	24021	31070	1372	13993
	7	24152	8656	8923	40863	23229	30733	1381	13871
Bracketing Standard	8	23631	8767	9302	41025	22159	32781	1474	14544
	Mean	23705.25	8874	8914.125	40809.63	23127.75	31176.25	1402.75	13794.25
	SD	689.70	230.33	281.80	613.19	944.71	773.80	34.33	354.63
	RSD %	**2.91**	**2.60**	**3.16**	**1.50**	**4.08**	**2.48**	**2.45**	**2.57**

Table 6. Summary of recovery experiments in metformin drug product using Method 1.

Nitrosamine Impurity	0.2 ng/mL (0.01 ppm) Spiked in Metformin 500 mg ER Tablet for Sample Size of 20 mg/mL	0.5 ng/mL (0.025 ppm) Spiked in Metformin 500 mg ER Tablet for Sample Size of 20 mg/mL	1 ng/mL (0.05 ppm) Spiked in Metformin 500 mg ER Tablet for Sample Size of 20 mg/mL
NDMA	97.68	98.83	99.18
NDEA	96.44	104.65	98.95
NMBA	100.2	116.2	98.7
NEIPA	98.31	101.04	95.9
NDIPA	101.82	101.14	96.96
NDPA	101.66	102.5	104.05
NMPA	105.78	110.56	107.72
NDBA	97.51	93.63	93.34

Note:
1. Recovery experiment performed in triplicate injections.
2. Since the Agilent 6470 LC/TQ is capable of very low limits of detection, the sample concentration of 20 mg/mL of metformin formulations is used, which is enough to reach regulatory limits. The sensitivity can be further improved by increasing the sample concentration subject to matrix effect and chromatographic separation.

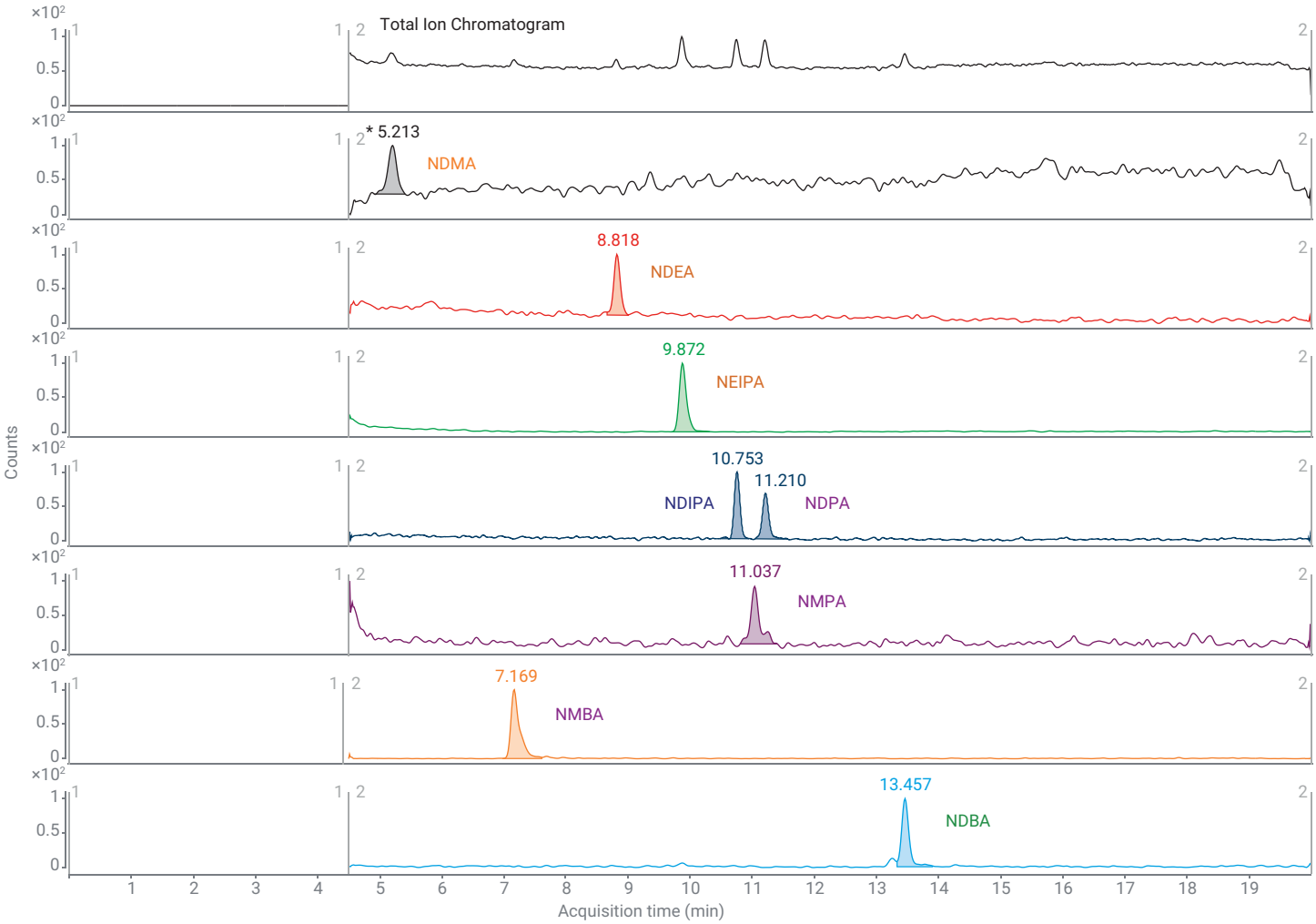


Figure 1. Representative MRM chromatogram of all the nitrosamine impurities at 0.5 ng/mL using Method 1.

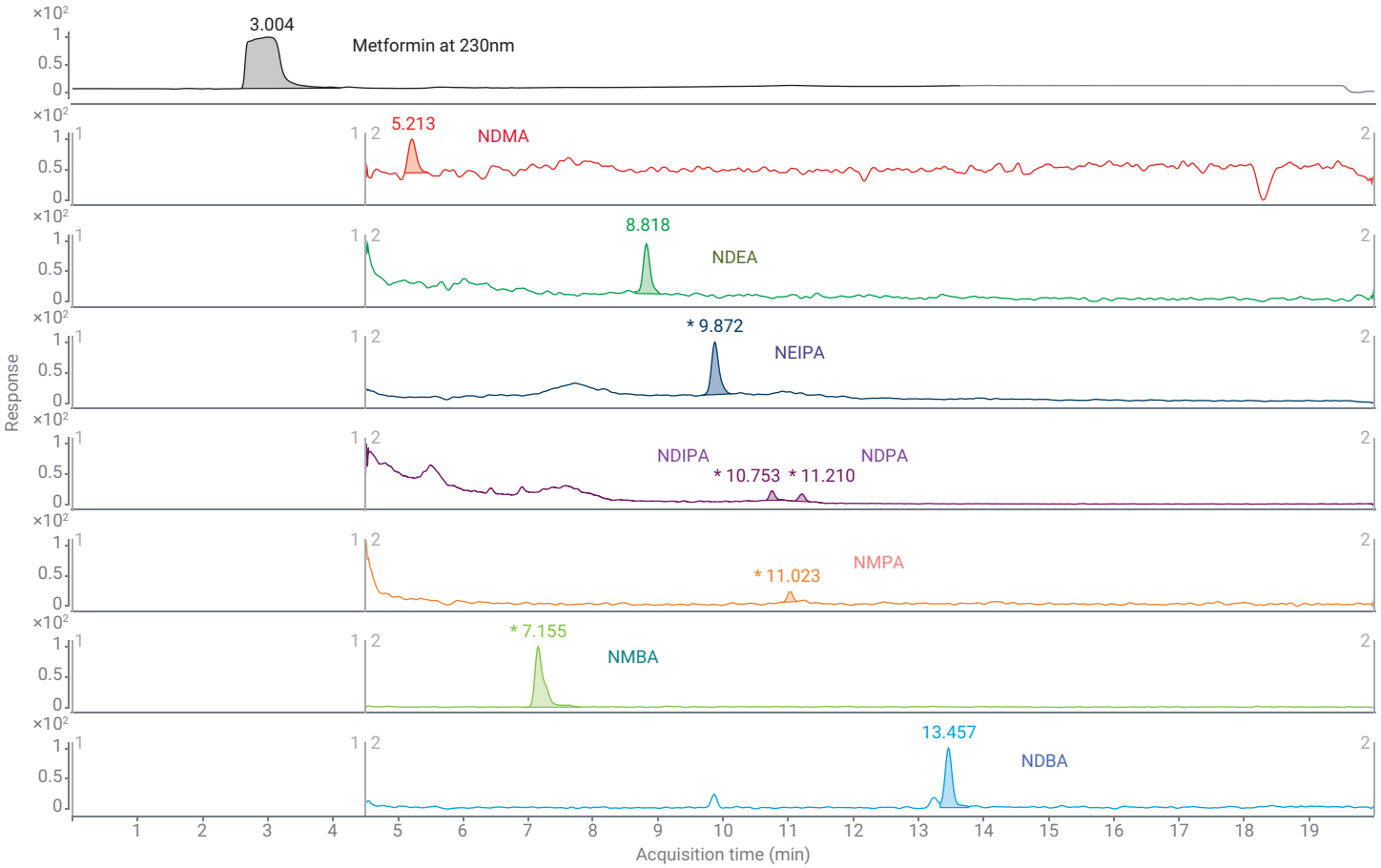


Figure 2. Representative MRM chromatogram of all eight nitrosamine impurities at 0.5 ng/mL (0.025 ppm) along with the metformin UV chromatogram using Method 1.

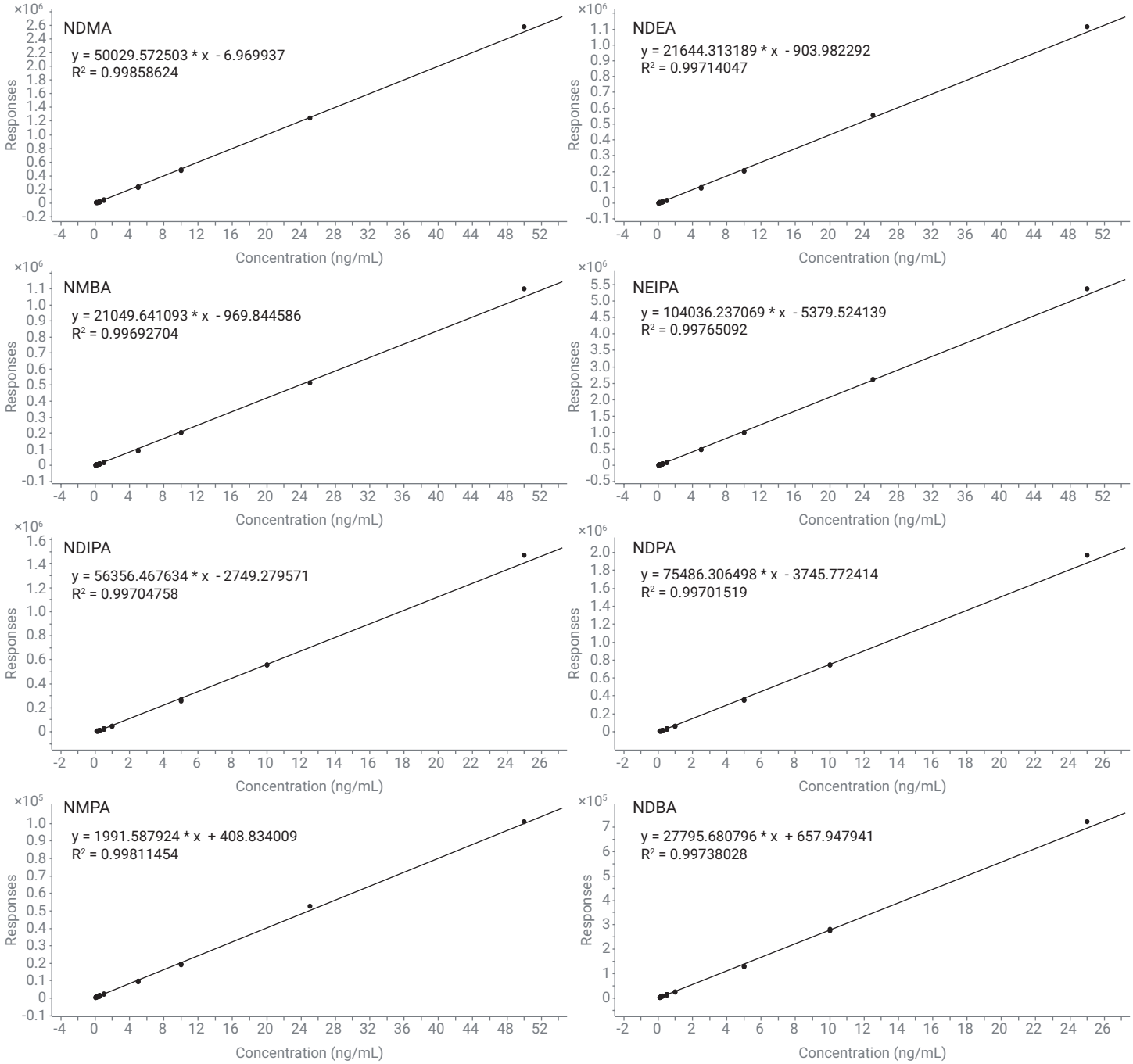


Figure 3. Representative calibration curves from an Agilent 6470 LC/TQ for all the nitrosamine impurities using a 1/x weighting factor and Method 1.

Method 2

Although Method 1 shows excellent recovery for metformin drug substance and those drug products that dissolve well in the sample diluent without converting into a thick suspension, Method 2 shows excellent recovery for both drug substance and drug products, as the solutions do not convert into suspension in their sample diluent. Critical parameters like specificity, reproducibility, linearity, recovery, LOQ, and LOD were characterized using this method to ensure method performance.

LOQ, LOD limits, and S/N values are captured in Table 7. The calibration concentrations ranged from 0.1 to 25 ng/mL or 0.25 to 50 ng/mL, with specific details mentioned in Table 7. 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). All eight calibration curves are shown in Figure 6. Reproducibility data for eight replicates, including bracketing standards, is captured in Tables 8A and 8B. This data shows excellent results at 1 and 3 ng/mL concentration levels. The recovery data at two different spiked concentration levels of 1 ng/mL (0.01 ppm) and 3 ng/mL (0.03 ppm) show excellent results as per guidelines, and are captured in Table 9.

Figures 4 and 5 are representative extracted-ion MRM chromatograms from the 6470 LC/TQ showing elution of all the eight nitrosamine impurities in a 3 ng/mL (0.03 ppm) standard solution and spiked in metformin (100 mg/mL), respectively.

Table 7. Result summary of Method 2. Data includes signal-to-noise (S/N), calculated LOD/ LOQ, coefficient of regression, and calibration curve fit. All standards used 1/x weighted calibration curves using Method 2.

	LOD		LOD	LOQ		LOQ		Linearity Range	
Compound	ng/mL	ppm**	(S/N)	ng/mL	ppm	(S/N)	R^2	ng/mL	ppm
NDMA	0.25	0.0025	11.29	0.5	0.005	17.58	0.998	0.5 to 25	0.005 to 0.25
NDEA	0.1	0.001	7.44	0.25	0.0025	13.8	0.998	0.25 to 25	0.0025 to 0.25
NMBA	0.1	0.001	22.62	0.25	0.0025	39.65	0.998	0.25 to 50	0.0025 to 0.5
NEIPA	0.05	0.0005	19.42	0.1	0.001	34.68	0.995	0.1 to 25	0.001 to 0.25
NDIPA	0.05	0.0005	15.15	0.25	0.0025	44.32	0.996	0.25 to 25	0.0025 to 0.25
NDPA	0.05	0.0005	17.51	0.25	0.0025	101.60	0.998	0.25 to 25	0.0025 to 0.25
NMPA	0.1	0.001	20.90	0.25	0.0025	56.06	0.997	0.25 to 25	0.0025 to 0.25
NDBA	0.25	0.0025	109.68	0.5	0.005	175.45	0.996	0.5 to 50	0.005 to 0.5

*S/N was calculated using the Auto-RMS algorithm, using Agilent MassHunter Quantitative Analysis 10 software.
**ppm concentration is the actual concentration with respect to test concentration 100 mg/mL.

Table 8A. Representative data for reproducibility of the method at 1 ng/mL (0.01 ppm) including bracketing standard using Method 2.

	S. No.	NDMA	NDEA	NMBA	NEIPA	NDIPA	NDPA	NMPA	NDBA
Initial Replicates	1	8995	4244	1459	15675	9891	12191	2366	5973
	2	8705	4098	1485	15818	10264	12737	2390	6007
	3	8721	4190	1485	15971	10402	12041	2393	5847
	4	9001	4119	1482	15812	9854	12609	2362	5883
	5	8886	4039	1493	15419	10757	12774	2337	6003
	6	9134	4226	1413	15883	10070	12184	2234	5840
	7	8776	4034	1441	16036	9719	12020	2322	5844
Bracketing Standard	8	8633	4178	1432	15740	10096	11902	2395	5587
	Mean	8856.38	4141.00	1461.25	15794.25	10131.63	12307.25	2349.88	5873.00
	SD	175.53	80.88	29.68	191.39	336.37	346.40	53.83	135.94
	RSD %	**1.98**	**1.95**	**2.03**	**1.21**	**3.32**	**2.81**	**2.29**	**2.31**

Table 8B. Representative data for reproducibility of the method at 3 ng/mL (0.03 ppm) including bracketing standards using Method 2.

	S. No.	NDMA	NDEA	NMBA	NEIPA	NDIPA	NDPA	NMPA	NDBA
Initial Replicates	1	25452	11751	4563	53145	30556	40473	6416	16512
	2	25478	11409	4593	51933	30564	40709	6411	16481
	3	25644	11775	4577	52877	30499	39644	6442	16776
	4	25549	11794	4571	53277	31244	40297	6290	16686
	5	25906	11402	4310	53368	31422	41661	6227	16653
	6	25678	11599	4445	53525	31497	40770	6434	16569
	7	25668	11198	4520	52916	31045	40716	6407	16581
Bracketing Standard	8	25618	11736	4373	52116	31132	39698	6397	16261
	Mean	25624.125	11583	4494	52894.625	30994.875	40496	6378	16564.875
	SD	142.09	221.85	106.20	580.90	403.93	646.85	77.01	155.59
	RSD %	**0.55**	**1.92**	**2.36**	**1.10**	**1.30**	**1.60**	**1.21**	**0.94**

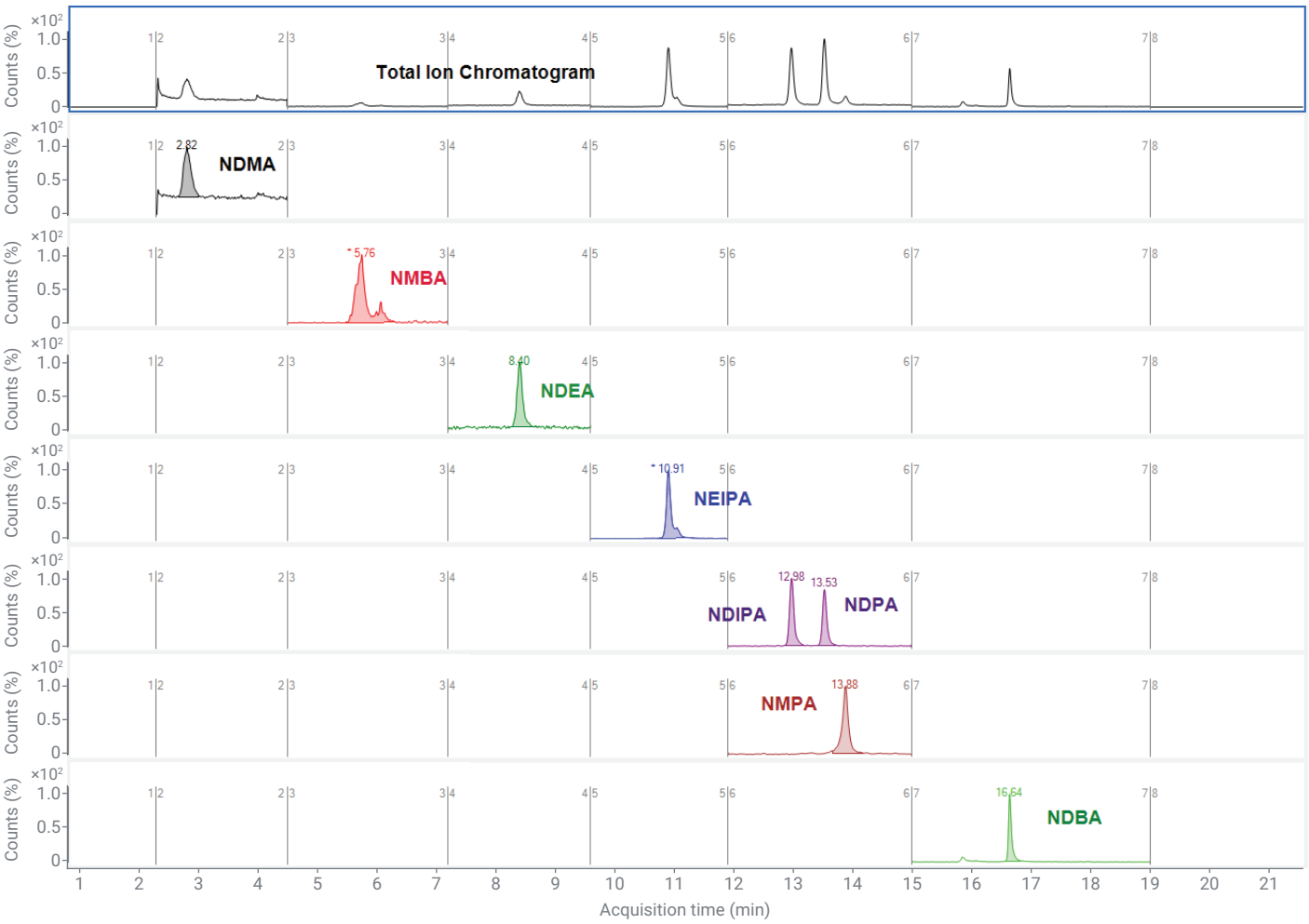


Figure 4. Representative MRM chromatogram of all eight nitrosamine impurities at 3 ng/mL using Method 2.

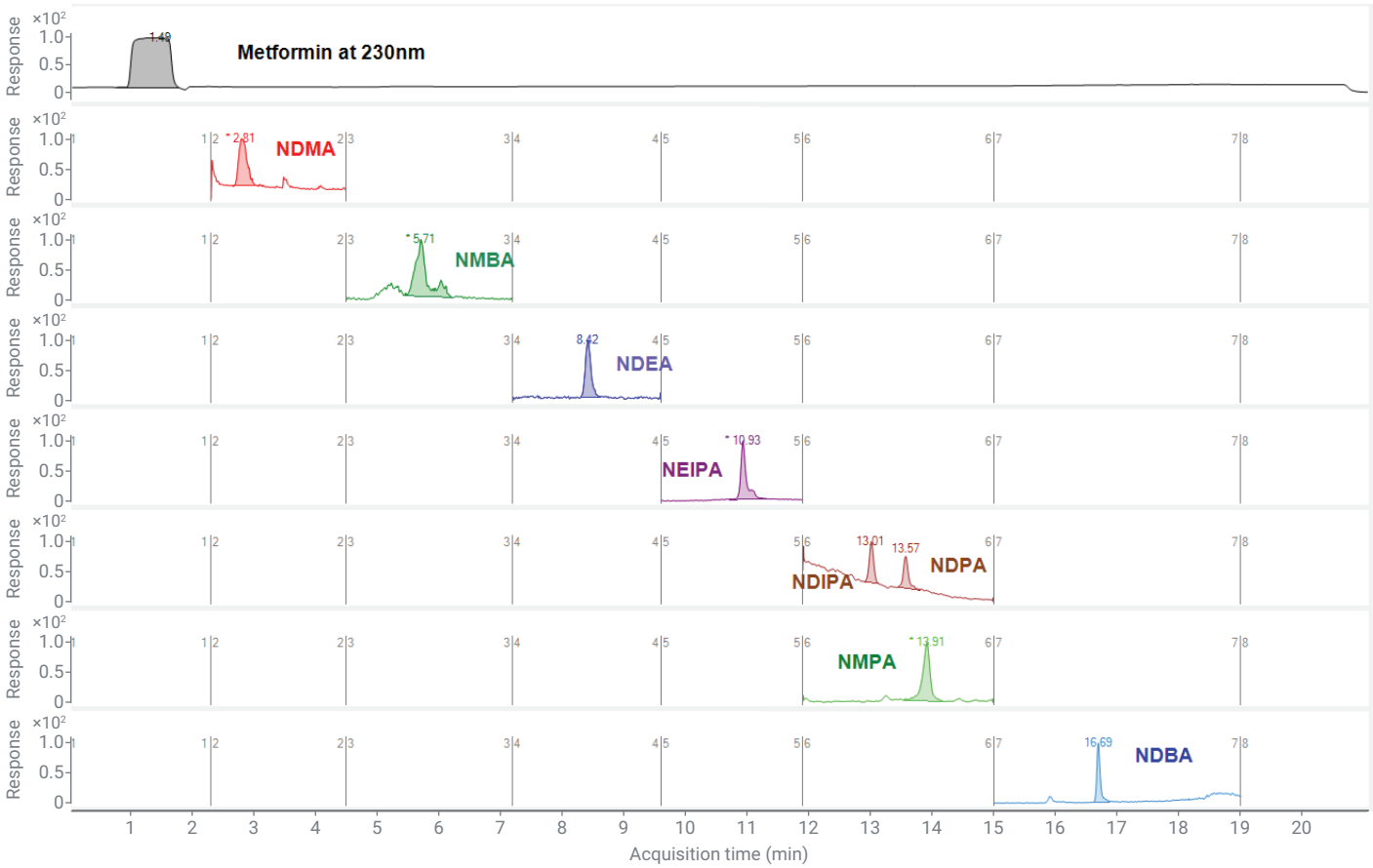


Figure 5. Representative MRM chromatogram of all eight nitrosamine impurities at 3 ng/mL (0.03 ppm) along with the metformin UV chromatogram using Method 2.

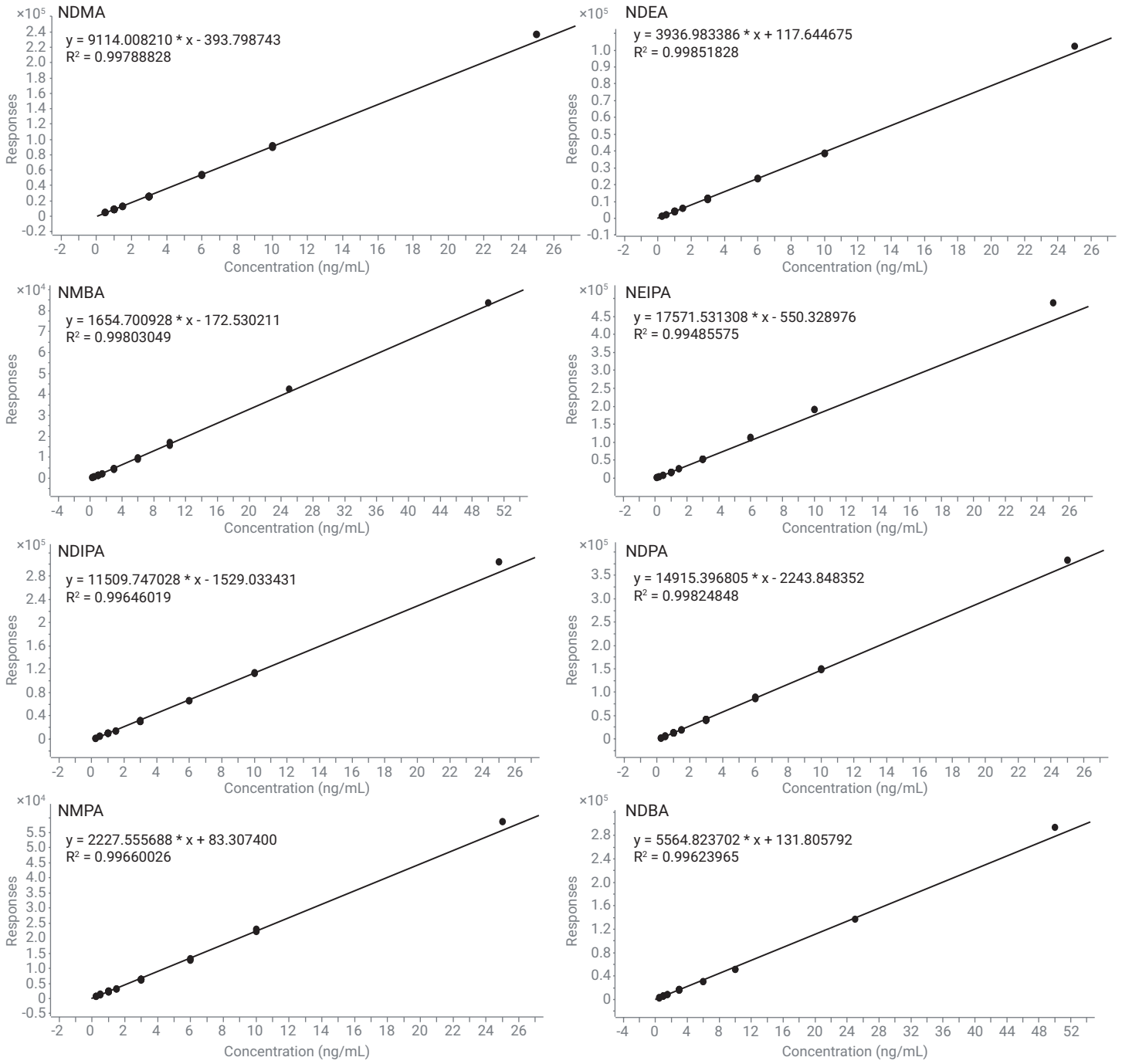


Figure 6. Representative calibration curves from an Agilent 6470 LC/TQ for all the nitrosamine impurities using a 1/x weighting factor.

Table 9. Summary of recovery experiments in metformin drug product using Method 2.

Nitrosamine Impurity	1 ng/mL (0.01 ppm) Spiked in Metformin 500 mg ER Tablet for Sample Size of 100 mg/mL	3 ng/mL (0.03 ppm) Spiked in Metformin 500 mg ER Tablet for Sample Size of 100 mg/mL
NDMA	103.70	104.60
NDEA	98.90	98.28
NMBA	109.16	108.78
NEIPA	97.58	91.91
NDIPA	102.32	95.87
NDPA	95.43	99.87
NMPA	91.43	94.64
NDBA	96.64	97.68

Note:
1. Recovery experiment performed in triplicate injections.
2. Sample size and sample preparation used here are same as mentioned in FDA-published method.

Accuracy and reproducibility

Calibration curve details for all the nitrosamine impurities are shown in Table 7. Each nitrosamine target demonstrated an accuracy rate within 15% of the expected concentration, and reproducibility across all levels exhibited CVs less than 15%. Figure 6 shows the calibration curves generated using Method 2 from a 6470 LC/TQ system.

Conclusion

The 6470 LC/TQ can analyze nitrosamine impurities at the very low concentration levels demanded by regulatory requirements. This is demonstrated using two different methods using different sample preparation and column chemistries. This application note is intended to demonstrate the complete solution to address the nitrosamine contamination challenge related to metformin, including reproducibility and sensitivity of the 6470 LC/TQ in the detection of eight nitrosamine impurities at low concentration levels in metformin drug substance and drug products.

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