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LC-MS/MS method for the quantification of 10 nitrosamine impurities in metformin

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Introduction

Since 2018, multiple batches of drug products, including angiotensin receptor blockers[1] (ARBs) and the histamine blocker ranitidine[2] (commonly known as Zantac™), have been recalled due to the presence of nitrosamines at unacceptable amounts. Scientists at the Center for Drug Evaluation and Research (CDER) division of the U.S. Food and Drug Administration (FDA) have published multiple methods to test these products for the presence of nitrosamines, including gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-MS (LC-MS) technologies. These methods are specific for detecting and quantifying up to eight different nitrosamines in these drug products, even if present at amounts well below the allowable intake level. The FDA was notified by international regulators of the presence of nitrosamines, particularly N-nitrosodimethylamine (NDMA) in metformin[3], a biguanides drug product and the first-line medication for the treatment of type 2 diabetes. Using the expertise gained in detecting nitrosamines in other products (e.g., ARBs and ranitidine), the FDA developed and validated two different and complementary methods to test for multiple nitrosamines in metformin products.

The regulator continues to investigate the presence of the NDMA impurity in metformin approved for sale in the U.S. Testing has found NDMA in certain lots of extended release (ER) metformin, and the regulator is recommending companies to recall batches with levels of NDMA above the acceptable intake limit of 96 nanograms per day[4]. As a consequence, several pharmaceutical companies are recalling metformin formulations owing to the significant levels of NDMA in them.

The FDA recently published an LC-MS method that was developed and validated in alignment with ICH Q2(R1) guidelines for the detection and quantitation of eight nitrosamine impurities[5], including N-nitrosodimethylamine (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) and N-nitroso-N-methyl-4-aminobutyric acid (NMBA) in metformin. However, the demand to quantify more nitrosamine impurities in pharmaceutical formulations requires developing an LC-MS/MS method that allows detection and quantification of additional nitrosamine impurities simultaneously. The LC-MS/MS method presented here has been developed and evaluated/tested for the simultaneous determination of 10 nitrosamine impurities in metformin drug substance, additionally including N-nitrosomorpholine (NMOR) and N-nitrosopiperidine (NPIP).

The separation of metformin and the 10 nitrosamine impurities is realized by reversed-phase chromatography; detection is achieved by a triple quadrupole mass spectrometer. High detection selectivity and sensitivity is achieved by taking advantage of selected reaction monitoring (SRM) of protonated impurity ions.

Experimental

Instruments

- Thermo Scientific™ Vanquish™ Flex Binary UHPLC system equipped with temperature-controlled autosampler and column compartment
- Thermo Scientific™ TSQ™ Quantis triple stage quadrupole mass spectrometer with atmospheric pressure chemical ionization (APCI)

Consumables/reagents

- Reference standards procured from Cleanchem Laboratories
  - N-nitrosodimethylamine (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-nitrosomorpholine (NMOR)
  - N-nitrosopiperidine (NPIP)
- Fisher Scientific™ Formic acid, Optima™ LC/MS grade (Fisher Scientific P/N A117-50 or equivalent)
- Fisher Scientific™ Methanol, Optima™ LC/MS grade (Fisher Scientific P/N A456-4 or equivalent)
- Fisher Scientific™ Water, Optima™ LC/MS grade (Fisher Scientific P/N AAB-W6-4 or equivalent)
- Invitrogen™ 2 mL microcentrifuge tubes (P/N AM12475)
- Thermo Scientific™ Titan3™ 0.2 µm PVDF syringe filters (P/N 42213-PV)
- Thermo Scientific™ Chromacol™ GOLD HPLC vials (2-SVG)
- Thermo Scientific™ Acclaim™ 120 C18 column, 4.6 × 150 mm, 3 µm (P/N 059133)

Diluent solution and blank preparation

Mix 80 mL of methanol and 20 mL of water and use as diluent solution and blank.

Stock standard preparation

Prepare an individual stock standard solution of 1,000 µg/mL in methanol for each nitrosamine impurity.

Standard preparation (3.0 ng/mL)

Transfer 10 µL of stock standard solution of each impurity into a 10 mL volumetric flask and dilute to volume using diluent solution to prepare the mixed intermediate dilution. Afterwards, transfer 30 µL aliquot volume of the mixed intermediate dilution into a 10 mL volumetric flask and dilute to volume with diluent solution. This standard solution can be used for system suitability experiment as well as recovery evaluation.

Preparation of linearity standards, LOQ and LOD

Prepare a mixed standard solution of all 10 nitrosamines at a concentration of 20 ng/mL for NPIP and NEIPA and 40 ng/mL for other nitrosamines by taking a suitable volume from individual stock standard solution (1,000 µg/mL) and diluting with diluent solution. This standard serves as the linearity standard of the highest concentration. From this mixed standard solution, take a suitable volume and serially dilute to achieve five more linearity standards. Dilute further to prepare LOQ solutions (0.5 ng/mL for NPIP and NEIPA and 1 ng/mL for others) and LOD solutions (0.2 ng/mL for NPIP and NEIPA and 0.4 ng/mL for others). LOQ serves as the linearity standard of the lowest concentration.

Drug product sample preparation

Crush the appropriate number of tablets to obtain a target concentration of 100 mg/mL of API. Dissolve in 2.0 mL of diluent solution in a 2 mL microcentrifuge tube. Mix the solution for about 5 minutes using a vortex mixer. Sonicate the samples for 10 minutes.

After extraction, centrifuge the sample for 10 minutes at 13,000 rpm and 6 °C. Filter the supernatant using a 0.2 µm PVDF syringe filter, discard the first 0.2 mL, and transfer the filtered sample into an HPLC vial for LC/MS analysis.

Chromatographic conditions

Table 1. HPLC conditions

Parameter	Value		
HPLC column	Acclaim 120 C18 4.6 x 150 mm, 3 µm		
Column temp.	40 °C		
Flow rate	0.400 mL/min		
Mobile phase A	0.1% formic acid in water		
Mobile phase B	0.1% formic acid in methanol		
Gradient	*Time (min)*	*Solvent A %)*	*Solvent B %*
	0	86	14
	5	86	14
	7	50	50
	11	33	67
	16	33	67
	19	20	80
	24	20	80
	24.5	86	14
	32	86	14
Injection volume	15 µL		
Autosampler temp.	10 °C		
Needle wash	80:20, methanol:water		

Mass spectrometer parameter settings

Table 2. Ion source settings

Parameter	Value
Ion source type	APCI
Polarity	Positive
Sheath gas flow rate	45 arbitrary units
Aux gas flow rate	5 arbitrary units
Sweep gas flow rate	0 arbitrary units
Corona discharge voltage	4 µA
Capillary temp.	275 °C
Aux gas heater temp.	350 °C

Table 3. Divert valve settings

Time (min)	Position	Remarks
0	1-6	Diverted
5.2	1-2	LC to MS
31	1-6	Diverted

Table 4. SRM settings

Compound	Start time (min)	End time (min)	Precursor (*m/z*)	Product (*m/z*)	Collision energy (V)	RF lens (V)	Source fragmentation (V)	Q1 resolution (FWHM)	Q3 resolution (FWHM)
NDMA	0	32	75.012	43	16.67	67	22	0.4	1.2
				*58.071	13.26				
NDEA	0	32	103.062	75.054	11.91	73	18	0.4	1.2
				*29.167	15.28				
NPIP	0	32	115.088	69.054	15.66	89	22	0.4	0.7
				*41.054	21.81				
NMOR	0	32	117.06	87.125	11.99	91	24	0.4	1.2
				*45	19.49				
NEIPA	0	32	117.1	75.054	10.56	57	16	0.4	0.7
				*43.071	17.81				
NDIPA	0	32	131.1	89.196	9.47	59	12	0.4	0.7
				*43.03	13.17				
NDPA	0	32	131.15	89.125	10.73	72	20	0.4	0.7
				*43.03	14.48				
NMPA	0	32	137.062	66	20.5	86	24	0.4	1.2
				*107	13.09				
NMBA	0	32	147.082	116.97	6.98	54	12	0.4	1.2
				*44.03	14.6				
NDBA	0	32	159.1	57.083	14.56	83	15	0.4	1.2
				*103	11.57				

*Qualifier ion

Injection order

- Blank injection at the beginning of a sequence
- Standard solution (3 ng/mL) for six consecutive injections
- Linearity standards, single injection each
- LOD and LOQ for six replicate injections
- Neat standard solutions for recovery at three different concentration levels starting from LLOQ concentration
- Standard spiked samples of metformin drug product for recovery evaluation at three different concentration levels starting from LLOQ concentration
- Three extra injections of blank and standard solution (bracketing standard) interspersed throughout the sequence to check system's robustness

System suitability requirements[1]

- The area of an interference peak for nitrosamine impurities in the blank injection, if present, should be no more than 5% of the peak area in the standard solution.
- The % RSD of the peak area for each nitrosamine impurity for the first six injections of standard solution should be no more than 10%.
- The cumulative % RSD of the peak area for each nitrosamine impurity should be no more than 15%. (Cumulative % RSD of the peak area is calculated by combining the initial six replicate injections of the standard solution and each subsequent bracketing standard.)

Observation shared by FDA method

- NMBA and NEIPA exist as syn and anti-conformers due to the restricted rotation of N-N bond,[1,2] and these conformers can be partially separated by the method's chromatographic conditions.
- The NMBA peak is observed as a doublet at a ratio of approximately 3:1.
- Integrate both peaks and use the combined peak area for NMBA.
- Depending on column and concentration of the sample, the NEIPA peak may appear as doublet or a single peak with a tailing shoulder. Include the resolved second peak or the tailing of the main peak when integrating the NEIPA peak(s).
- The retention time difference of any impurity in the analyzed samples should not be more than 2% of the retention time of the corresponding standard in the standard solution.
- Report the nitrosamine impurity content in ppm with three significant figures if the value is ≥ LOD.
- Report "not detected" if no nitrosamine impurity is detected or the value is < LOD.

Calculation as per FDA

Drug substance:

$$\text{Nitrosamine impurity (ppm)} = \frac{A_{spl}}{As} \times C_s \times \frac{1\ mg}{1 \times 10^6\ ng} \times \frac{V}{W} \times 10^6$$

Where: Nitrosamine impurity refers to NDMA, NDEA, NEIPA, NDIPA, NDPA, NMPA, NDBA, NMOR, NPIP, or NMBA

A_{spl} = Area of the nitrosamine impurity peak in the sample solution

As = Average area (n = 6) of the nitrosamine impurity peak from the first six consecutive injections of the standard solution

C_s = Concentration of the nitrosamine impurity in the standard solution (3.0 ng/mL)

W = Weight of drug substance (mg)

V = Volume of the diluent in the sample solution (mL)

Drug product:

$$\text{Nitrosamine impurity (ppm)} = \frac{A_{spl}}{As} \times C_s \times \frac{1\ mg}{1 \times 10^6\ ng} \times \frac{1}{100\ mg/mL} \times 10^6$$

Where: Nitrosamine impurity refers to NDMA, NDEA, NEIPA, NDIPA, NDPA, NMPA, NDBA, NMOR, NPIP, or NMBA

A_{spl} = Area of the nitrosamine impurity peak in the sample solution

As = Average area (n = 6) of the nitrosamine impurity peak from the first six consecutive injections of the standard solution

C_s = Concentration of the nitrosamine impurity in the standard solution (3.0 ng/mL)

Data analysis

Data analysis has been performed using Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) software, version 7.3.

Results and discussion

During the course of method development, it was observed that when multiple nitrosamines are analyzed in SRM mode using a triple quadrupole LC-MS system, there is a huge impact of the ionization mode. The APCI ionization mode gives the desired sensitivity for all nitrosamines in this method, whereas ESI is suitable only for a few of them.

System suitability was performed by injecting six replicate injections of standard solution at 3 ng/mL to evaluate the performance of the LC-MS/MS instrument. Table 5 shows the results of %RSD calculated for retention times and peak area responses of all impurity standards.

Table 5. System suitability (%RSD) of nitrosamine impurities

System Suitability (%RSD) for Nitrosamine Impurities

NDMA

Sr. No.	Injection Name	Name	Ret. Time (min)	Area counts*sec
1	STANDARD-3NG-1	NDMA	7.31	57027
2	STANDARD-3NG-2	NDMA	7.31	62683
3	STANDARD-3NG-3	NDMA	7.31	61608
4	STANDARD-3NG-4	NDMA	7.31	58362
5	STANDARD-3NG-5	NDMA	7.31	61495
6	STANDARD-3NG-6	NDMA	7.31	61162
		MEAN	7.310	60389.5
		SD	0.0000	2190.02
		%RSD	*0.0*	*3.6*

NMOR

Sr. No.	Injection Name	Name	Ret. Time (min)	Area counts*sec
1	STANDARD-3NG-1	NMOR	9.79	80395
2	STANDARD-3NG-2	NMOR	9.79	81103
3	STANDARD-3NG-3	NMOR	9.79	79310
4	STANDARD-3NG-4	NMOR	9.79	80426
5	STANDARD-3NG-5	NMOR	9.79	85434
6	STANDARD-3NG-6	NMOR	9.79	78705
		MEAN	9.790	80895.5
		SD	0.0000	2384.28
		%RSD	*0.0*	*2.9*

NMBA

Sr. No.	Injection Name	Name	Ret. Time (min)	Area counts*sec
1	STANDARD-3NG-1	NMBA	11.11	64960
2	STANDARD-3NG-2	NMBA	11.11	68500
3	STANDARD-3NG-3	NMBA	11.11	64717
4	STANDARD-3NG-4	NMBA	11.11	64555
5	STANDARD-3NG-5	NMBA	11.05	65837
6	STANDARD-3NG-6	NMBA	11.05	68350
		MEAN	11.090	66153.2
		SD	0.0310	1815.16
		%RSD	*0.3*	*2.7*

NDEA

Sr. No.	Injection Name	Name	Ret. Time (min)	Area counts*sec
1	STANDARD-3NG-1	NDEA	13.35	85920
2	STANDARD-3NG-2	NDEA	13.35	83224
3	STANDARD-3NG-3	NDEA	13.35	82385
4	STANDARD-3NG-4	NDEA	13.35	81516
5	STANDARD-3NG-5	NDEA	13.35	84557
6	STANDARD-3NG-6	NDEA	13.35	83253
		MEAN	13.350	83475.8
		SD	0.0000	1567.60
		%RSD	*0.0*	*1.9*

NPIP

Sr. No.	Injection Name	Name	Ret. Time (min)	Area counts*sec
1	STANDARD-3NG-1	NPIP	13.91	124334
2	STANDARD-3NG-2	NPIP	13.91	123294
3	STANDARD-3NG-3	NPIP	13.91	126834
4	STANDARD-3NG-4	NPIP	13.91	132014
5	STANDARD-3NG-5	NPIP	13.91	129950
6	STANDARD-3NG-6	NPIP	13.91	121314
		MEAN	13.910	126290.0
		SD	0.0000	4099.23
		%RSD	*0.0*	*3.2*

NEIPA

Sr. No.	Injection Name	Name	Ret. Time (min)	Area counts*sec
1	STANDARD-3NG-1	NEIPA	14.78	253852
2	STANDARD-3NG-2	NEIPA	14.78	243740
3	STANDARD-3NG-3	NEIPA	14.78	239167
4	STANDARD-3NG-4	NEIPA	14.78	239081
5	STANDARD-3NG-5	NEIPA	14.78	253008
6	STANDARD-3NG-6	NEIPA	14.78	246183
		MEAN	14.780	245838.5
		SD	0.0000	6485.57
		%RSD	*0.0*	*2.6*

NDIPA

Sr. No.	Injection Name	Name	Ret. Time (min)	Area counts*sec
1	STANDARD-3NG-1	NDIPA	16.26	98690
2	STANDARD-3NG-2	NDIPA	16.26	97778
3	STANDARD-3NG-3	NDIPA	16.26	98203
4	STANDARD-3NG-4	NDIPA	16.26	103096
5	STANDARD-3NG-5	NDIPA	16.26	97228
6	STANDARD-3NG-6	NDIPA	16.26	101040
		MEAN	16.260	99339.2
		SD	0.0000	2263.32
		%RSD	*0.0*	*2.3*

NMPA

Sr. No.	Injection Name	Name	Ret. Time (min)	Area counts*sec
1	STANDARD-3NG-1	NMPA	16.94	95098
2	STANDARD-3NG-2	NMPA	16.94	93786
3	STANDARD-3NG-3	NMPA	17.01	92801
4	STANDARD-3NG-4	NMPA	17.01	94986
5	STANDARD-3NG-5	NMPA	17.01	91952
6	STANDARD-3NG-6	NMPA	17.01	96360
		MEAN	16.987	94163.8
		SD	0.0361	1628.75
		%RSD	*0.2*	*1.7*

NDPA

Sr. No.	Injection Name	Name	Ret. Time (min)	Area counts*sec
1	STANDARD-3NG-1	NDPA	17.19	112416
2	STANDARD-3NG-2	NDPA	17.19	108293
3	STANDARD-3NG-3	NDPA	17.19	111569
4	STANDARD-3NG-4	NDPA	17.19	112703
5	STANDARD-3NG-5	NDPA	17.19	108097
6	STANDARD-3NG-6	NDPA	17.19	113959
		MEAN	17.190	111172.8
		SD	0.0000	2431.49
		%RSD	*0.0*	*2.2*

NDBA

Sr. No.	Injection Name	Name	Ret. Time (min)	Area counts*sec
1	STANDARD-3NG-1	NDBA	23.53	51579
2	STANDARD-3NG-2	NDBA	23.53	52797
3	STANDARD-3NG-3	NDBA	23.53	50136
4	STANDARD-3NG-4	NDBA	23.53	47511
5	STANDARD-3NG-5	NDBA	23.53	51557
6	STANDARD-3NG-6	NDBA	23.53	47915
		MEAN	23.530	50249.2
		SD	0.0000	2141.45
		%RSD	*0.0*	*4.3*

Linearity was determined by injection of low to high calibration standards of the desired concentration range. Figure 1 shows the linearity of nitrosamine standards.

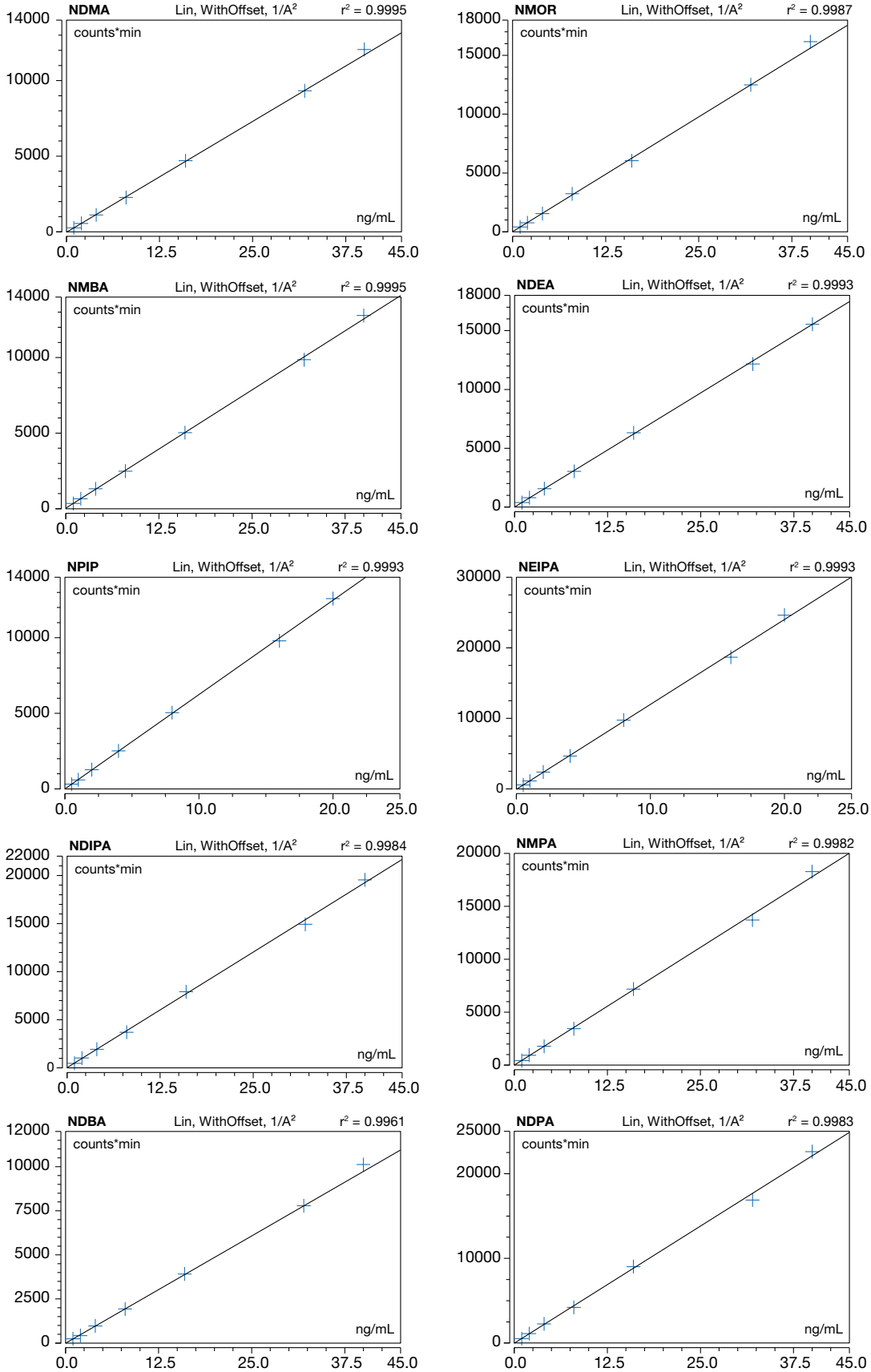

Figure 1. Calibration plots

The chromatograms of the solvent blanks are shown in Figure 2.

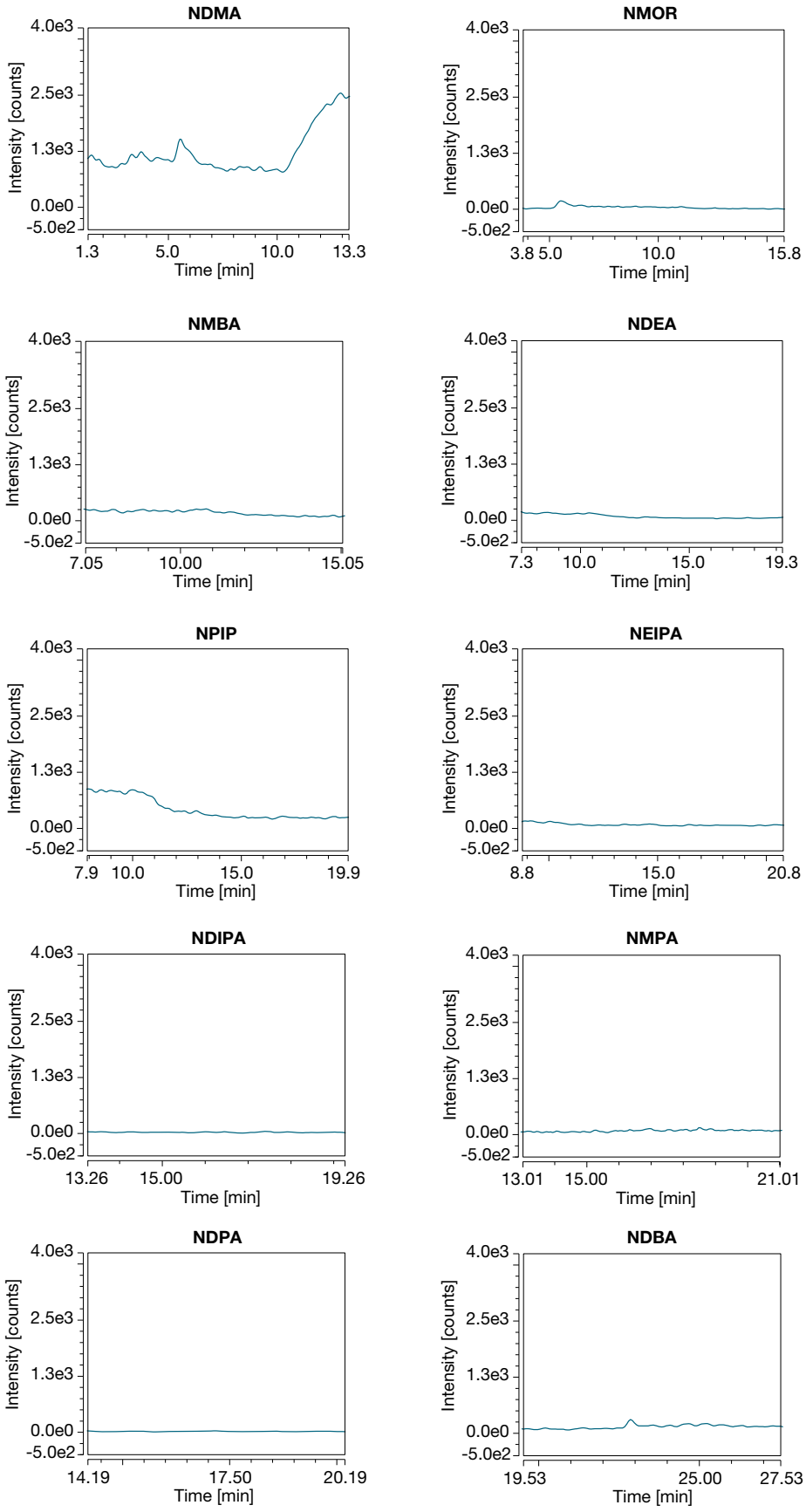

Figure 2. Chromatograms of solvent blanks

The chromatograms of the ten nitrosamine impurities at the LOQ are shown in Figure 3.

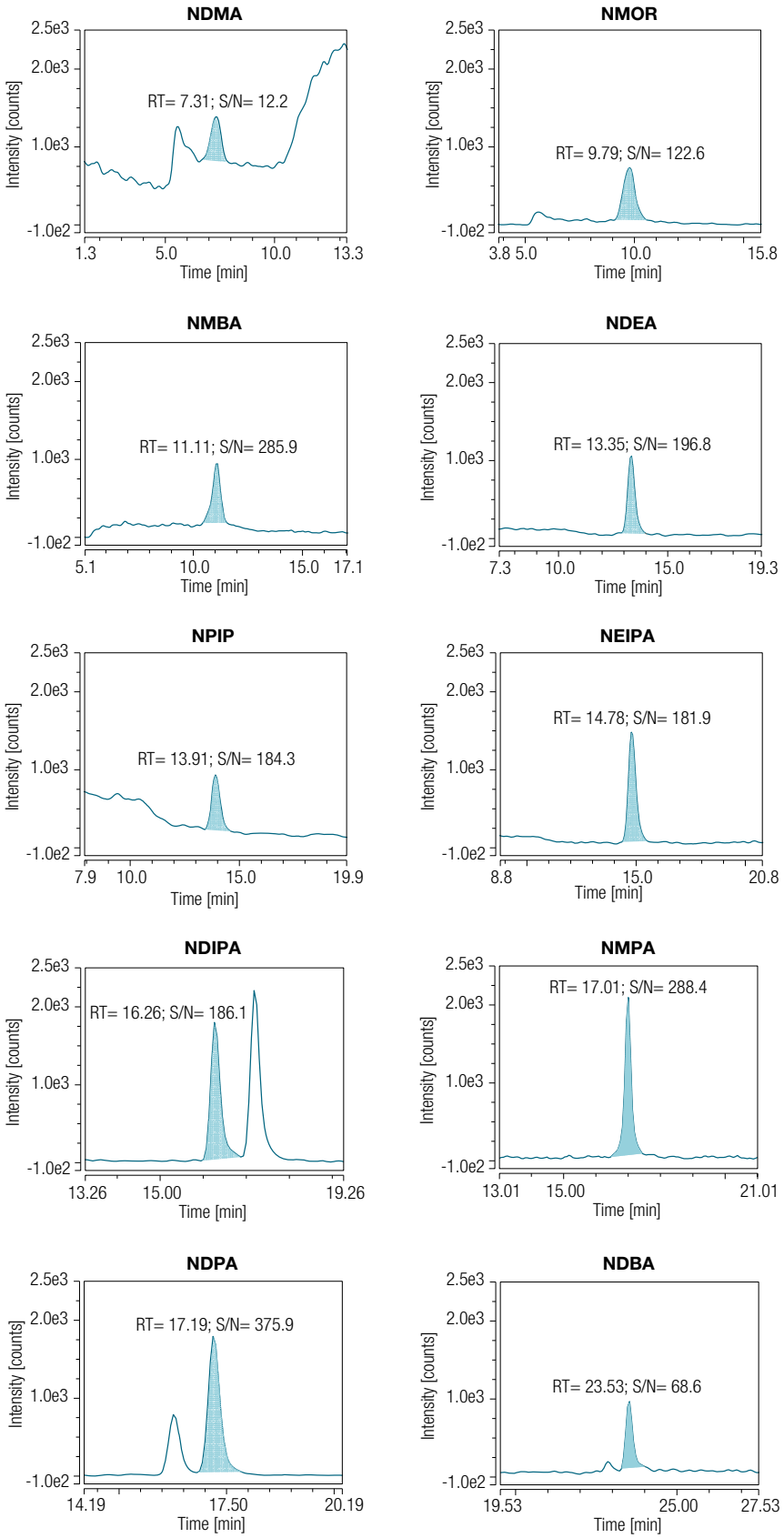

Figure 3. Chromatograms at the LOQ

Figure 4 shows the chromatograms of the standard at 3.0 ng/mL (0.03 ppm w.r.t. sample concentration)

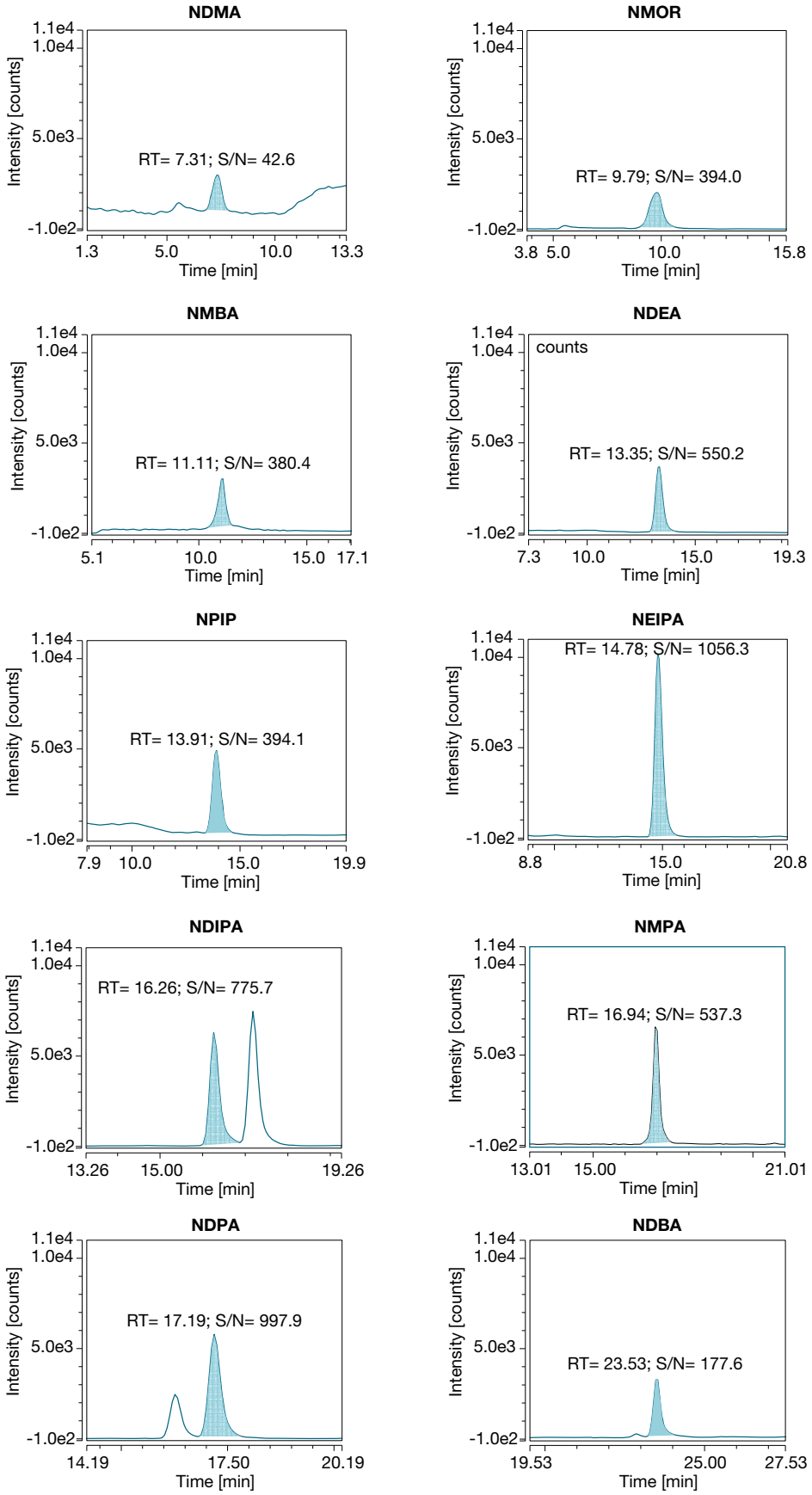

Figure 4. Chromatograms of the standard at 3.0 ng/mL (0.03 ppm w.r.t. sample concentration)

Table 6 summarizes the LOD, LOQ, S/N ratio, and linearity range for the 10 nitrosamine impurities.

The recovery evaluation data at low, mid, and high concentrations are presented in Table 7.

Table 6. Results summary

S. no.	Compound name	Limit of detection (LOD)	Limit of quantification (LOQ)	Signal-to-noise ratio at LOQ	Linearity range (ppb)
		Nominal conc. (ppb)	Nominal conc. (ppb)		
1	NDMA	0.4	1	12.2	1-40
2	NMOR	0.4	1	122.6	1-40
3	NMBA	0.4	1	285.9	1-40
4	NDEA	0.4	1	196.8	1-40
5	NPIP	0.2	0.5	184.3	0.5-20
6	NEIPA	0.2	0.5	181.9	0.5-20
7	NDIPA	0.4	1	186.1	1-40
8	NMPA	0.4	1	288.4	1-40
9	NDPA	0.4	1	375.9	1-40
10	NDBA	0.4	1	68.6	1-40

Table 7. Recovery results

Recovery at Low concentration level										
Impurity	**NDMA**	**NMOR**	**NMBA**	**NDEA**	**NPIP**	**NEIPA**	**NDIPA**	**NMPA**	**NDPA**	**NDBA**
Concentration	1 ng/mL	1 ng/mL	1 ng/mL	1 ng/mL	0.5 ng/mL	0.5 ng/mL	1 ng/mL	1 ng/mL	1 ng/mL	1 ng/mL
% Recovery*	*104.8*	*99.7*	*107.5*	*89.5*	*86.8*	*93.8*	*87.5*	*84.6*	*94.6*	*108.5*
Recovery at Mid concentration level										
Impurity	**NDMA**	**NMOR**	**NMBA**	**NDEA**	**NPIP**	**NEIPA**	**NDIPA**	**NMPA**	**NDPA**	**NDBA**
Concentration	3 ng/mL	3 ng/mL	3 ng/mL	3 ng/mL	3 ng/mL	3 ng/mL	3 ng/mL	3 ng/mL	3 ng/mL	3 ng/mL
% Recovery*	*87.8*	*92.7*	*105.4*	*89.0*	*90.7*	*88.0*	*86.3*	*82.0*	*88.9*	*96.0*
Recovery at High concentration level										
Impurity	**NDMA**	**NMOR**	**NMBA**	**NDEA**	**NPIP**	**NEIPA**	**NDIPA**	**NMPA**	**NDPA**	**NDBA**
Concentration	16 ng/mL	16 ng/mL	16 ng/mL	16 ng/mL	8 ng/mL	8 ng/mL	16 ng/mL	16 ng/mL	16 ng/mL	16 ng/mL
% Recovery*	*87.3*	*97.7*	*118.0*	*92.6*	*97.0*	*92.3*	*92.0*	*84.9*	*92.9*	*95.4*

* % Recovery calculation for Nitrosamines = (Area in Spiked Drug Sample-Area in Unspiked Drug Sample/Area in Neat Standard) × 100

Conclusion

1. A LC-MS/MS method for the analysis of 10 nitrosamine impurities in metformin drug product has been successfully developed with excellent reproducibility, linearity, and recovery. The method showed capabilities to meet expectations even lower than currently required concentrations. The R^2 reported for all compounds is >0.99.

2. The LOQ for all ten impurities was established lower than currently required by regulatory expectations. The %RSD at desired standard concentration (0.030 ppm with respect to sample concentration) was less than 5% for all compounds, which meets reproducibility requirements.

3. %Recovery was determined at 0.03 ppm and found to be within the permissible limit (80 to 120%). Recovery was determined at two additional concentration levels, i.e., LLOQ level and slightly higher concentration level than standard concentration, and the results were found to be within 80–120%.

4. Cumulative %RSD (i.e., including system suitability standard and bracketing standards) was found to be within 6.1% for all ten impurities when the whole sequence was analyzed for 35 hours continuously, which reflects the robustness of the system and method.

5. The TSQ Quantis LC-MS/MS system is capable of successfully achieving the desired concentration limits for the nitrosamines. The sensitivity and reproducibility of the instrument and method meets expectations for the analysis of metformin tablet samples.

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