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Low-level Quantification of 10 Mutagenic Nitrosamine Impurities in Pioglitazone Hydrochloride Using Accurate Mass Spectrometry

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Introduction

Pioglitazone is used to treat type 2 diabetes because it increases the effectiveness of insulin produced by the body to help maintain blood sugar levels and alleviate symptoms. It is essential to ensure that drug products used to treat disease are free from contamination and safe to use. As a result, medicines such as Pioglitazone have been scrutinized since the Nitrosamine crisis began in 2018. The recommended limit for total Nitrosamines in most drug products is currently 30 ng/g, which is derived from a maximum daily dose of less than 880 mg/day. Pioglitazone Hydrochloride has a maximum daily dose of 45 mg and falls well below this threshold where a 30 ng/g limit can be implemented.

In this technical note, we present an accurate mass spectrometry method for quantifying 10 mutagenic Nitrosamines in Pioglitazone Hydrochloride, including NDMA. Excellent chromatographic separation was achieved for all 10 Nitrosamines and the Pioglitazone Hydrochloride active pharmaceutical ingredient (API) (**Figure 1**), using a Kinetex 2.6 µm Biphenyl HPLC column. This column chemistry was selected because the polar nature of the phase provides selectivity that allows separation of the Nitrosamine impurities and ensures that the Pioglitazone Hydrochloride elutes after the Nitrosamine peaks. Due to the extremely low levels of the Nitrosamines, it is necessary to load a higher concentration of the API which would overload the mass spectrometer. By ensuring the API elutes after the Nitrosamine impurities, the Pioglitazone Hydrochloride is diverted to waste and the cleanliness of the mass spectrometer is maintained. Statistically significant quantitative performance and linearity were achieved using accurate mass spectrometry at low concentration levels.

Sample Preparation

Standard preparation: A stock solution containing 10 µg/mL of each Nitrosamine was prepared in water from standard solutions. Serial dilutions in water were performed to generate calibration solutions with concentrations of 100, 50, 25, 5, 1, 0.4, 0.2, 0.1, 0.050, 0.025, and 0.010 ng/mL.

Spiked sample preparation: A 200 mg sample of Pioglitazone Hydrochloride API was weighed into a suitable vessel. A 5 mL aliquot of a 1 ng/mL Nitrosamine mixed standard solution was added and vortexed for 30 seconds. The solution was sonicated for 15 minutes and then centrifuged at 4500 rpm for 5 minutes. The supernatant was removed and filtered through a 0.2 µm PTFE filter and transferred to a HPLC vial for analysis. The resulting solution had a sample concentration of 40 mg/mL with a spike concentration of 1 ng/mL of Nitrosamine mix. This was equivalent to the 25 ng/g spike concentration of the sample.

LC Conditions

Column: Kinetex™ 2.6 µm Biphenyl
Dimensions: 100 x 2.1 mm
Part No.: 00D-4622-AN
Mobile Phase: A: 0.1 % Formic Acid in Water
B: 0.1 % Formic Acid in Methanol

Gradient:

Time (min)	%B
0.00	5
0.50	10
14.0	45
16.0	95
20.0	95
20.1	5
22.0	5

Flow Rate: 0.4 mL/min
Injection Volume: 25 µL
Temperature: 30 °C
LC System: SCIEX® ExionLC™
Detection: TOF MS and MRM^{HR}
Detector: SCIEX X500R QTOF

MRM Conditions

Polarity: Positive (APCI ionization)
Source Temperature: 300 °C
GS1: 55 psi
CUR: 30 psi
CAD: 7 psi
Nebulizing Current: 5 µA

MRM Transitions and Parameters

	TOF MS	MRM^{HR}		
Analyte	Precursor Ion (m/z)	Q1 (m/z)	Q3 (m/z)	CE (V)
N-Nitrosodimethylamine (NDMA)	75.0552	75.0	43.0297	22
N-Nitrosodi-n-propylamine (NDIPA)	131.1178	131.1	43.0543	14
N-Nitrosomethylethylamine (NMEA)	89.0709	89.0	61.0403	14
N-Nitrosodiethylamine (NDEA)	103.0865	103.0	75.0555	14
1-Nitrosopyrrolidine (NPYR)	101.0709	101.1	55.0546	34
1-Nitrosopiperidine (NPIP)	115.0865	115.1	41.0386	30
4-Nitrosomorpholine (NMO)	117.0658	117.1	87.068	14
N-Nitrosoethylisopropylamine (NEIPA)	117.1022	117.0	75.0557	12
N-Nitrosomethylaminobutyric Acid (NMBA)	147.0764	147.0	117.079	8
N-Nitrosodiisopropylamine (NDPA)	131.1178	131.1	43.0543	16

Results and Discussion

The accuracy of mass measurement is crucial when using an accurate mass spectrometer. This is increasingly important for compounds that have low molecular weights, such as Nitrosamines. Consequently, **Table 2** shows the high levels of mass accuracy that the X500R QTOF system can achieve with precursor and fragment ions that are used for the quantification of Nitrosamines in spiked samples at 1 ng/mL, equivalent to 25 ng/g of Pioglitazone Hydrochloride API.

The calibration curves for 10 Nitrosamines were plotted across a concentration range of 0.010-100 ng/mL (**Figures 3** and **4**). A linear dynamic range of 3 orders of magnitude was achieved for most Nitrosamines. No carryover was observed within the blank injection following the highest concentration. A high level of accuracy was achieved across the calibration range, meeting the requirements for Nitrosamine impurities in Pioglitazone Hydrochloride. For both precursor ion and MRM^{HR} based quantification of Nitrosamines, the r^2 value was >0.98 (**Table 1**).

With accurate mass spectrometry, users can choose a workflow that best meets their needs. With TOF MS, method setup is straightforward and requires minimal method development. The MRM^{HR} workflow adds another layer of selectivity with the flexibility to choose the most sensitive and selective fragments for quantification.

The extracted ion chromatograms (XICs) at the LOQ levels for 2 representative Nitrosamines using TOF MS and MRM^{HR} quantification are shown in **Figure 5**. Based on the experimental observations, the relative sensitivity of the MS method versus the targeted MS/MS method depends on the nature of the interference from the matrix. For example, an 8-fold improvement in the LOQ was observed using TOF MS for NDEA. However, in the case of NMBA, a 50-fold improvement in LOQ was reached with MRM^{HR}.

Accuracy and precision metrics were evaluated in standard solutions and spiked samples. A 1 ng/mL concentration in spiked solution (equivalent to 25 ng/g in sample concentration) was used for the assessment. The acceptable criteria for accuracy and precision at this concentration level were ±30 % and <25 % of the nominal concentration, respectively.

The spiked Pioglitazone Hydrochloride API sample met the specified requirements for all Nitrosamine impurities (**Table 2**). Overall, the %CV was <13.5 % and <13.3 % for precursor ion and MRM^{HR} quantification experiments, respectively. The percent accuracy was within ±15 % of the nominal concentration for both quantification workflows. The mass error was <1 ppm in spiked samples, demonstrating high mass accuracy for Nitrosamine impurity analysis in the API. The LOQ for NMBA was 5 ng/mL using the precursor ion TOF MS workflow which was found to be BLQ in spiked samples. **Figures 6** and **7** show representative chromatograms of 10 Nitrosamines in 1 ng/mL spiked samples using MRM^{HR} and 9 Nitrosamines using TOF MS experiments for quantification.

Figure 1. Representative XIC for 10 Nitrosamines and UV Chromatogram for Pioglitazone.

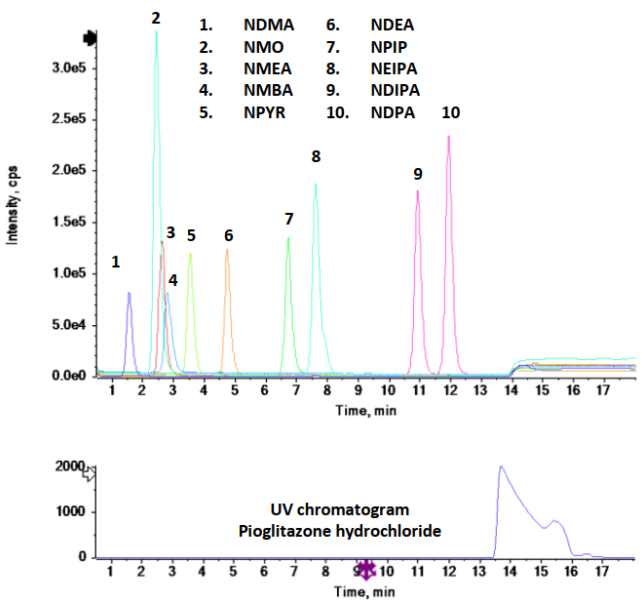


Figure 3. Calibration Curves Representing the Quantitative Results from 10 Nitrosamines using Precursor Ions in Full Scan TOF MS Mode.

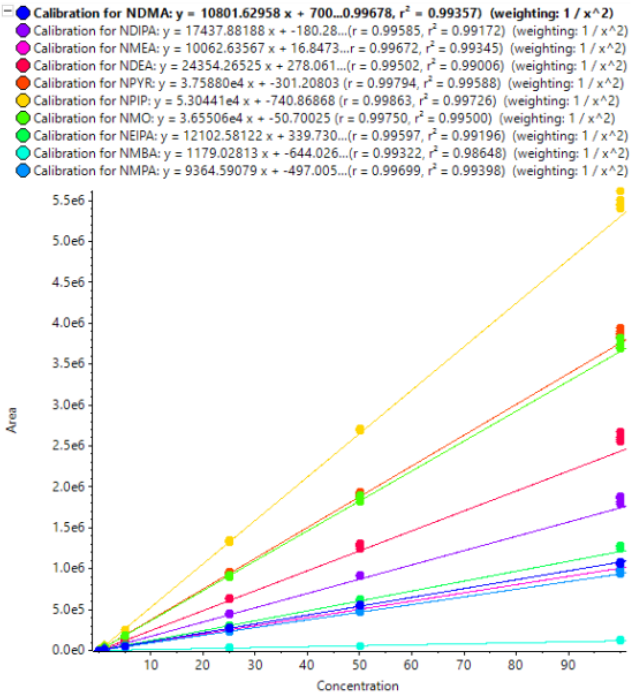


Figure 4. Calibration Curves Representing the Quantitative Results from 10 Nitrosamines using MRM^{HR}.

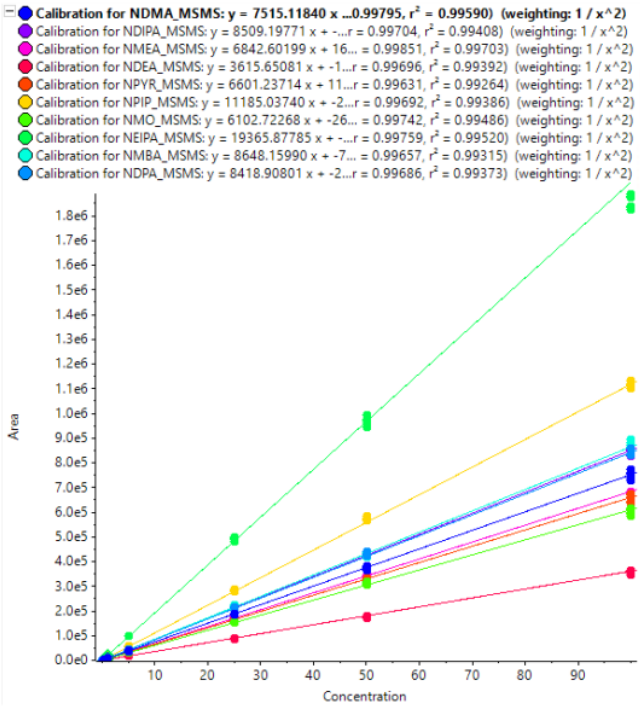


Figure 5. XICs at the LOQs of 2 Nitrosamines using Precursor Ion (TOF MS) and MRM^{HR}-based Quantification.

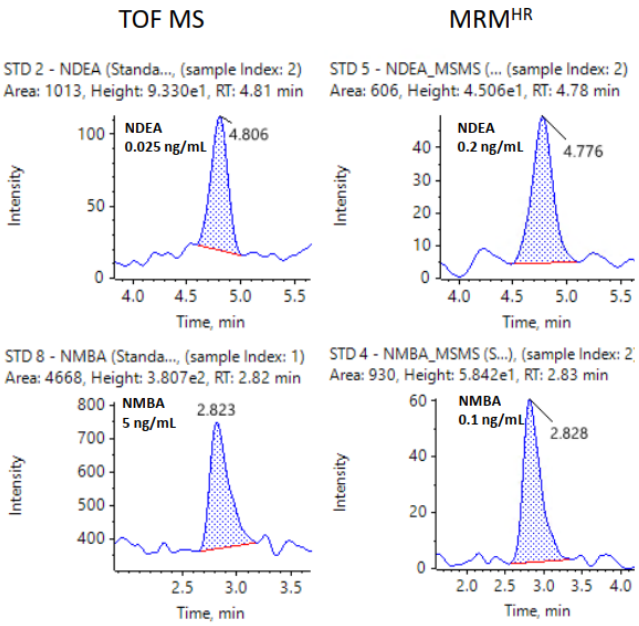


Table 1. Mass Error, %CV, and % Accuracy for Spiked Samples.

	TOF MS			MRM^{HR}		
Compound	Mass Error (PPM)	CV (%)	Accuracy (%)	Mass Error (PPM)	CV (%)	Accuracy (%)
NDMA	-0.679	13.5	85.9	-0.538	3.40	101
NDIPA	-0.258	1.80	112	-0.093	5.70	114
NMEA	0.537	7.60	85.2	0.560	4.40	110
NDEA	0.040	3.40	109	0.543	9.70	110
NPYR	-0.384	5.80	112	0.622	13.3	105
NPIP	0.396	3.00	100	0.679	11.1	102
NMO	0.301	5.40	112	-0.045	3.30	107
NEIPA	-0.971	4.10	99.1	0.508	3.10	107
NMBA	BLQ*	BLQ*	BLQ*	-0.452	6.10	98.9
NDPA	-0.326	1.80	95.6	0.176	5.60	101

**BLQ = Below the Limit of Quantification.*

Table 2. Linearity Range, %CV, and Correlation Coefficient (r^2) for 10 Nitrosamines.

	TOF MS			MRM^{HR}		
Compound	Linearity Range (ng/mL)	%CV at LOQ (n=6)	Correlation Coefficient (r^2)	Linearity Range (ng/mL)	%CV at LOQ (n=6)	Correlation Coefficient (r^2)
NDMA	0.1-100	10.5	0.993	0.2-100	8.90	0.995
NDIPA	0.05-100	11.7	0.991	0.1-100	9.30	0.994
NMEA	0.1-100	13.1	0.993	0.2-100	6.40	0.997
NDEA	0.025-100	8.60	0.990	0.2-100	11.0	0.993
NPYR	0.1-100	8.70	0.995	0.4-100	16.1	0.992
NPIP	0.1-100	8.80	0.997	0.4-100	11.8	0.993
NMO	0.05-100	17.1	0.995	0.2-100	8.60	0.994
NEIPA	0.2-100	12.3	0.991	0.1-100	13.6	0.995
NMBA	5-100	16.7	0.986	0.1-100	14.0	0.993
NDPA	0.05-100	11.7	0.991	0.1-100	13.4	0.993

Figure 6. Representative Chromatograms for 10 Nitrosamines in Spiked Samples using MRM^{HR}.

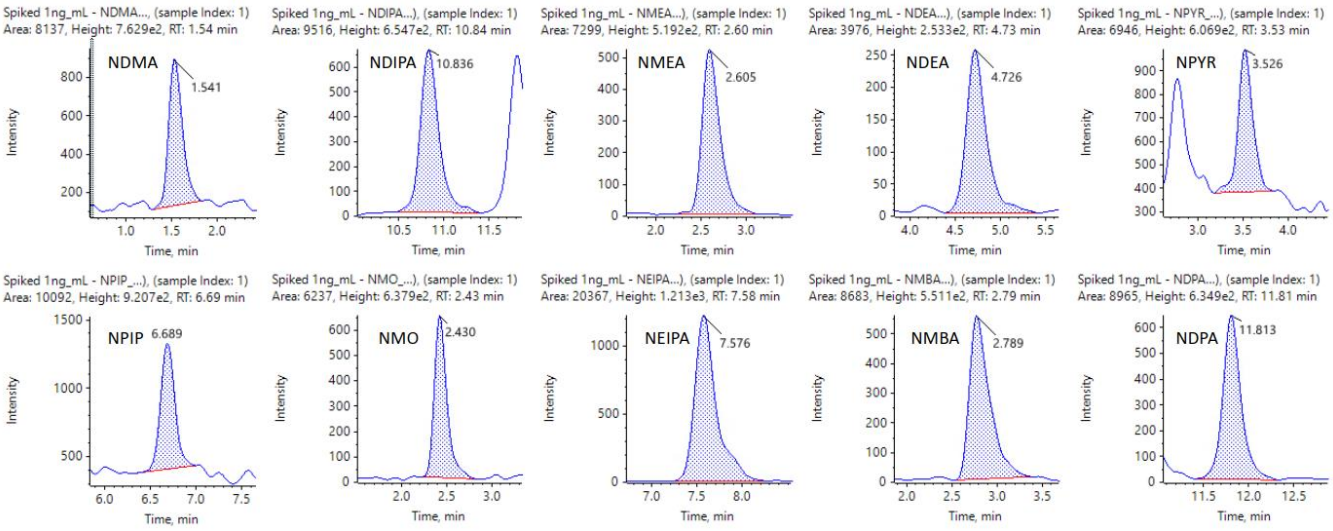
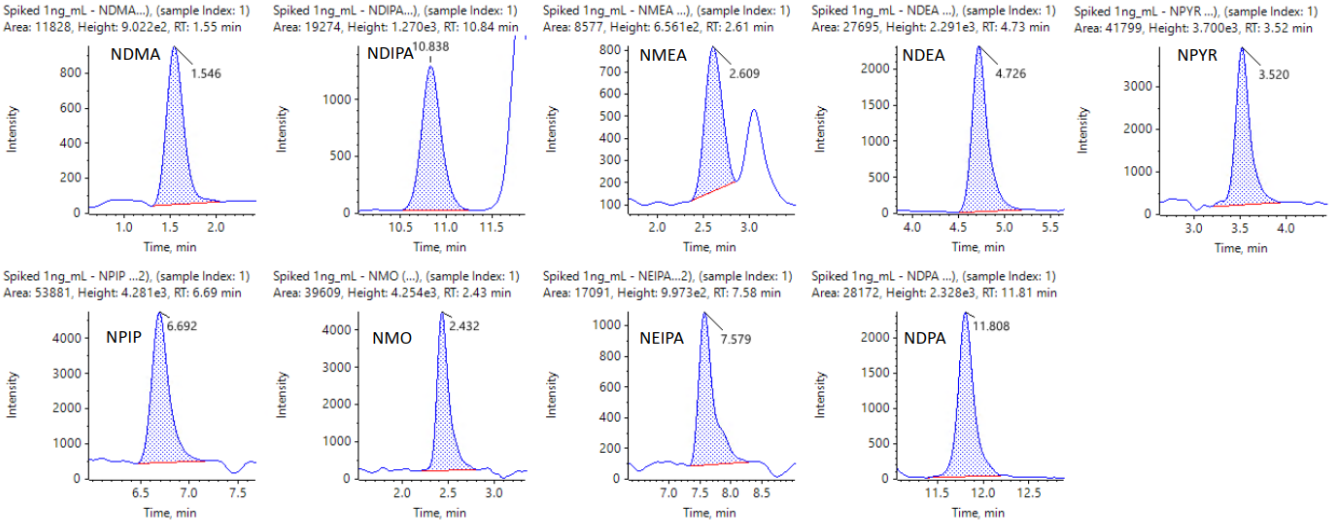


Figure 7. Representative Chromatograms for 9 Nitrosamines in Spiked Samples using the Precursor Ion in Full Scan TOF MS Mode.



Conclusions

Low-level quantification was achieved for 10 Nitrosamines in spiked samples using a Kinetex™ 2.6 µm Biphenyl HPLC column in conjunction with the SCIEX® X500R QTOF system. Due to the high degree of polar and aromatic selectivities provided, the Biphenyl stationary phase has proven an appropriate choice for chromatographic resolution for all 10 Nitrosamines. Excellent linearity and precision were reached for the analysis of Nitrosamines, demonstrating exceptional quantitative performance. High mass accuracy (<1 ppm) for low molecular weight Nitrosamines was accomplished, minimizing false positive results. Utilizing the fast-scanning speed of the X500R QTOF system, simultaneous monitoring was performed on 10 Nitrosamines in precursor ion in full scan TOF MS mode and MRM^{HR} experiments. The method demonstrated the quantification of Nitrosamine impurities below the current recommended limit (30 ng/g) in the Pioglitazone Hydrochloride drug product.

Kinetex Ordering Information

2.6 µm Minibore Columns (mm)						SecurityGuard™ ULTRA Cartridges (mm)‡
Phases	**30 x 2.1**	**50 x 2.1**	**75 x 2.1**	**100 x 2.1**	**150 x 2.1**	**3/pk**
EVO C18	00A-4725-AN	00B-4725-AN	—	00D-4725-AN	00F-4725-AN	AJ0-9298
PS C18	00A-4780-AN	00B-4780-AN	—	00D-4780-AN	00F-4780-AN	AJ0-8951
Polar C18	00A-4759-AN	00B-4759-AN	—	00D-4759-AN	00F-4759-AN	AJ0-9532
Biphenyl	00A-4622-AN	00B-4622-AN	—	00D-4622-AN	00F-4622-AN	AJ0-9209
XB-C18	00A-4496-AN	00B-4496-AN	00C-4496-AN	00D-4496-AN	00F-4496-AN	AJ0-8782
C18	00A-4462-AN	00B-4462-AN	00C-4462-AN	00D-4462-AN	00F-4462-AN	AJ0-8782
C8	00A-4497-AN	00B-4497-AN	00C-4497-AN	00D-4497-AN	00F-4497-AN	AJ0-8784
HILIC	00A-4461-AN	00B-4461-AN	00C-4461-AN	00D-4461-AN	00F-4461-AN	AJ0-8786
Phenyl-Hexyl	00A-4495-AN	00B-4495-AN	00C-4495-AN	00D-4495-AN	00F-4495-AN	AJ0-8788
F5	00A-4723-AN	00B-4723-AN	—	00D-4723-AN	00F-4723-AN	AJ0-9322

for 2.1 mm ID

‡SecurityGuard ULTRA Cartridges require holder, Part No.: AJ0-9000

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