Application Note

Pharmaceuticals

Quantitation of N-Nitroso Sitagliptin Impurity (NTTP) in Sitagliptin and Metformin Combination Drug Product Using the Agilent 6475 LC/TQ

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Abstract

Nitrosamine impurities are nitroso derivatives where the nitroso group is attached to an amine. The active pharmaceutical ingredient (API) derived class of nitrosamines are known as nitrosamine drug-substance-related impurities (NDSRI). They are structurally related to APIs as there could be degradant products or byproducts generated during the storage period or during the manufacturing process of the drug product. The acceptable intake for N-nitroso sitagliptin impurity (3-nitroso-1-(3-(trifluoromethyl)-5,6-dihydro- [1,2,4] triazolo[4,3-a] pyrazin-7(8H)-yl)-4-(2,4,5-trifluorophenyl) butane-1-one) is 37 ng/day as per the European Medicines Agency (EMA) regulation.

This application note describes the procedure for the multiple reaction monitoring (MRM)-based low-level quantitation of nitroso sitagliptin using an Agilent 1290 Infinity II liquid chromatograph (LC) coupled to an Agilent 6475 triple quadrupole mass spectrometer (LC/TQ). The method described shows excellent linearity over the range of 0.04 to 1.3 ng/mL (0.013 to 0.43 ppm with respect to an API load of 3 mg/mL) with R^2 values greater than 0.99. The lower limit of quantitation (LLOQ) of 0.04 ng/mL in a neat standard and the limit of quantitation (LOQ) of 0.08 ng/mL in a spiked sample have been achieved for targeted NDSRI. The method is demonstrated to be accurate (recovery in a range of 70 to 130%) and precise (RSD < 5.0 % at limit of quantitation and specification limit) for the N-nitroso sitagliptin impurity. The signal-to-noise ratio (S/N) is reported with the specified limits. This method provides a limit of detection (LOD) of 0.008 ng/mL (S/N > 30:1), lower limit of quantitation (LLOQ) of 0.04 ng/mL (S/N) of > 300:1), and a limit of quantitation (LOQ) of 0.088 ng/mL (S/N >600:1) using Agilent 6475A LC/TQ. The described method can be implemented in preapproval drug development and post approval risk assessment to prevent unexpected NDSRI findings in the oral solid dosage formulation.

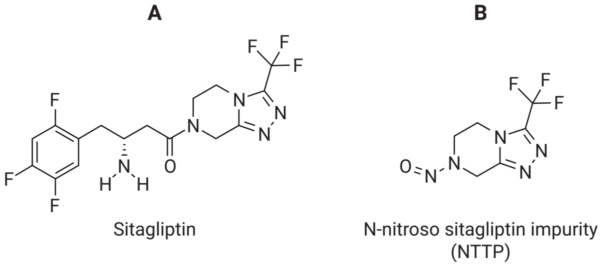
Introduction

Nitrosamines are members of a "cohort-of-concern" by the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) concerning the synthesis of drug substances involving the use of reactive chemicals, reagents, solvents, catalysts, and other processing aids (M7 (R1)). Nitrosamine drug-substance-related impurities (NDSRIs) are categorized as potent carcinogens by the International Agency for Research on Cancer/World Health Organization (IARC/WHO). NDSRIs can be formed due to the presence of secondary amine or tertiary amine (only with alpha hydrogen) in the substrate structure when reacting with any nitrosating agent under acidic conditions.[1]

The nitrosamines carcinogenicity is based on an acceptable intake limit (AI). The AI corresponds to a theoretical 1:100,000 risk to developing cancer during a lifetime. However, for most of the NDSRIs, there is no carcinogenicity data available. For such nitrosamines, health authorities have set a guidance that recommends a risk-based safety assessment of NDSRI and defines the AI for NDSRIs in drug products and APIs.

API-sitagliptin, a dipeptidyl peptidase-4 (DPP-4) inhibitor, serves as an adjunct to diet and exercise in managing type 2 diabetes mellitus. Recently, the FDA identified the presence of a nitrosamine impurity, nitroso-sitagliptin impurity (NTTP), in select samples of sitagliptin, raising concerns due to the potential carcinogenicity of nitrosamine compounds. While NTTP falls within this class of compounds, its specific carcinogenic potential remains unexplored.[2] However, considering data on closely related nitrosamines, the FDA established temporary distribution guidelines allowing sitagliptin containing NTTP above the acceptable intake limit (37 ng per day), in efforts to prevent medicine shortages and ensure patient access. The permissible limit extends up to 246.7 ng per day, while closely monitoring potential risks associated with NTTP exposure. This regulatory approach seeks to balance patient safety with the continued availability of essential diabetes management medications.

N-nitroso sitagliptin (NTTP) is an example of cyclic tertiary nitrosamine that has undergone nitrosation to form an NDSRI impurity.



Sitagliptin

N-nitroso sitagliptin impurity (NTTP)

Figure 1. (A) Sitagliptin. (B) N-nitroso sitagliptin impurity (NTTP).

Various analytical methods have been published by the United States Food and Drug Administration (US FDA) and other regulatory bodies for the estimation of nitrosamines in drug products.[3]

For most NDSRIs, analytical methods are not yet available. Therefore, manufacturers will need to develop and qualify a dedicated analytical method that is suitable for their sample matrix and specific for the NDSRIs.

Analytical methods that are developed must be sensitive enough to quantify the nitrosamine in question down to a level that corresponds to 10% of the specification limit. LC/MS/MS is an inherently selective and sensitive analytical technique that is well suited to identify and quantify mutagenic impurities at extremely low levels and has been widely adopted in the pharmaceutical industry.[4]

Experimental

Chemicals and reagents

An N-nitroso sitagliptin impurity standard, tablet formulation, and placebo were obtained from a pharmaceutical company. Other LC/MS-grade solvents (methanol, water) were purchased from Honeywell (Charlotte, NC, USA) and formic acid was purchased from Sigma-Aldrich (Kansas, USA).

Instrument configuration

Table 1. LC configurations and parameters.

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 (90:10)
Sample Diluent	100% methanol
Injection Volume	10 µL
Multisampler Temperature	5 °C
Analytical Column	Biphenyl column (4.6 × 150 mm, 2.7 µm)
Column Temperature	50 °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 rate (mL/min) 0 95 5 0.5 7 95 5 0.5 8 70 30 0.5 12 70 30 0.5 12.5 5 95 0.5 21 5 95 0.5 21.5 95 5 0.5 25 95 5 0.5
Stop Time	25 min
Wavelength	230 nm for sitagliptin

Table 2. Triple quadrupole MS source parameters.

Parameter	Value
Instrument	Agilent 6475 triple quadrupole mass spectrometer (G6475A)
Ion Source	Agilent atmospheric pressure chemical ionization (APCI)
MS/MS Mode	MRM
Ionization Mode (Polarity)	Positive
Gas Temperature	300 °C
Gas Flow	7 L/min
Nebulizer Pressure	30 psi
Vaporizer Temperature	300 °C
Nebulizer Current	6 µA
Capillary Voltage	3,000 V
MS1/MS2 Resolution	0.7/0.7 (unit/unit)

Table 3. Compound-related parameters in MRM mode.

Parameter	Value
ID	N-Nitroso triazolopyrazine (NTTP)
Precursor Ion (*m/z*)	222
Product Ion (*m/z*)	192
Dwell Time (ms)	200
Fragmentor (V)	80
Collision Energy (V)	7
Cell Accelerator Voltage (V)	5
Polarity	Positive

Table 4. Working standard solution preparation for the plotting calibration curve.

Working Standard (ng/mL)	Volume Taken (mL)	Volume of Diluent (mL)	Total Volume (mL)	Resultant Concentration (ng/mL)	Levels
5.5	1.20	3.800	5.0	1.30	120% SPEC
5.5	1.00	4.000	5.0	1.10	100% SPEC
5.5	0.80	4.200	5.0	0.88	80% SPEC
5.5	0.50	4.500	5.0	0.56	50% SPEC
5.5	0.250	4.750	5.0	0.27	25% SPEC
5.5	0.110	4.900	5.0	0.11	10% SPEC
5.5	0.080	4.920	5.0	0.088	LOQ
5.5	0.037	4.960	5.0	0.04	LLOQ
0.08	0.500	4.500	5.0	0.008	LOD

Table 5. Diverter valve program.

Start Time (min)	Scan Type	Diverter Valve
0	MRM	To waste
8	MRM	To MS
11	MRM	To waste

Sample preparation

Specification limit

The following calculation was used to determine the specification (spec) limit, where "MDD" represents the maximum daily dose in mg, and "AI" represents acceptable intake:

Specification limit = AI ÷ MDD

The AI for N-nitroso sitagliptin impurity is 37 ng/day. The MDD of sitagliptin in the sitagliptin and metformin combination tablet is 100 mg. Therefore, the limit of N-nitroso sitagliptin impurity (ppm) was calculated as 37 ng/day ÷ 100 mg/day = 0.37 ppm.

This calculation of 0.37 ppm corresponds to 1.11 ng/mL absolute concentration with the API test concentration of 3 mg/mL.

Preparation of working standard solution

Working standards were prepared with the diluent as specified in Table 1 to give the final concentration range of 0.088 to 1.3 ng/mL for the calibration plot.

Formulation sample preparation

Using the labeled concentration (50 mg of sitagliptin/tablet), crush enough tablets to obtain at least three times the API target weight. Calculate the average weight per tablet, accurately weigh 15 mg of sitagliptin equivalent tablet-crushed powder, and transfer it to the 15 mL centrifuge tube. Then, add 5 mL of diluent.[5] Vortex the sample for one minute, followed by 40 minutes of shaking using a Rotospin shaker. After effective extraction, centrifuge the samples at 4,000 rpm, at 10 °C for 20 minutes. Filter the supernatant solution using a 0.22 µm PVDF syringe filter. Transfer into an HPLC sample vial for LC/TQ analysis.

Sample preparation for recovery

Recovery samples were prepared in triplicate by spiking at a specification limit of 1.1 ng/mL (0.366 ppm), following the previously discussed procedure.

Data acquisition and data analysis

All samples were acquired using Agilent MassHunter Data Acquisition software, version 12.0.

MRM transitions were obtained and optimized using the Agilent MassHunter Optimizer tool. This tool automatically optimizes fragmentor voltages for the precursor ion, and collision energies for the product ions. A standard solution with a 50 ng/mL concentration was introduced to the MS by flow injection analysis with an injection volume of 5 µL through the automated workflow. The MRM mode was used for quantification, as it selectively filters the precursor ion and product ions of the compound of interest, thereby increasing the S/N and selectivity.

Instrument source parameters (Table 2) and MRM parameters (Table 3) were optimized to maximize sensitivity, while maintaining consistency in the method performance for large batches.

Once the chromatographic separation between the API and impurities was established, a time program was set to divert the API to waste with the help of an integrated diverter valve, as mentioned in Table 5.

The limit of detection (LOD) and limit of quantitation (LOQ) were calculated automatically using slope and intercept in the formula $y = mx + c$, where m is the slope and c is the y-intercept. Chromatograms were viewed through MassHunter Qualitative Analysis software, version 12.0. Quantitation of each batch was carried out using MassHunter Quantitative Analysis software, version 12.0.

Results and discussion

For chromatography separation column selection screening, different solvents were tested. These tests resulted in methanol being chosen over acetonitrile due to methanol's higher polarity and improved retention of the impurity. The mobile phases initially included 0.1% formic acid buffer for ionization. A column temperature of 50 °C was necessary for effective separation between the impurity and the API.

Ultimately, the biphenyl column was selected as the optimal choice. The effectiveness of the column in retaining nitrosamine impurities was attributed to the pi-pi interactions of nitroso groups with the stationary phase, which provided selectivity and retention not observed in other columns.[6]

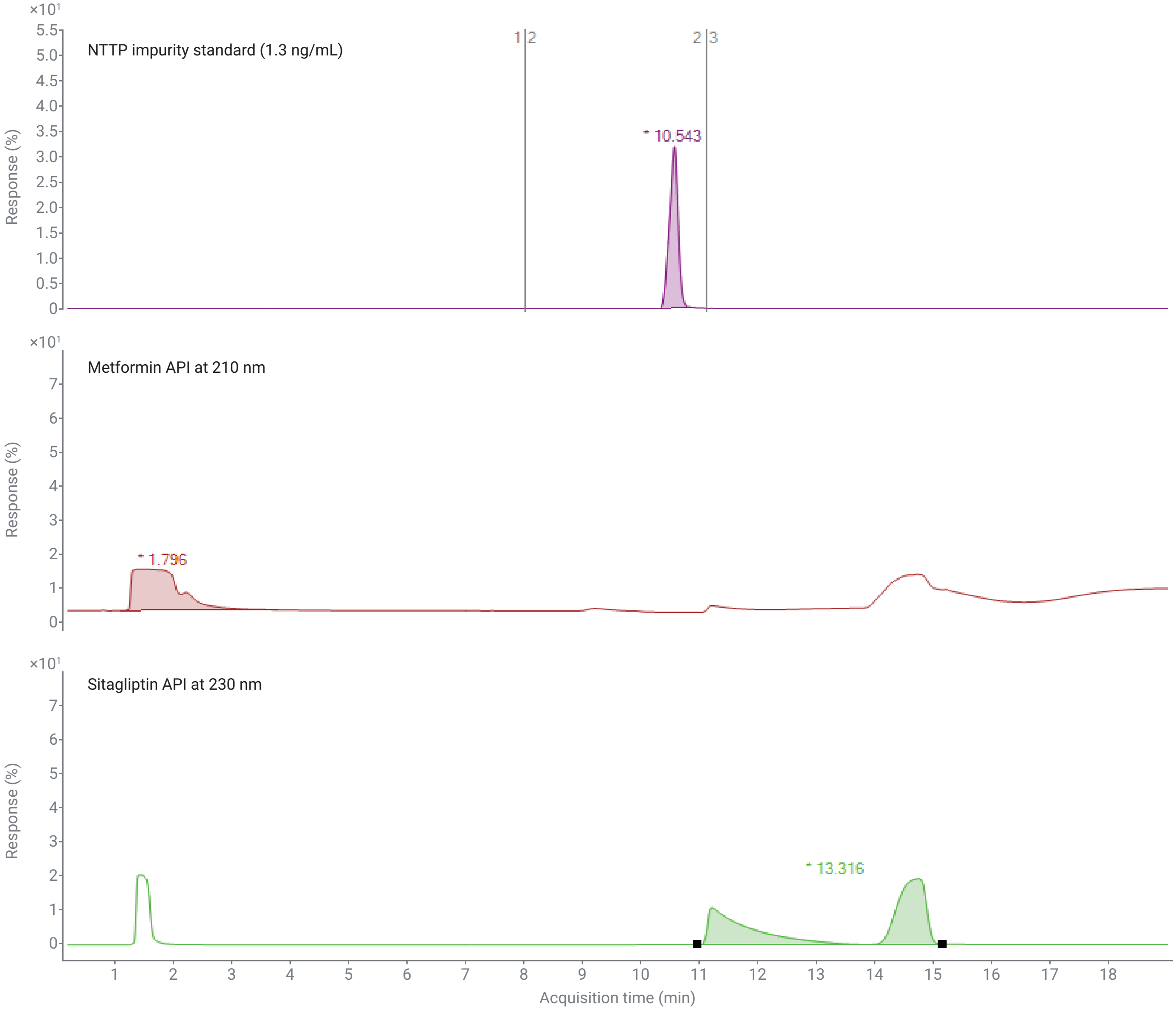


Figure 2. Representative MRM chromatogram of standard (STD) 1.3 ng/mL and DAD chromatogram for metformin and sitagliptin highlighting separation between NTTP impurity and APIs in extended-release tablet formulation.

Critical parameters like specificity, reproducibility, linearity, recovery, LOQ, and sensitivity were established. The sensitivity parameter included limit of detection (LOD), lower limit of quantitation (LLOQ) and limit of quantitation (LOQ) with S/N calculated using a root mean square (RMS) algorithm as described in Figures 3A, 3B, and 3C, respectively.[7]

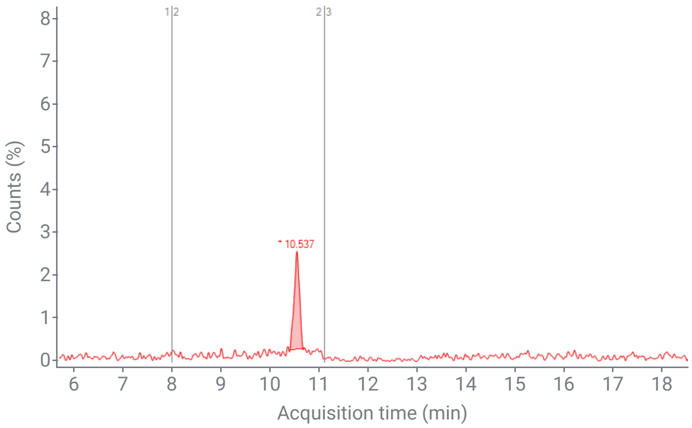


Figure 3A. Representative MRM chromatogram of NTTP impurity at LOD 0.008 ng/mL with S/N > 30:1 calculated using RMS.

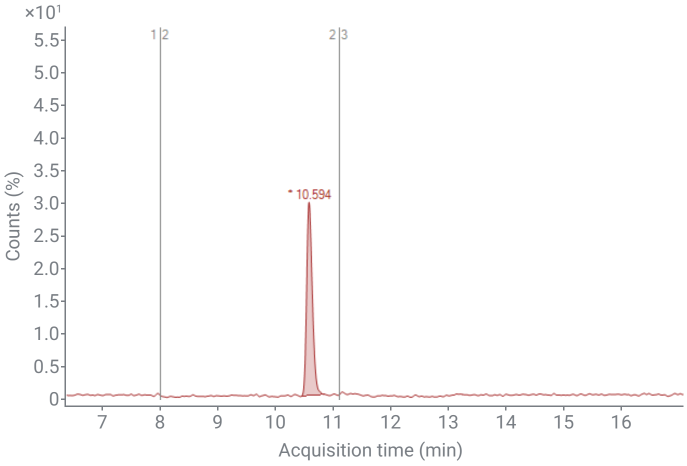


Figure 3B. Representative MRM chromatogram of NTTP impurity at LLOQ 0.04 ng/mL (LOQ established in aqueous standard) with S/N > 300:1 calculated using RMS.

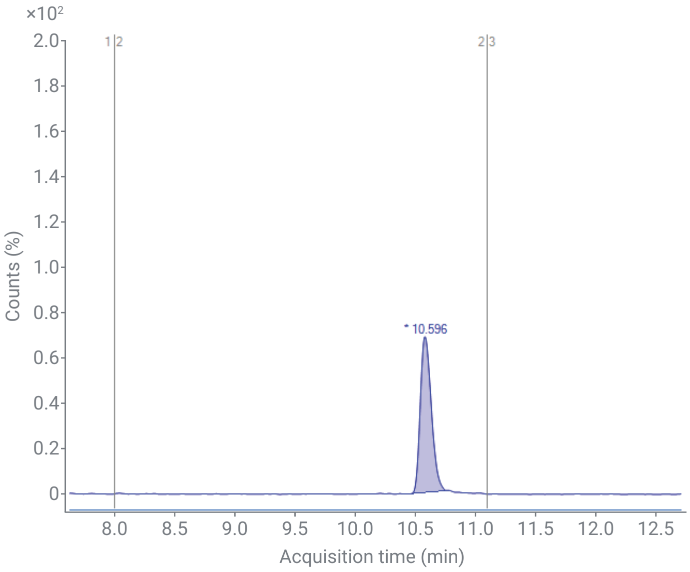


Figure 3C. Representative MRM chromatogram of NTTP impurity at LOQ 0.088 ng/mL (LOQ established in sample) with S/N > 600:1 calculated using RMS. The noise level was reduced with optimized conditions, which resulted in high S/Ns for the NTTP impurity at LLOQ and LOQ concentration levels.

Parameters for analytical method development such as range, including established LOD (0.008 ng/mL), LLOQ (0.04 ng/mL), and LOQ (0.088 ng/mL) are described in Table 6.

Linear regression, weightin g factor 1/x with specific details as mentioned in Figure 5. The regression coefficient (R^2) had a value of 0.999 for N-nitroso sitagliptin using equation y = mx + c, displaying linear responses throughout the concentration range of 0.04 to 1.3 ng/mL (0.013 to 0.43 ppm w.r.t drug load of 3 mg/mL).[8]

Table 6. Representing LOD, LLOQ, LOQ, and a range for NTTP impurity.

Sr. No.	Compound	LOD		LLOQ		LOQ		R²	Linearity Range	
		ng/mL	ppm	ng/mL	ppm	ng/mL	ppm		ng/mL	ppm
1	N-Nitroso sitagliptin (NTTP)	0.008	0.0026	0.04	0.0133	0.088	0.029	0.994	0.04 to 1.3	0.0133 to 0.433

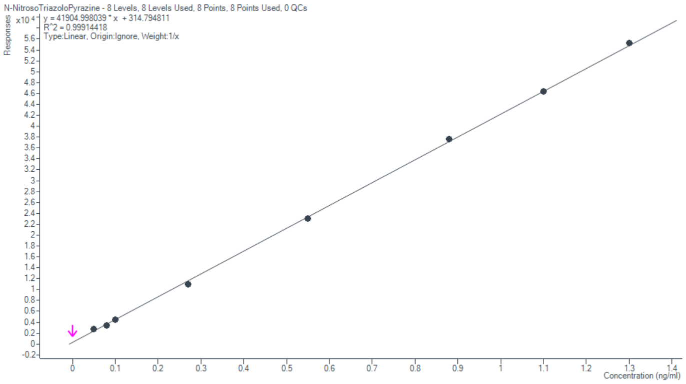
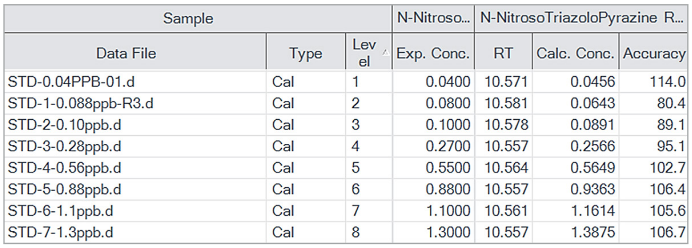


Figure 4. The calibration curve generated for N-nitroso sitagliptin (NTTP) from 0.04 to 1.3 ng/mL.

Accuracy of the standard used to generate the calibration curve was determined and found to be within 80 to 120% for N-nitroso sitagliptin impurity (NTTP) as shown in Figure 6.



Sample			N-Nitroso...	N-NitrosoTriazoloPyrazine R...		
Data File	Type	Level	Exp. Conc.	RT	Calc. Conc.	Accuracy
STD-0.04PPB-01.d	Cal	1	0.0400	10.571	0.0456	114.0
STD-1-0.088ppb-R3.d	Cal	2	0.0800	10.581	0.0643	80.4
STD-2-0.10ppb.d	Cal	3	0.1000	10.578	0.0891	89.1
STD-3-0.28ppb.d	Cal	4	0.2700	10.557	0.2566	95.1
STD-4-0.56ppb.d	Cal	5	0.5500	10.564	0.5649	102.7
STD-5-0.88ppb.d	Cal	6	0.8800	10.557	0.9363	106.4
STD-6-1.1ppb.d	Cal	7	1.1000	10.561	1.1614	105.6
STD-7-1.3ppb.d	Cal	8	1.3000	10.557	1.3875	106.7

Figure 5. Concentration of standards (0.04 to 1.3 ng/mL) used to plot the calibration curve.

Specificity was also established with sample preparation, optimized in terms of extraction solvent and time. The method achieved the recovery between 80 to 120% for N-nitroso sitagliptin impurity in sitagliptin and metformin extended-release formulation as described in Figure 6.

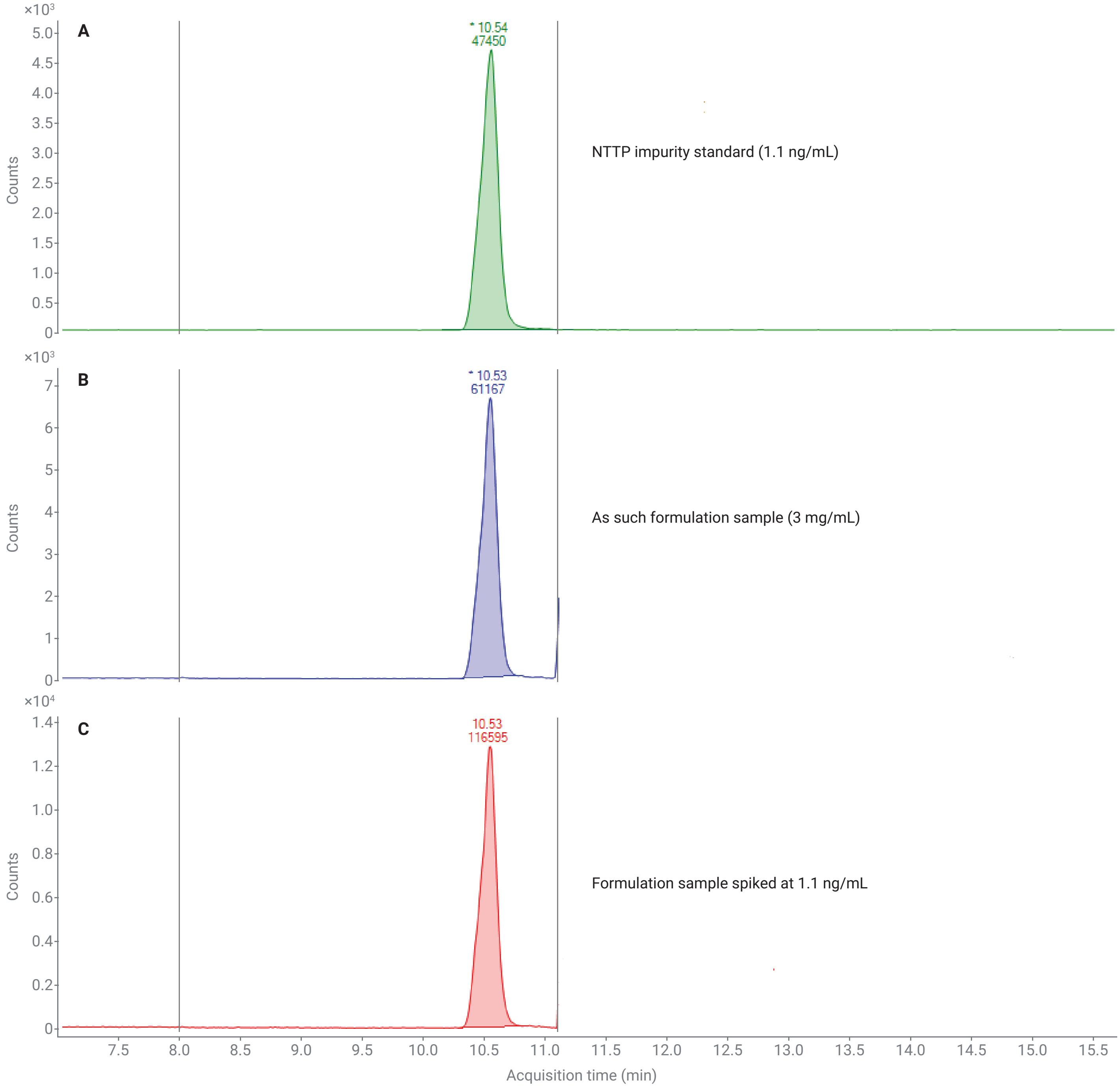


Figure 6. Representative chromatogram of N-nitroso sitagliptin (NTTP) impurity in aqueous standard (A), the control formulation sample (B), and spiked sample at a concentration of 1.1 ng/mL with sitagliptin drug load of 3 mg/mL (C).

The repeatability experiment was performed as illustrated in Table 7 at three different concentration levels with six replicates in aqueous standard, including LLOQ of 0.04 ng/mL, LOQ of 0.088 ng/mL, spec limit of 1.1 ng/mL (six replicates), and relative standard deviations (RSD) of 1.7, 2.7, and 1.24%, respectively.

Table 7. Representative diagram highlighting precision at LOD, LLOQ, and LOQ with RSDs of 1.7, 2.7, and 1.24%, respectively.

Injection No.	Area Count Observed for Standard Samples		
	(0.04 ng/mL; 0.013 ppm)	(0.088 ng/mL; 0.029 ppm)	(1.1 ng/mL; 0.37 ppm)
1	1,640	3,360	45,214
2	1,638	3,541	46,588
3	1,701	3,321	45,902
4	1,688	3,391	46,112
5	1,691	3,480	45,315
Average	1,671.6	3,418.6	45,929.5
SD	30.15	90.10	570.94
RSD (%)	1.7	2.7	1.24

Method accuracy was assessed for obtaining the recovery of 80 to 120%. RSD between parallel preparations was found to be no more than 5%.[8]

Recovery was calculated using the formula:

$$\text{Recovery (\%)} = \frac{\text{Response in spiked sample} - \text{Response in control sample}}{\text{Response in standard preparation}} \times 100$$

Method precision and specificity were evaluated by performing the recovery at the specification limit with three different preparations (n = 3) in a sample matrix, as described in Figure 7.

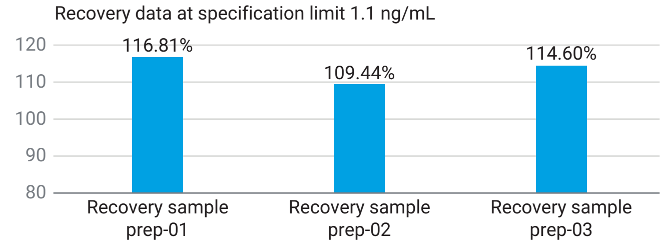


Figure 7. Recovery at specification level in triplicate performed in the drug product.

Conclusion

A highly sensitive and selective method was developed for the quantitation of N-nitroso sitagliptin in an extended-release tablet formulation. This method could establish all critical parameters of method performance using an Agilent 1290 Infinity II LC coupled to an Agilent 6475 triple quadrupole mass spectrometer. This application note demonstrates the N-nitroso sitagliptin quantification using the 6475 LC/TQ with high precision, accuracy, and reproducibility. This is demonstrated using the method specified in the application note for quantification of N-nitroso sitagliptin (NDSRIs). The method provides a linear response from 0.04 to 1.3 ng/mL, with accuracy and precision at the LOQ and specification limit, with an RSD within 5%. The method demonstrated can provide an LOD of 0.008 ng/mL (S/N > 30:1), an LLOQ of 0.04 ng/mL (S/N > 300:1), and an LOQ of 0.088 ng/mL (S/N > 600:1). The method is highly reproducible, including bracketing standards, which show the applicability of the method for easy transfer to routine batch analysis of N-nitroso sitagliptin. It also addresses the reproducibility where recovery in tablet samples were obtained within ±20% and sensitivity of the 6475 LC/TQ in the detection of NDSRI (N-nitroso sitagliptin) nitrosamine impurities at low concentration levels in drug products. The method provides chromatographically resolved NDSRI with the API (sitagliptin). This method provides excellent sensitivity as per the latest US FDA guidance on control of nitrosamines, such as NDSRI, in drug substances. The method developed was found to be accurate and selective for the quantitation of NTTP impurity.

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