Application Note

Pharmaceuticals

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Determination of Genotoxic Nitrosamine Impurity in Bumetanide API and Tablets Using the Agilent 6470 Triple Quadrupole LC/MS

Figure 1. Agilent 1290 Infinity II LC coupled to an Agilent 6470 triple quadrupole LC/MS.

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Abstract

This application note describes an LC/MS/MS based method for the quantitation of 'N-nitroso Bumetanide impurity' in Bumetanide API and low dose tablets. Quantitation of this impurity in low dose tablets of Bumetanide is extremely challenging because of the high amounts of excipient material in each tablet. High amounts of excipients can cause matrix effects (ionization suppression or enhancement) resulting in inaccurate quantitation. Adding more complexity to the analysis, chromatographic separation of the impurity from the API is required.

This application note also suggests a tMRM confirmatory technique – as it is important to avoid false-positive results. Triggered MRM (tMRM) based generation of product ion spectra can be used as an extra tool for the confirmation of N-nitroso Bumetanide impurity in both drug substance and drug formulation. MRM ion ratios of the sample can be compared to nitrosamine impurity standards.

Introduction

Bumetanide is drug administered orally to treat swelling caused by congestive heart failure, liver disease, or kidney disease, including a condition called nephrotic syndrome.[1] During the manufacturing process of tablets/drug formulation, a side product called N-nitroso Bumetanide may be formed as an impurity. Due to it's structural similarity to other nitrosamine genotoxic impurities, it is potentially genotoxic in nature.[2]

The determination of genotoxic impurities in drug substances and drug products is a critical regulatory requirement as they may increase the risk of cancer.[3] The allowable daily intake would provide a basis for estimating an appropriate quantitation limit required for an analytical method for these impurities.

In this application note, a highly selective Multiple Reaction Monitoring (MRM) based LC/MS/MS method was developed using an Agilent 6470 triple quadrupole LC/MS (LC/TQ). The sensitivity of the 6470 LC/TQ can easily detect compounds at the required limits of detection. The special design of the ion optics and stable electronics of the system provides consistent results across multiple batches.

Experimental

Chemicals and reagents

N-nitroso Bumetanide standard was purchased from Clearsynth lab, India. LC/MS-grade solvents such as methanol and water were purchased from Honeywell (Charlotte, NC, USA). Formic acid, MS grade was purchased from Fluka (now of Honeywell).

Instrument configuration

- 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 diode array detector (G7117A)
- Agilent 6470 triple quadrupole LC/MS (G6470B)

Table 1. Chromatography conditions.

Parameter	Value
Mobile Phase A	0.5% formic acid in water
Mobile Phase B	Methanol: 0.5% formic acid in water (95/5)
Flow Rate	0.5 mL/min
Injection Volume	20 µL
Column Temperature	30 °C
Sample Diluent	Methanol/Water (40/60)
Needle Wash	Methanol/Water (60/40)
UV Wavelength	254 nm
Gradient	Time (min) %A %B 0 95 5 2 95 5 2.1 53 47 16 22 78 17 22 78 18 10 90 20 10 90 20.1 95 5 22 95 5
Column	Agilent InfinityLab Poroshell HPH-C18 3 × 150 mm, 4 µm (p/n 693970-502T)

Table 2. MRM parameters.

Precursor Ion (*m/z*)	Product Ion (*m/z*)	Dwell Time (ms)	Fragmentor (V)	Collision Energy (V)	Cell Accelerator Voltage (V)	Resolution Q1/Q3
394.1	321.0	200	108	16	4	Unit/Unit
394.1	240.0	200	108	24	4	Unit/Unit

Table 3. MS source parameters.

Parameter	Value
Ionization Source	AJS ESI
Ionization Mode	ESI Positive
Gas Temperature	325 °C
Gas Flow	12 L/min
Nebulizer	42 psi
Sheath Gas	200 °C
Sheath Gas Flow	12 L/min
Capillary Voltage	5,900 V
Nozzle Voltage	1,900 V

Table 4. Diverter valve program.

Start Time (min)	Scan Type	Diverter Valve
0	MRM	To Waste
13	MRM	To MS
15	MRM	To Waste

Sample preparation

API preparation

25.0 mg of the Bumetanide API was accurately weighed and dissolved in 60 mL of methanol in a 100 mL volumetric flask. After 15 minutes of sonication, the flask was filled to volume with water then mixed well. A portion of the sample was then centrifuged at 6,000 rpm for 10 minutes, followed by filtration through a PVDF syringe filter into a clean 2 mL sample vial.

Placebo preparation

42.5 mg of placebo was accurately weighed into a 15 mL centrifuge tube, and 0.4 mL of methanol added. After 15 minutes sonication, 0.6 mL of water was added and the sample was vortexed. The sample was then centrifuged at 6,000 rpm for 10 minutes, followed by filtration through a PVDF syringe filter into a clean 2 mL sample vial.

Tablet preparation

42.5 mg (equivalent to 0.25 mg API) of crushed tablet powder was weighed accurately and transferred to a 15 mL centrifuge tube. 0.4 mL of methanol was added and the centrifuge tube was sonicated for 15 minutes with an extra vortex step every 5 minutes. After sonication, 0.6 mL of water was added to the contents and vortexed again for 1 minute. Contents were then centrifuged at 6,000 rpm for 10 minutes. After centrifugation, contents were filtered through a PVDF filter into a clean 2 mL sample vial. 20 µL was injected into the LC/MS/MS system.

Data acquisition and data analysis

All samples were acquired using the Agilent MassHunter Data Acquisition software version 10.1. MRM transitions were obtained and optimized using the Agilent MassHunter Acquisition optimizer software. This tool automatically optimized fragmentor voltages for the Q1 precursor ions and collision energies for the Q3 product ions.

A standard solution with a concentration of 500 ng/mL was introduced to the MS by Flow Injection Analysis with an injection volume of 5 µL. Through the automated workflow, 10 product ions from each impurity were selected for creation to MRM transitions.

Chromatograms were viewed through MassHunter Qualitative Analysis software version 10.0. Quantitation of each batch was carried out using MassHunter Quantitative Analysis software version 10.1.

Results and discussion

Chromatographic conditions were developed to achieve maximum separation between the impurity, API, and excipients present in tablet samples. Separation was critical to reduce the matrix effects and charge competition from excipients and the API.

Instrument MRM parameters and source parameters were optimized to maximize sensitivity while maintaining consistency in the method performance for large batches.

API was diverted to waste to avoid severe contamination of the MS using the integrated diverter valve. As per the diverter valve time program, only eluent with retention times between 13.00 and 15.00 minutes proceeded to the MS.

A linear concentration curve spanning three orders of magnitude was produced from 0.1 ng/mL to 100 ng/mL (R^2 value of 0.9957 with $1/X^2$ weighing). The lowest concentration of 0.1 ng/mL demonstrated a S/N>120:1 using the peak to peak algorithm for noise calculation, demonstrating the sensitivity of the 6470 LC/TQ and the possibility to analyze lower concentrations.

Validation parameters such as linearity, reproducibility, recovery, specificity, and sensitivity in terms of LOQ and LOD were characterized to ensure good method performance. Accuracies for calibration points were within ±20% of the expected concentration and all bracketing standards were within ±20%. No manual integration was needed. Ion ratios of the impurity in standards and positive formulations matched within ±10%, confirming the presence of this impurity in the formulation.

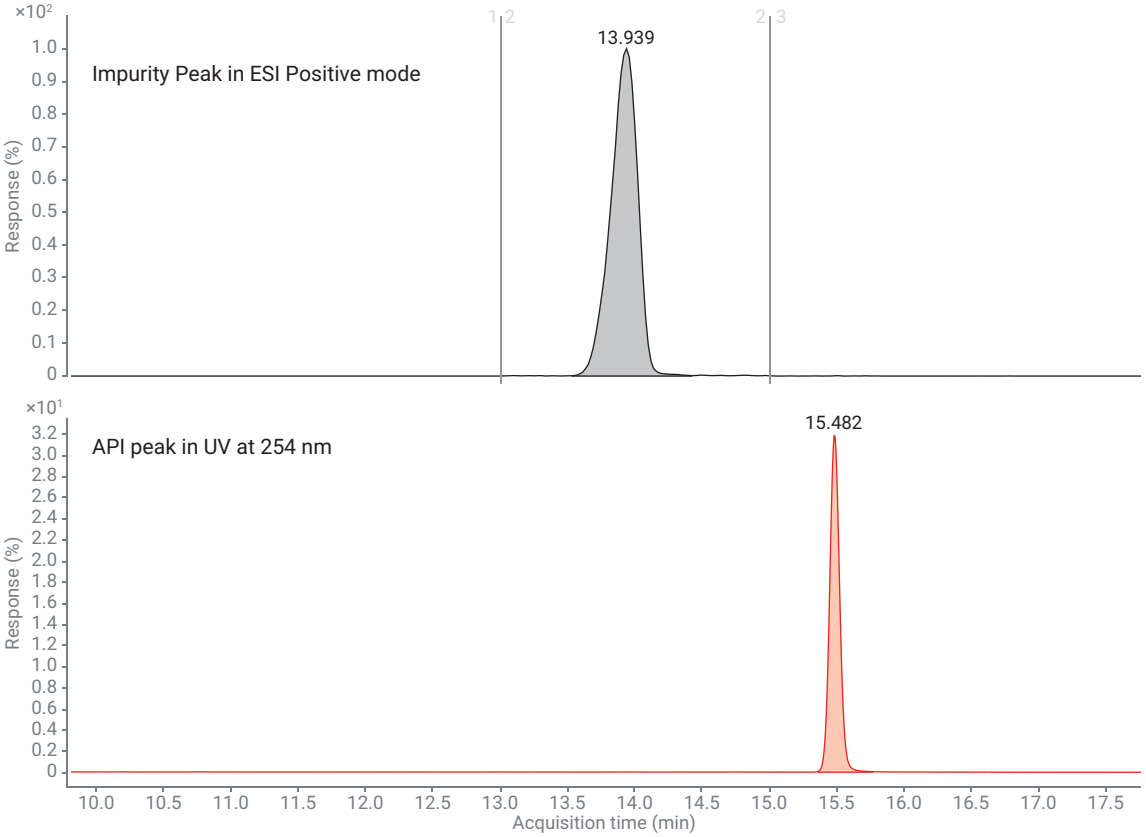

Figure 2. Chromatographic separation between the API and the impurity. The API is diverted from the MS and detected by the DAD.

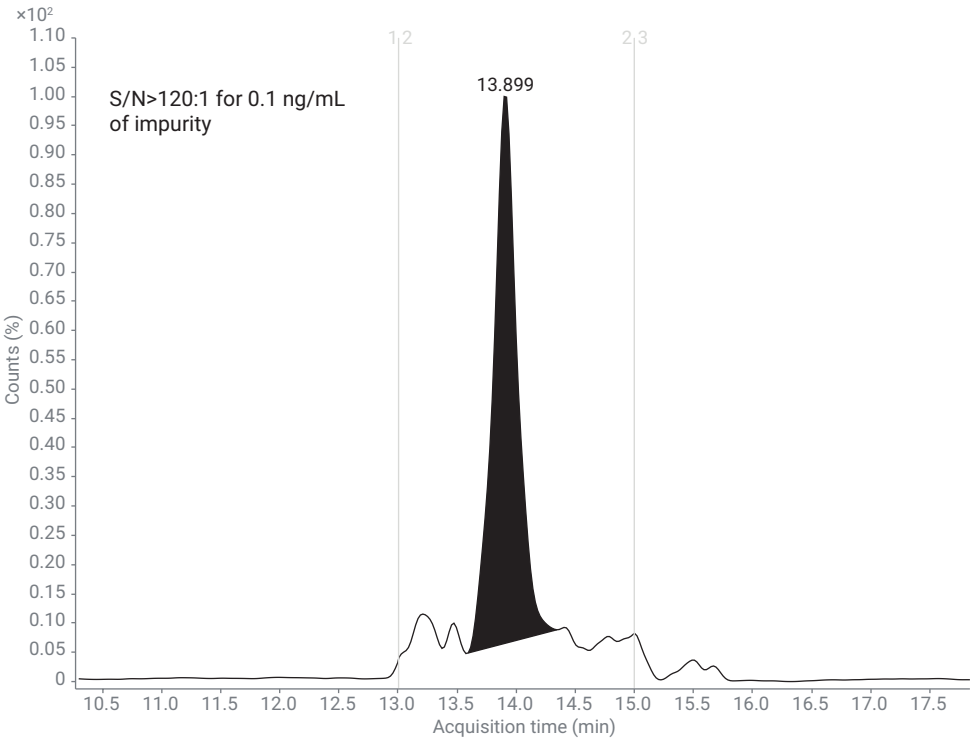

Figure 3. MRM chromatogram of a 0.1 ng/mL injection of impurity.

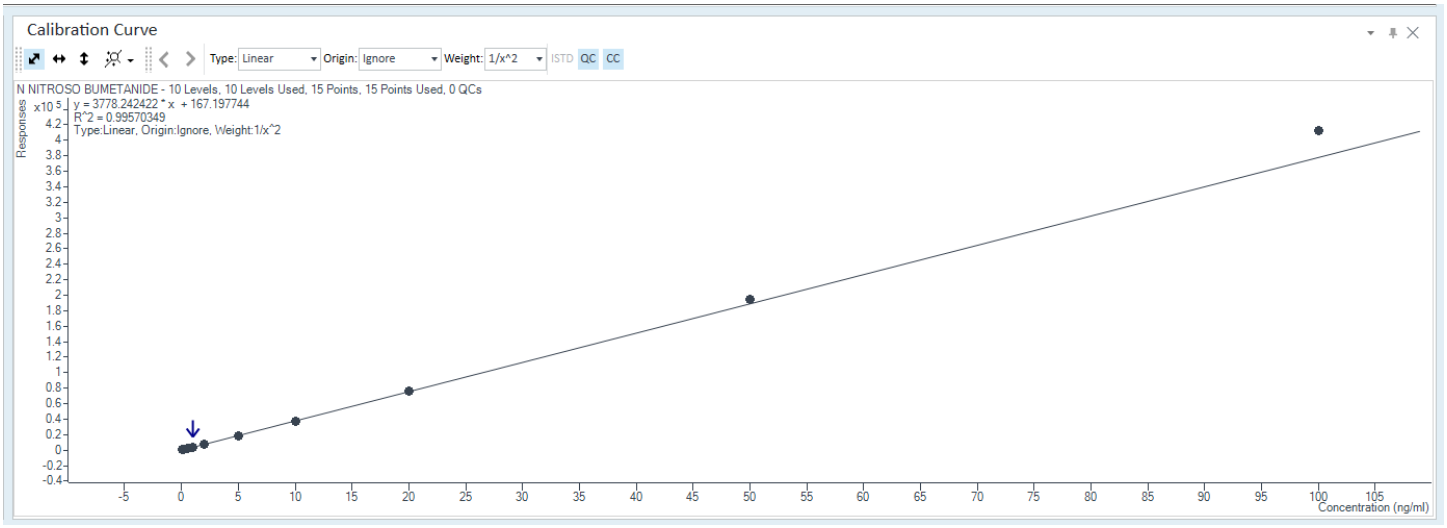

Figure 4. Calibration curve spanning three orders of magnitude from 0.1 ng/mL to 100 ng/mL. (R^2: 0.9957 with linear regression with $1/X^2$ weighing).

Batch Table

Sample: BLANK | Sample Type: <All> | Compound: N NITROSO BUMETANIDE | ISTD:

	Sample			N NITRO...	N NITROSO BUMETANIDE Results						Qualifier (394.1 -> 240.0)...	
	Data File	Type	Level	Exp. Conc.	RT	Resp.	MI	Calc. Conc.	Final Conc.	Accuracy	Ratio	MI
▶	BLANK1-r002-r002.d	Blank			13.818	117	☐	0.0000	0.0000			☐
	0.1 NG_ML.d	Cal	1	0.1000	13.919	528	☐	0.0955	0.0955	95.5	67.6	☐
	0.2 NG_ML.d	Cal	2	0.2000	13.919	1008	☐	0.2224	0.2224	111.2	54.2	☐
	0.5 NG_ML.d	Cal	3	0.5000	13.939	2102	☐	0.5114	0.5114	102.3	71.7	☐
	1 NG_ML-r001.d	Cal	4	1.0000	13.919	3790	☐	0.9576	0.9576	95.8	69.6	☐
	1 NG_ML-r002.d	Cal	4	1.0000	13.939	4087	☐	1.0360	1.0360	103.6	62.7	☐
	1 NG_ML-r003.d	Cal	4	1.0000	13.939	3900	☐	0.9867	0.9867	98.7	61.2	☐
	1 NG_ML-r004.d	Cal	4	1.0000	13.919	3951	☐	1.0001	1.0001	100.0	59.0	☐
	1 NG_ML-r005.d	Cal	4	1.0000	13.919	3469	☐	0.8726	0.8726	87.3	73.0	☐
	1 NG_ML-r006.d	Cal	4	1.0000	13.939	3934	☐	0.9956	0.9956	99.6	61.5	☐
	2 NG_ML.d	Cal	5	2.0000	13.919	7451	☐	1.9251	1.9251	96.3	67.4	☐
	5 NG_ML.d	Cal	6	5.0000	13.919	18874	☐	4.9441	4.9441	98.9	64.9	☐
	10 NG_ML.d	Cal	7	10.0000	13.919	36715	☐	9.6595	9.6595	96.6	65.7	☐
	20 NG_ML.d	Cal	8	20.0000	13.939	76314	☐	20.1250	20.1250	100.6	63.8	☐
	50 NG_ML.d	Cal	9	50.0000	13.939	194684	☐	51.4095	51.4095	102.8	65.1	☐
	100 NG_ML.d	Cal	10	100.0000	13.939	412954	☐	109.0964	109.0964	109.1	64.1	☐
●	BLANK2.d	Sample			13.980	198	☐		0.0083			☐
	BLANK3.d	Sample			13.959	127	☐		0.0000			☐
	PLACEBO.d	Sample			13.959	233	☐		0.0175			☐
	API.d	Sample			13.838	113	☐		0.0000		63.9	☐
	TABLET 0.5 MG.d	Sample			13.939	90770	☐	23.9458	23.9458		64.7	☐
	TABLET 1 MG.d	Sample			13.939	78562	☐	20.7191	20.7191		66.0	☐
	TABLET 2 MG.d	Sample			13.939	82250	☐	21.6940	21.6940		65.9	☐
	BRACKETING STD_1 NG_ML.d	Cal	4	1.0000	13.939	4089	☐	1.0366	1.0366	103.7	68.4	☐
	RECOVERY STD_API.d	Sample			13.959	945	☐	0.2056	0.2056		58.1	☐
	BLANK4.d	Sample			13.919	248	☐		0.0216		63.3	☐
	API SPIKE.d	Sample			13.939	849	☐	0.1803	0.1803		70.8	☐
	BRACKETING STD_1 NG_ML_2.d	Cal	4	1.0000	13.939	3883	☐	0.9823	0.9823	98.2	65.6	☐
	BLANK6.d	Sample			13.919	132	☐		0.0000		69.0	☐
	RECOVERY STD LEVEL 1.d	Sample			13.939	35051	☐	9.2195	9.2195		64.9	☐
	RECOVERY STD LEVEL 2.d	Sample			13.939	73502	☐	19.3819	19.3819		64.5	☐
	TABLET 2 MG SPIKE LEVEL 1.d	Sample			13.939	126960	☐	33.5106	33.5106		65.0	☐
	TABLET 2 MG as such.d	Sample			13.939	85421	☐	22.5320	22.5320		64.2	☐
	TABLET 2 MG SPIKE LEVEL 2.d	Sample			13.939	172459	☐	45.5354	45.5354		64.8	☐

Figure 5. A snapshot of the precision and accuracy results presented in MassHunter Quantitative Analysis software.

Triggered MRM (tMRM) confirmation

tMRM acquisition leverages the sensitivity of MRM while providing a targeted product ion spectrum, which can be used for library identification and enhanced confirmation. As a result, tMRM increases throughput by allowing for fast, sensitive, quantitative, and qualitative analysis in a single analytical run.

In tMRM analysis, up to 10 MRM transitions (primary and secondary) can be defined for each target analyte in the method. Primary transitions are always acquired for the analytes and can be used for quantitation. If the signal of the primary transitions exceeds a user-defined threshold, the secondary transitions are triggered and acquired for a specified number of scans.

tMRM spectra of N-nitroso Bumetanide impurity were acquired for all positive formulations. These spectra provided a 99.9% match with the tMRM spectra acquired from impurity standards.

The results shown in Figure 7 show consistent performance throughout the sample batch. Reproducibility was checked by injecting 1 ng/mL of standard six times and by two extra injections of the same concentration as bracketing standards. The Relative Standard Deviation (%CV) of the eight injections was calculated as 5%. A metrics plot of area response and retention time of all eight injections is shown in Figure 7B, demonstrating the consistency in the result.

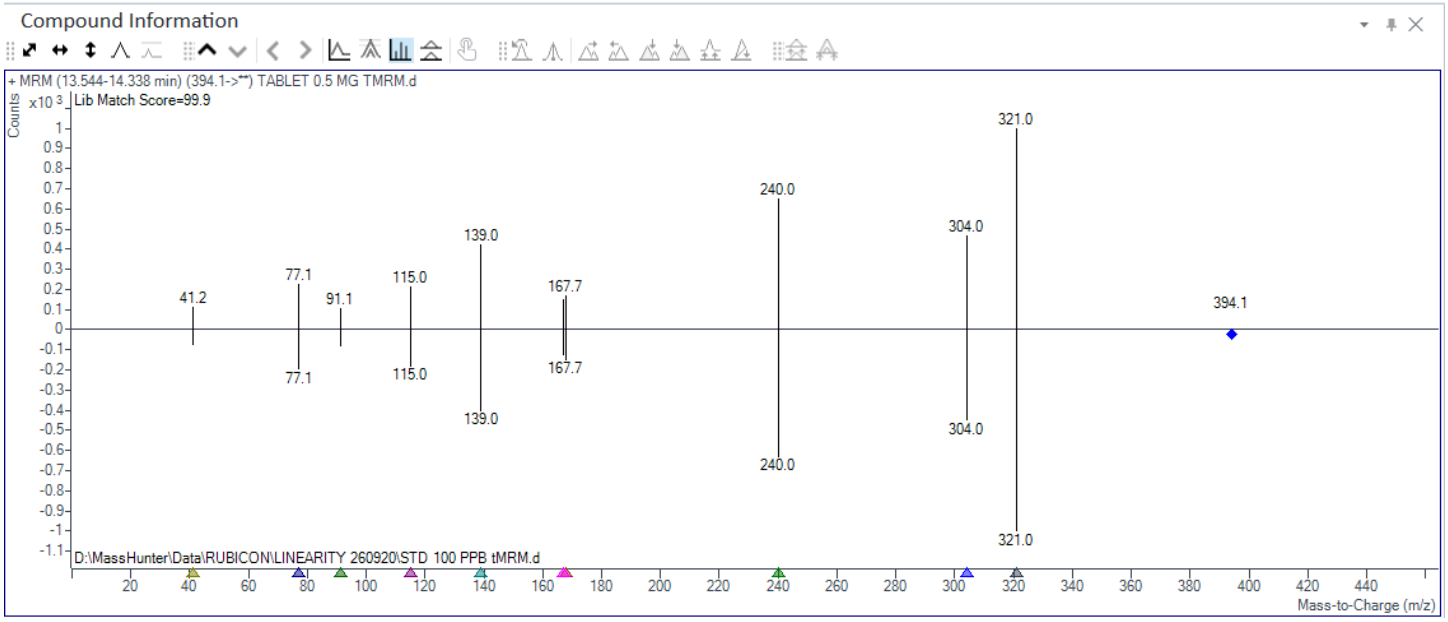

Figure 6. tMRM spectra comparison of N-nitroso Bumetanide impurity from a 0.5 mg tablet (top) and standard library spectra (bottom).

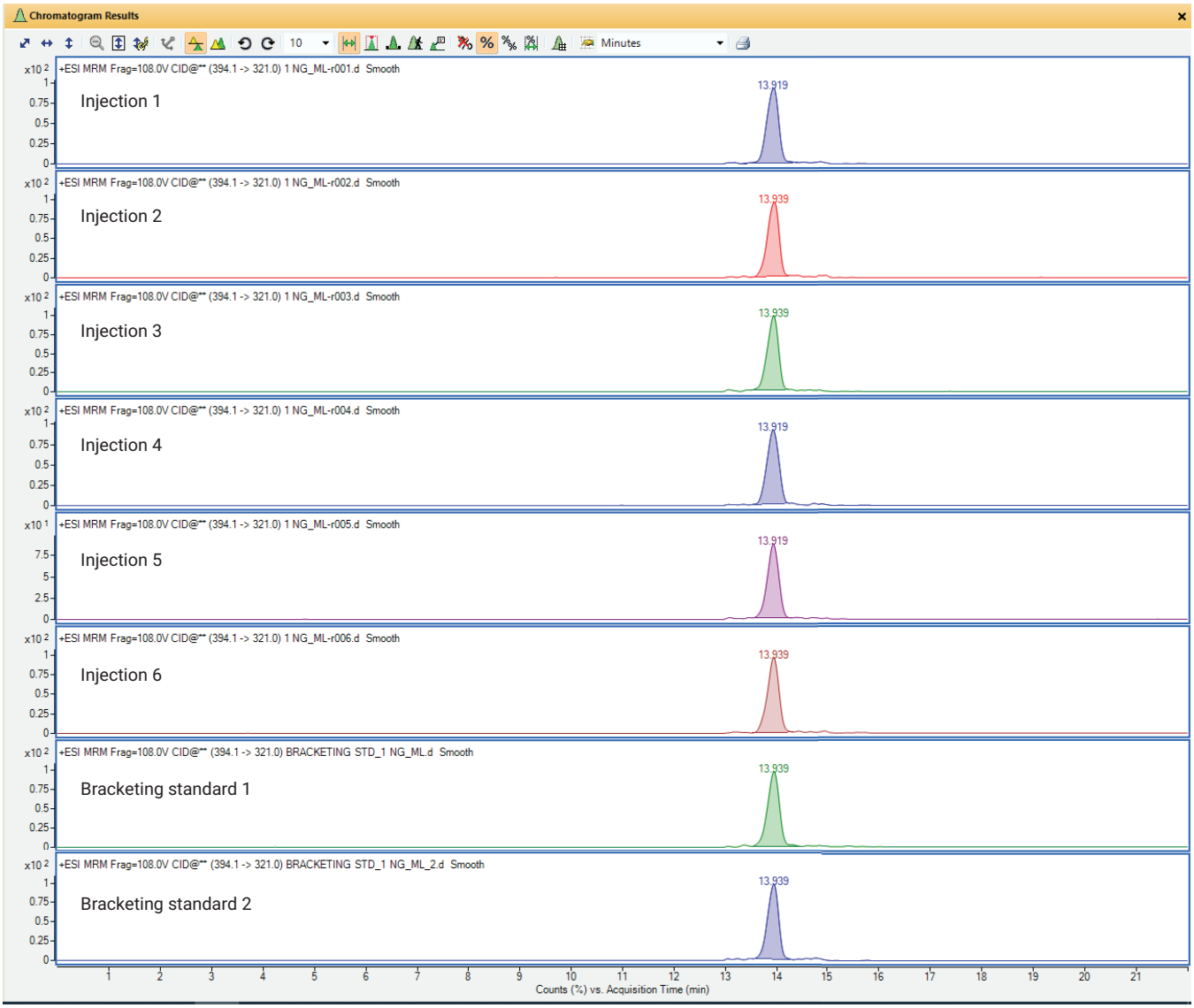

Figure 7A. Area response and retention time reproducibility for eight consecutive injections (including two bracketing standards) of 1 ng/mL of N-nitroso Bumetanide impurity.

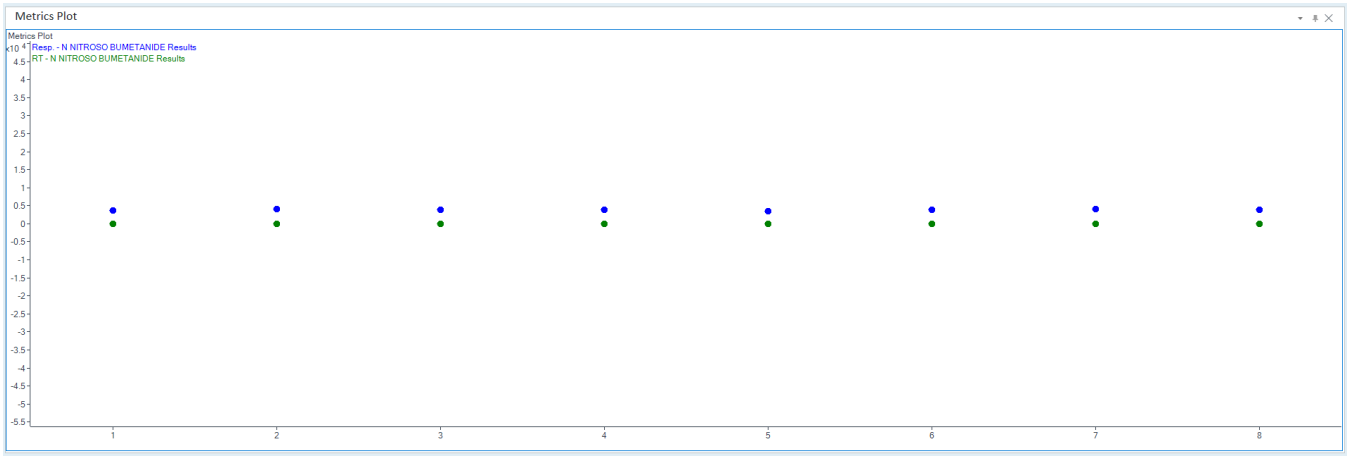

Figure 7B. Metrics plot of area response and retention time of eight injections of 1 ng/mL concentration of N-nitroso Bumetanide impurity.

Concentration of N-Nitroso Bumetanide with respect to the concentration

Calculation for 0.5 mg Tablet	
Response of Impurity in 0.5 mg Tablet	90,770 counts
Average Response of 1 ng/mL Impurity Standard of Six Injections	3,855 counts
Weight of the Tablet	0.25 mg/mL
Impurity (ppm) = (Area of sample/ [average area of standard, n = 6] × concentration of impurity in standard (ng/mL)/10^6 × (1/0.25 mg/mL) × 10^6 Reference FY19-177-DPA-S, USFDA	
Impurity (ppm) = (90,770/3,855) × (1/10^6) × (1/0.25) × 10^6 = 94.18 ppm	

Calculation for 1.0 mg Tablet	
Response of Impurity in 0.5 mg Tablet	78,562 counts
Average Response of 1 ng/mL Impurity Standard of Six Injections	3,855 counts
Weight of the Tablet	0.25 mg /mL
Impurity (ppm) = (Area of sample/ [average area of standard, n = 6] × concentration of impurity in standard (ng/mL)/10^6 × (1/0.25 mg/mL) × 10^6 Reference FY19-177-DPA-S, USFDA	
Impurity (ppm) = (78,562/3,855) × (1/10^6) × (1/0.25) × 10^6 = 81.52 ppm	

Calculation for 2.0 mg Tablet	
Response of Impurity in 0.5 mg Tablet	82,250 counts
Average Response of 1 ng/mL Impurity Standard of Six Injections	3,855 counts
Weight of the Tablet	0.25 mg/mL
Impurity (ppm) = (Area of sample/ [average area of standard, n = 6] × concentration of impurity in standard (ng/mL)/10^6 × (1/0.25 mg/mL) × 10^6 Reference FY19-177-DPA-S, USFDA	
Impurity (ppm) = (82,250/3,855) × (1/10^6) × (1/0.25) × 10^6 = 85.34 ppm	

Recovery study

Spiked recovery study in API at 0.2 ppb level

25 mg of Bumetanide standard was accurately weighed and transferred to a 100 mL volumetric flask. 60 mL of methanol was added and the sample was sonicated for 15 minutes, cooled to room temperature then diluted to volume with water. This solution was spiked with 100 µL of 100 ppm stock (100 ng/mL) N-nitroso bumetanide impurity standard. From the previous solution 200.0 µL was accurately transferred to a 10 mL volumetric flask then diluted with water/methanol (60/40) to volume. The resultant impurity concentration in the solution was 2 ng/mL. From the standard stock solution, 1.0 mL was pipetted into a 10 mL volumetric flask then diluted to the mark with water/methanol (60/40). The resultant impurity concentration of the final solution was 0.2 ng/mL.

In a similar fashion, a recovery standard was prepared without API.

Parameter	Value
Recovery Standard Area of Impurity Obtained From the Quantitation Table	945 counts
Area of the Impurity in the API Spike Solution	849 counts
% Recovery	(849/945) × 100 = 89.8%

Spike recovery study in commercial tablets (2 mg)

Spike at 10 ng/mL level

The average weight of 20 tablets was first determined. Those 20 tablets were then crushed into a fine powder using a mortar and pestle. 42.5 mg of sample powder was weighed (calculated to be equivalent to 0.25 mg of bumetanide) then transferred into a dry 15 mL centrifuge tube. The powder was spiked with 10 µL of 1 ppm impurity standard and mixed with 0.4 mL of methanol. The solution was sonicated for 15 minutes with intermittent shaking after every 5 minutes. After cooling to room temperature, the solution was diluted up to 1.0 mL with 0.6 mL water and shaken for 5 minutes. The solution was filtered through a 0.45 µm PVDF membrane filter. (The resultant solution contained 10 ng/mL of N-nitroso Bumetanide impurity)

Similarly, a recovery standard was prepared without tablet powder.

Parameter	Value
Recovery Standard Area of Impurity Peak Obtained from Quantitation Table	35,051 counts
Area of Impurity in Tablet	85,241
Area of the Impurity in the Tablet (2 mg) Spike Solution	126,960 counts
% Recovery	[(126,960–85,421)/ 35,051] × 100 = 118.5%

Spike at 20 ng/mL level

The average weight of 20 tablets was first determined. Those 20 tablets were then crushed into a fine powder using a mortar and pestle. 42.5 mg of sample powder was weighed (calculated to be equivalent to 0.25 mg of bumetanide) then transferred into a dry 15 mL centrifuge tube. The powder was spiked with 10 µL of 2 ppm impurity standard. 0.4 mL of methanol was added and sonicated for 15 minutes with intermittent shaking after every 5 minutes. After cooling to room temperature, the solution was diluted to 1.0 mL with 0.6 mL water and shaken for 5 minutes. The solution was filtered through a 0.45 µm PVDF membrane filter. (The resultant solution contained 10 ng/mL of N-nitroso Bumetanide impurity)

Similarly, a recovery standard was prepared without tablet powder.

Parameter	Value
Recovery Standard Area of Impurity Peak Obtained from Quantitation Table	73,502 counts
Area of Impurity in Tablet	85,241
Area of the Impurity in the Tablet (2 mg) Spike Solution	172,459 counts
% Recovery	[(172,459–85,421)/ 73,502] × 100 = 118.4%

Conclusion

A highly sensitive and robust MRM method was developed to quantify N-nitroso Bumetanide impurity in Bumetanide API (drug substance) and Bumetanide tablets (drug product).

The chromatographic method provided excellent separation between the impurity and the API to avoid interference. To avoid contamination of the MS, an integrated diverter valve program was included to divert high concentration API as it elutes.

Analysis of the results demonstrates that the placebo and API do not contain the N-nitroso Bumetanide impurity, but it was found to be present in the tablets. This result was further confirmed by MRM ion ratios and tMRM spectral comparison with standards.

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