U.S. FOOD & DRUG ADMINISTRATION

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Liquid Chromatography-High Resolution Mass Spectrometry (LC-ESI-HRMS) Method for the Determination of N-Nitroso-Bumetanide in Bumetanide Drug Products and Drug Substance

Background: Bumetanide is a diuretic drug. The potential for the presence or formation of *N*-nitroso-bumetanide has been identified in several bumetanide drug products. To help ensure the safety and quality of bumetanide drug products and drug substance, the agency has developed and validated a method to determine the presence or absence of bumetanide Nitrosamine Drug Substance-Related Impurity (NDSRI). The structure for *N*-nitroso-bumetanide is shown in Figure 1 below.

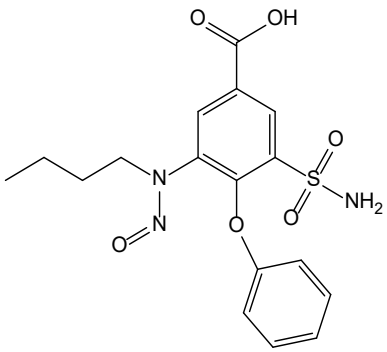

Figure 1: *N*-Nitroso-Bumetanide

Conclusions:

A reverse phase LC method with HRMS detection was developed and validated for the determination of *N*-nitroso-bumetanide in bumetanide drug products and drug substance. The method was validated according to ICH Q2 (R1). Method verification and/or re-validation is recommended prior to use to demonstrate that the method is suitable for its intended purpose. The limit of detection (LOD), limit of quantitation (LOQ) and range of the method are summarized below:

	***N*-Nitroso-Bumetanide**
Limit of Detection (LOD)	0.5 ppm
Limit of Quantitation (LOQ)	1.0 ppm
Range	1.0 – 1000 ppm

LC-ESI-HRMS Method for the Determination of *N*-Nitroso-Bumetanide in Bumetanide Drug Products and Drug Substance

Purpose

The LC-ESI-HRMS method was developed and validated to quantitate *N*-nitroso-bumetanide in bumetanide drug products and drug substance.

Principle

N-nitroso-bumetanide impurity is separated from bumetanide in bumetanide drug products by reverse phase chromatography and is detected by a high-resolution and high-mass accuracy (HRAM) mass spectrometer. High sensitivity and selectivity are achieved by using parallel reaction monitoring (PRM) method. The target analyte is fragmented, and the accurate *m/z* value of the product ions are monitored. Quantitation is performed by comparing the peak area of the *N*-nitroso-bumetanide in extracted ion chromatogram (with *m/z* tolerance of ± 15 ppm) of the samples, to the peak area of the *N*-nitroso-bumetanide reference standard in an external standard calibration.

Reagents

- *N*-Nitroso-Bumetanide Reference Standard
- Water, LC/MS grade or equivalent
- Methanol, LC/MS grade
- Formic Acid, LC/MS grade

Equipment/Instrument

- HPLC or UHPLC system equipped with temperature-controlled autosampler and column compartment
- Q Exactive™ mass spectrometer (Thermo-Fisher Scientific) or equivalent
- HPLC column: InfinityLab Poroshell 120 EC-C18, 2.7 µm 120 Å, 50 x 3.0 mm (Agilent, Part No. 699975-302(T) or equivalent)
- Analytical Balance
- Vortex Mixer
- 15 mL glass centrifuge tubes
- Sonicator
- 0.22 µm PVDF syringe filters
- Centrifuge
- HPLC vials

Mobile Phase A: Water, 0.1% Formic Acid

Mobile Phase B: Methanol, 0.1% Formic Acid

Diluent and Blank: Methanol

Stock Standard Preparation
Accurately weigh 10 ± 3 mg of *N*-nitroso-bumetanide standard and transfer into a 100 mL volumetric flask. Dilute to volume with methanol and mix using a stir bar and plate until dissolved.

Intermediate Stock Standard
Transfer the appropriate aliquot volume of the stock standard into a volumetric flask to get a target concentration of 1000 ng/mL. Dilute to volume with methanol.

Working Standard (50 ng/mL)
Transfer 5.0 mL aliquot volume of the intermediate stock standard into a 100 mL volumetric flask and dilute to volume with methanol. Prepare fresh daily.

Drug substance sample preparation
Accurately weigh 20 mg of drug substance and quantitatively transfer into a 20 mL volumetric flask. Dilute to volume with methanol and mix the solution using a stir bar and plate until fully dissolved. Filter the solution using a 0.22 µm PVDF syringe filter 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 1.0 mg/mL of API in methanol, and transfer into a 15 mL glass 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 mechanical wrist action shaker.

After extraction, centrifuge the sample for 15 minutes at 3000 rpm. Filter the supernate using a 0.22 µm PVDF syringe filter into an hplc vial for LC/MS analysis.

Chromatographic Conditions

HPLC Column	InfinityLab Poroshell 120 EC-C18, 2.7 µm 120 Å, 50 x 3.0 mm (Agilent, Part No. 699975-302(T) or equivalent)		
Column Temp.	30 °C		
Flow Rate	0.5 mL/min		
Mobile Phase A	Water, 0.1% Formic Acid		
Mobile Phase B	Methanol, 0.1% Formic Acid		
Gradient	**Time (min)**	**A%**	**B%**
	0	95	5
	0.5	95	5
	0.7	50	50
	5.0	30	70
	6.5	10	90
	8.0	10	90
	8.1	95	5
	11.0	95	5
Injection Volume	5 µL		
Autosampler Temp.	4 - 8 °C		

Needle Wash	Methanol

Mass spectrometer conditions

- Instrument
  Q Exactive™ mass spectrometer (Thermo-Fisher)
- ESI Ion Source Settings

Sheath Gas Flow Rate	50 arbitrary units
Aux Gas Flow Rate	15 arbitrary units
Sweep Gas Flow Rate	0 units
Spray Voltage	3.5 kV
Capillary Temp.	300 °C
Aux Gas Heater Temp.	350 °C

- Scan Settings

Parameters	***N*-Nitroso-Bumetanide**
Scan Type	PRM
Polarity	Positive
Scan Start – End (min)	4.6 – 5.7
m/z Isolated for PRM	394.1067
NCE	20
Isolation Window	1.0 *m/z*
Microscans	1
Resolution	140,000
AGC target	1e6
Maximum IT	200 ms

Inclusion List

Mass (*m/z*)	**Polarity**	**Start (min)**	**End (min)**	**Comment**
394.1067	Positive	4.6	5.7	*N*-nitroso-bumetanide

Injection Sequence

- Inject Blank at least once at the beginning of a sequence
- Inject the Working Standard for six consecutive times
- Inject the Working Standard once every six injections of the samples and at the end of a sequence.

Example:

Order	**Solution**	**No. of Injections**
1	Blank	2
2	Working Standard	6
3	Blank	1
4	Sample 1	1

5	Sample 2	1
6	Sample 3	1
7	Sample 4	1
8	Sample 5	1
9	Sample 6	1
10	Working Standard	1
…	…	…

System Suitability

- The % RSD (n = 6) of the *N*-nitroso-bumetanide peak area for the first six injections of the Working Standard solution should not be more than 10%.
- The cumulative % RSD of the *N*-nitroso-bumetanide peak area in the Working Standards 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.

Data Processing

- The *N*-nitroso-bumetanide peak areas from the extracted ion chromatograms (EIC) with a *m/z* tolerance of ±15 ppm is used for quantitation. The *N*-nitroso-bumetanide *m/z* values to be extracted are listed below:

Compound	m/z to be extracted
N-nitroso-bumetanide	321.0534, 364.1081

- The retention time difference of the *N*-nitroso-bumetanide peak in the analyzed samples should not be more than 2% of the retention time of the corresponding nitroso-bumetanide peak in the reference standard solution.

Calculation

Drug Substance:

N-nitroso-bumetanide (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: A_{spl} = Area of the *N*-nitroso-bumetanide peak in the sample solution
As = Average area (n = 6) of the *N*-nitroso-bumetanide peak from the first six consecutive injections of the working standard
C_s = Concentration of the *N*-nitroso-bumetanide in the working standard (ng/mL)
W = Weight of drug substance (mg)
V = Volume of the diluent in the sample solution (mL)

Drug Product:

N-nitroso-bumetanide (ppm) = $\frac{A_{spl}}{As} \times C_s \times \frac{1\ mg}{1 \times 10^6\ ng} \times \frac{1}{1\ mg/mL} \times 10^6$

where: A_{spl} = Area of the *N*-nitroso-bumetanide peak in the sample solution
As = Average area (n = 6) of the *N*-nitroso bumetanide peak from the first six consecutive injections of the working standard
C_s = Concentration of the *N*-nitroso-bumetanide in the standard solution (ng/mL)