IDENTIFICATION OF SIX NITROSAMINE IMPURITIES IN WESTERN MEDICINES BY LC-HRMS

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Disclaimer: The testing method below provide option and guidance for the users to detect nitrosamine impurities in western medicines. The method should be validated by users to ensure it is fit for its intended use.

1 Scope

This document outlines the test method for the identification of six nitrosamine impurities namely N-nitroso-di-methylamine (NDMA), N-nitroso-di-ethylamine (NDEA), N-nitroso-N-methylamino butyric acid (NMBA), N-ethyl-N-nitroso-2-propanamine (NEIPA), N-nitroso-diisopropylamine (NDIPA) and N-nitroso-di-n-butylamine (NDBA) in western medicines by LC-Q-Exactive Hybrid Orbitrap Mass Spectrometer (LC- HRMS).

2 Identification of Six Nitrosamine by Liquid Chromatography-Q-Exactive Hybrid Orbitrap MassSpectrometer (LC-HRMS)

2.1 Reagents and Chemicals

N-nitroso-di-methylamine (NDMA)
N-nitroso-di-ethylamine (NDEA)
N-nitroso-N-methylamino butyric acid (NMBA)
N-ethyl-N-nitroso-2-propanamine (NEIPA)
N-nitroso-diisopropylamine (NDIPA)
N-nitroso-di-n-butylamine (NDBA)
Methanol(MeOH), HPLC grade (sample preparation); LC/MS grade (mobile phase)
Formic acid, MS grade
Deionized water (DI water)
Diluent: MeOH / DI water (80/20)

2.2 Instruments and Apparatus

Q Exactive Plus Hybrid Quadrupole-Orbitrap MS coupled with UltiMate 3000
Centrifuge
Ultrasonic bath
Volumetric flask (Class A, 10 mL)
Membrane filter (PTFE 0.2 µm)
Micropipette
2 mL vials
1.5 mL Eppendorf tube
Conical centrifuge tube, Polypropylene (PP)

2.3 LC-HRMS Method

HPLC

Column:	Phenomenex® Gemini C18 analytical column (4.6 mm x 100 mm, 3 µm) or equivalent
Column oven temperature:	40 °C
Injection volume:	5 µL
Mobile phase A:	0.1% formic acid in DI water
Mobile phase B:	0.1% formic acid in Methanol

Flow rate:	0.35 mL/min		
Gradient:	Time (min)	Mobile phase A (%)	Mobile phase B (%)
	0	80	20
	1	80	20
	12	5	95
	16	5	95
	16.1	80	20
	20	80	20

[Note: The flow rate or run time may be varied to obtain optimum separation.]

HRMS

Ionization mode:	ESI
ESI parameter:	Spray voltage: 3.5 kV (positive) and 3.0 kV (negative); Capillary temperature: 350 ºC; Sheath gas: 35; Aux Gas: 5; Auxiliary Gas Heater Temp: 300 ºC
Target SIM 1	3.5-6.0 min; Resolution: 35,000; AGC target: 2e5; Max IT: 500 ms; Isolation window: 1.0 m/z; Polarity: Positive
Target SIM 2	5.0-7.0 min; Resolution: 35,000; AGC target: 2e5; Max IT: 500 ms; Isolation window: 1.0 m/z; Polarity: Negative
Target SIM 3	7.0-16 min; Resolution: 35,000; AGC target: 2e5; Max IT: 500 ms; Isolation window: 1.0 m/z; Polarity: Positive
Full Scan	7.0-16 min; Resolution: 35,000; AGC target: 1e6; Max IT: 200 ms; Scan range: 65-180 m/z; Polarity: Positive

Inclusion List:	m/z	Formula	Species	Polarity	Comment
Target SIM 1	75.05529	$C_2H_6N_2O$	+H	Positive	NDMA
Target SIM 3	103.08659	$C_4H_{10}N_2O$	+H	Positive	NDEA
Target SIM 3	117.10224	$C_5H_{12}N_2O$	+H	Positive	NEIPA
Target SIM 3	131.11789	$C_6H_{14}N_2O$	+H	Positive	NDIPA
Target SIM 2	145.06187	$C_5H_{10}N_2O_3$	-H	Negative	NMBA
Target SIM 3	159.14919	$C_8H_{18}N_2O$	+H	Positive	NDBA

[Note: (i) Valve switches window may adjust depends on the different system to avoid excessive contamination of MS detector from API and excipients (subject to the RT of the target analyte); (ii) The MS parameter may need to adjust depends on different sensitivity of Q-Exactive.]

2.4 Standard, Sample, Sample Blank and Spiked Sample Preparation

2.4.1 Standard Preparation

1. Stock Standard Solution (20 mg/L): Prepare from commercially available standards (solid or liquid form) in 10 mL volumetric flask, top up to volume with MeOH individually.
2. Mix Stock Standard Solution (1 mg/L): accurately transfer 500 µL of Stock Standard Solution to a 10 mL volumetric flask and top up to volume with Diluent.
3. Working Standard Solution:
   Working Standard Solution I (1 µg/L): accurately transfer 10 µL of Mix Stock Standard Solution (1 mg/L) to a 10 mL volumetric flask and top up to volume with Diluent.

Working Standard Solution II (5 µg/L): accurately transfer 50 µL of Mix Stock Standard Solution (1 mg/L) to a 10 mL volumetric flask and top up to volume with Diluent

[Note: Protect all Standard Solutions from light.]

2.4.2 Sample Preparation

1 Weigh 10 tablets together and calculate the average mass of one tablet.
2 Accurately weigh an amount of powdered sample, corresponding to 500 mg of API into a 15 mL PP conical bottom centrifuge tube.
3 Add 10 mL of diluent, vortex to mix well and sonicate for 10 min. [Scale down the sample amount proportionally if sample is insufficient].
4 Transfer about 1 mL mixture to a 2 mL Eppendorf tube, centrifuge the mixture at 15000 rpm for 5 min at room temperature.
5 Filter the supernatant into HPLC vial through a 0.2 µm PTFE Membrane filter.

[Note: Protect sample solutions from light.]

2.4.3 Spiked Sample Preparation

Spiked Sample Solution I

1. Accurately weigh an amount of powdered sample, corresponding to 500 mg of API into a 15 mL PP Conical bottom centrifuge tube.
2. Transfer 10 µL Mix Stock Standard Solution into the tube.
3. Repeat step 3-5 as in Section 2.4.2 to obtain Spiked Sample Solution I [1 ng/mL in Spiked Sample Solution, corresponding to 0.02 µg/g in sample, LODs of NDEA, NMBA, NEIPA, NDIPA and NDBA].

Spiked Sample Solution II

1. Accurately weigh an amount of powdered sample, corresponding to 500 mg of ARB API into a 15 mL PP Conical bottom centrifuge tube.
2. Transfer 45 µL Mix Stock Standard Solution into the tube.
   Repeat step 3-5 as in Section 2.4.2 to obtain Spike Sample Solution I [4.5 ng/mL in Spiked Sample Solution, corresponding to 0.09 µg/g in sample, LOD of NDMA].

2.4.4 Sample Blank Preparation

Sample Blank Solution: Prepare the Sample Blank as described for the Sample Preparation in Section 2.4.2 but without the sample addition.

2.5 Test Procedure

1. Select method: Nitrosamine 6 in 1_HRMS
2. Inject solvent blank (Diluent).

3. Inject Working Standard Solution.
4. Inject solvent blank.
5. Inject Sample Blank.
6. Inject Sample Solution.
7. Inject Spiked Sample Solution.
8. Inject solvent blank.
9. Flush LC-HRMS system immediately after the analysis.

2.6 Interpretation of Results

1. The positive identification result is valid only if:
   i. The peaks corresponding to NDMA, NDEA, NEIPA, NDIPA, NDBA and NMBA in the chromatogram from the Sample Solution have close retention time (±0.3 min) to the peak from the Standard Solution chromatogram;
   ii. The Mass error of the peak corresponding to NDMA obtained from the Standard Solution and Sample Solution must be within the ±10 ppm tolerance window to the theoretical mass of NDMA. The Mass error of the peak corresponding to NDEA, NEIPA, NDIPA, NDBA or NMBA obtained from the Standard Solution and Sample Solution must be within the ±5 ppm tolerance window to the theoretical mass of analytes.
2. For negative identification, the result is valid only if:
   i. No peak corresponding to NDMA, NDEA, NEIPA, NDIPA, NDBA or NMBA was observed in the chromatogram obtained from the Sample Solution. Positive results are observed in Spiked Sample Solution for NDMA, NDEA, NEIPA, NDIPA, NDBA or NMBA;

   ii. Report as 'Not Detected' and indicate the LOD accordingly.

	NDMA	NDEA	NEIPA	NDIPA	NDBA	NMBA
Scan type	SIM (+)	SIM (+)	SIM (+)	SIM (+)	SIM (+)	SIM (-)
Instrument LOD (ng/mL)	4.5	1	1	1	1	1
Method LOD (µg/g)	0.09	0.02	0.02	0.02	0.02	0.02

[Note: The calculation of LOD for this method was based on 500 mg of API in the drug substance. The LOD was determined based on the peak S/N of the standards spiked into the sample matrix.]

Example Chromatograms of NDMA, NDEA, NEIPA, NDIPA, NDBA and NMBA (Concentration: 5 ng/mL)