DETERMINATION OF SIX NITROSAMINE IMPURITIES IN WESTERN MEDICINES BY LC-MS/MS

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Disclaimer: The testing method below provides option and guidance for the users to determine 6 nitrosamines 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 determination of six nitrosamine impurities including *N*-nitroso-*di*-methylamine (NDMA), *N*-nitroso-*di*-ethylamine (NDEA), *N*-ethyl-N-nitroso-2-propanamine (NEIPA), *N*-nitroso-*di*-isopropylamine (NDIPA), *N*-nitroso-*di*-n-butylamine (NDBA) and *N*-Nitroso-*N*-methylamino butyric acid (NMBA) in western medicines by Liquid Chromatography Hybrid Tandem Mass Spectrometry (Q-Trap LC-MS/MS).

2 Determination of Six Nitrosamine by LC-MS/MS

2.1 Reagents and Chemicals

N-Nitroso-*di*-methylamine (NDMA)
N-Nitroso-*di*-methylamine-D6 (NDMA-D6)
N-Nitroso-*di*-ethylamine (NDEA)
N-Ethyl-n-nitroso-2-propanamine (NEIPA)
N-Nitroso-*di*-isopropylamine (NDIPA)
N-Nitroso-*di*-n-butylamine (NDBA)
N-Nitroso-*di*-n-butylamine-D18 (NDBA-D18)
N-Nitroso-n-methylamino butyric acid (NMBA)
N-Nitroso-n-methylamino butyric acid-D3 (NMBA-D3)
Methanol (MeOH), HPLC grade
Formic acid, MS grade
Deionized water (DI water)
Diluent (with IS): 80% methanol in DI water containing 10 ng/mL of IS (NDMA-D6, NDBA-D18 and/or NMBA-D3)

2.2 Instruments and Apparatus

Liquid Chromatography Tandem Mass Spectrometry (QTRAP 6500+ MS/MS coupled with Agilent 1290 Infinity LC)
Centrifuge
Ultrasonic bath
Volumetric flask (Class A, 10 mL)
Membrane syringe filter (PTFE 0.2 µm)
Micropipette
2 mL vials
1.5 mL Eppendorf tube
Conical bottom centrifuge tube, Polypropylene (PP)

2.3 LC-MS/MS 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
	16.5	80	20
	20	80	20

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

MS/MS

MS:	QTRAP 6500+					
Polarity:	Positive					
Ionization mode:	APCI (Atmospheric Pressure Chemical Ionization)					
MS parameter:	CUR: 20 psi; CAD: Medium; TEP: 400 °C; GSI: 40 psi; CXP: 11					
Valve switches*:	Time (min)			Position	Remark	
	0.0-3.7			A	To waste	
	3.7-6.0			B	To MS	
	6.0-7.2			A	To waste	
	7.2-17.0			B	To MS	
MRM:	ID	Q1	Q3	DP	EP	CE
	NDMA 1	75.0	43.0	60	7	21
	NDMA 2	75.0	58.0	60	7	16
	NDMA IS	81.0	46.0	60	7	21
	NDEA 1	103.1	75.1	45	10	14
	NDEA 2	103.1	47.1	45	10	21
	NEIPA 1	117.0	75.0	40	12	14
	NEIPA 2	117.0	47.0	40	12	24
	NEIPA 3	117.0	41.0	40	12	35
	NDIPA 1	131.1	89.1	34	10	12
	NDIPA 2	131.1	47.0	34	10	17
	NDIPA 3	131.1	43.1	34	10	20
	NMBA 1	147.1	117.0	17	13	8
	NMBA 2	147.1	87.0	17	13	12
	NMBA 3	147.1	102.0	17	13	12
	NMBA IS	150.0	120.0	17	8	9
	NDBA 1	159.1	103.0	40	11	15
	NDBA 2	159.1	57.1	40	11	17
	NDBA 3	159.1	41.0	40	11	32
	NDBA IS	177.2	66.2	57	11	21

[*Note: 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)]

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. *Stock Internal Standard Solution* (20 mg/L): Prepared from commercially available NDMA-D6, NDBA-D18 and NMBA-D3 standard and dilute with MeOH.
3. *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 80% methanol.
4. *Mix Stock Internal Standard Solution* (1 mg/L): Accurately transfer 500 µL of NDMA-D6, NDBA-D18 and NMBA-D3 *Stock Internal Standard Solution* to a 10 mL volumetric flask and top up to volume with MeOH.
5. *Working Standard Solutions* (with 10 µg/L IS; prepared in 10 mL Volumetric flask individually):

Working Standard Solution	Standards Conc.	Vol of *Mix Stock Standard solution* (1 mg/L)	Vol of *Mix Stock IS Solution* (1 mg/L)	
1	0	0	100 µL	Top up to volume (10 mL) using 80% methanol
2	1 µg/L	10 µL	100 µL	
3	2 µg/L	20 µL	100 µL	
4	5 µg/L	50 µL	100 µL	
5	10 µg/L	100 µL	100 µL	
6	20 µg/L	200 µL	100 µL	
7	50 µg/L	500 µL	100 µL	
8	100 µg/L	1000 µL	100 µL	

[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, equivalent to 500 mg of API into a 15 mL PP conical bottom centrifuge tube.
3 Add 10 mL of *Diluent (with IS)*, vortex to mix well and sonicate for 10 min [Note: Scale down the sample amount if necessary].
4 Transfer about 1 mL mixture to a 1.5 mL Eppendorf tube, centrifuge the mixture at 15000 rpm for 5 min at room temperature.
5 Filter the supernatant into a HPLC vial through a 0.2 µm PTFE Membrane filter.

[Note: Protect sample solutions from light. In the situation when the amount of powder is too much for effective sample extraction, please reduce the powder amount or increase the extraction solvent volume. In this case, the LOD of the method will be affected and needed to be recalculated accordingly.]

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, LOD of NDEA, NEIPA, NDIPA and NDBA].

Spiked Sample Solution II

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 20 µL *Mix Stock Standard Solution* into the tube.
3. Repeat step 3-5 as in Section 2.4.2 to obtain *Spike Sample Solution II* [2 ng/mL in *Spiked Sample Solution*, corresponding to 0.04 µg/g in sample, LOD of NDMA and NMBA].

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: *Nitrosamins_6 in 1_MRM_APCI*;
2. Inject solvent blank (80% methanol).
3. Inject *Working Standard Solution 1-8*.
4. Inject solvent blank.
5. Inject *Sample Blank*.
6. Inject *Sample Solution* (Duplicate) [Note: dilute sample with *Diluent (with IS)* accordingly when the concentration of the *Sample Solution* exceeds the calibration range.].
7. Inject *Spiked Sample Solution 1* and *II*.
8. Inject solvent blank.
9. Flush LC-MS/MS system immediately after the analysis.

2.6 Interpretation of Results

1. For negative identification, the result is valid only if:
   i. No peaks corresponding to NDMA, NDEA, NEIPA, NDIPA, NDBA and NMBA was observed in the chromatogram obtained from the *Sample Solution*. Positive results are obtained in *Spiked Sample Solution I* for NDEA, NEIPA, NDIPA and NDBA and *Spiked Sample Solution II* for NDMA and NMB;

   ii. Report as 'Not Detected' and indicate the LOD of the substances.

   The LOD for the 5 nitrosamines are listed as below:

	NDMA	NDEA	NEIPA	NDIPA	NDBA	NMBA
Instrument LOD (ng/mL)	2	1	1	1	1	2
Method LOD (µg/g)	0.04	0.02	0.02	0.02	0.02	0.04
Method LOQ (µg/g)	0.10	0.05	0.05	0.05	0.05	0.10

   [Note: The LOD were based on the 500 mg of API used in the drug substance].

2. For positive identification, the 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 peaks from the *Standard Solutions* chromatogram;

ii. The deviation of the ion pair ratios of NDMA, NDEA, NEIPA, NDIPA, NDBA and NMBA obtained from the *Standard Solutions* and *Sample Solution* for the two MRM transitions are not more than 20%.

3. The quantification is performed using the peak area ratios from ion pair of NDMA: (75.0/43.0)/(81.0/46.0), (NDMA 1/ NDMA IS); NDEA: (103.1/75.1) /(81.0/46.0), (NDEA 1/NDMA IS); NEIPA: (117.0/75.0)/(177.2/66.2), (NEIPA 1/NDBA IS); NDIPA: (131.1/89.1)/(177.2/66.2), (NDIPA 1/NDBA IS); NDBA: (159.1/103.1)/(177.2/66.2), (NDBA 1/NDBA IS) and NMBA: (147.1/117.0)/(150.0/120.0), (NMBA 1/NMBA IS) through linearity plot from *Working Standard Solutions 1, 4-8* (for NDMA and NMBA) and *Working Standard Solutions 1, 3-8* (for NDEA, NEIPA, NDIPA and NDBA). The quantification result is valid only if:
   i. The deviation of the peak area ratios for NDMA, NDEA, NEIPA, NDIPA, NDBA and NMBA obtained from duplicated sample solution are not more than 20%;
   ii. The linearity coefficient of the calibration plot is greater than 0.99;
   iii. Report as 'Less than 0.10 µg/g' and indicate the LOQ of the substances if the peak area ratio of NDMA and NMBA is above the peak area ratio of *Standard Solution* of 2 ng/mL but less than the peak area ratio of *Standard Solution* of 5 ng/g;
   iv. Report as 'Less than 0.05 µg/g' and indicate the LOQ of the substances if the peak area ratio of NDEA, NEIPA, NDIPA and NDBA is above the peak area ratio of *Standard Solution* of 1 ng/mL but less than the peak area ratio of *Standard Solution* of 2.5 ng/g.

2.7 Calculation

1. Calculation of nitrosamine content in sample with respect to drug products (per unit)

Content of nitrosamine in drug product = $[(C_{Spl} \times V_{Spl} \times Dil) / W_{Spl}] \times W_{Unit}$

Where:

Content of nitrosamine in drug product (ng/unit)

C_{Spl} : Concentration of nitrosamine obtained from liner plot, ng/mL
V_{Spl} : Volume of *Sample Solution*, mL (e.g. 10 mL)
W_{Spl} : Weight of sample used for sample preparation, g
W_{Unit} : Weight of each unit of sample, g (e.g. g/tab or g/cap etc.)
Dil : Dilution factor

2. Calculation of nitrosamine content in sample with respect to API

Content of nitrosamine with respect to API = $[(C_{Spl} \times V_{Spl} \times Dil)/W_{Spl}] \times W_{Unit} / S_{Unit}$

Where:

Content of nitrosamine with respect to API (ng/g)

C_{Spl} : Concentration of nitrosamine obtained from liner plot, ng/mL
V_{Spl} : Volume of *Sample Solution*, mL (e.g. 10 mL)
W_{Spl} : Weight of sample used for sample preparation, g
W_{Unit} : Weight of each unit of sample, g (e.g. g/tab or g/cap etc.)
S_{Unit} : Strength of the drug product per unit, g (e.g. 0.1 for 100 mg/tab)
Dil : Dilution factor

Example Chromatograms of NDMA, NDEA, NEIPA, NDIPA, NDBA and NMBA (Concentration: 20 ng/mL)