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Method of Test for Nitrosamines in Medicines - Multiple Analysis (GC-MS/MS Method)

1. Scope

This method is applicable to the determination of 12 nitrosamines such as *N*-nitrosodibutylamine (NDBA) in medicines. If the testing method is not available for some medicine, it can be adjusted, but should be validated before use.

2. Method

After extraction, nitrosamines are determined by gas chromatography/tandem mass spectrometry (GC-MS/MS).

2.1. Equipment

2.1.1. Gas chromatograph/tandem mass spectrometer.

2.1.1.1. Ion source: electron ionization, EI.

2.1.1.2. Column: DB-WAX UI, 0.25 μm, 0.25 mm i.d. × 30 m, or an equivalent product.

2.1.1.3. Liner: Agilent 5190-2293, inert, splitless, single taper, containing glass wool, or an equivalent product.

2.1.2. Vortex mixer.

2.1.3. Ultrasonicator.

2.1.4. Centrifuge: centrifugal force ≧ 3000 ×g.

2.2. Chemicals

Methanol, HPLC grade;

N-Nitrosodibutylamine (NDBA), 2000 μg/mL in dichloromethane, reference standard;

N-Nitrosodicyclohexylamine (NDCHA), reference standard;

N-Nitrosodiethylamine (NDEA), 5000 μg/mL in methanol, reference standard;

N-Nitrosodiisobutylamine (NDiBA), reference standard;

N-Nitrosodiisononylamine (NDiNA), reference standard;

N-Nitrosodiisopropylamine (NDiPA), reference standard;

N-Nitrosodimethylamine (NDMA), 5000 μg/mL in methanol, reference standard;

N-Nitrosodiphenylamine (NDPhA), 5000 μg/mL in methanol, reference standard;

N-Nitrosodipropylamine (NDPA), 5000 μg/mL in methanol, reference standard;

N-Nitrosomethylethylamine (NMEA), reference standard;
N-Nitrosomorpholine (NMOR), reference standard;
N-Nitrosopiperidine (NPIP), 5000 μg/mL in methanol, reference standard;
N-Nitrosodimethylamine-d_6 (NDMA-d_6), 1000 μg/mL in dichloromethane, isotope-labeled internal standard;
N-Nitrosodiethylamine-d_4 (NDEA-d_4), isotope-labeled internal standard;
N-Nitrosodiphenylamine-d_{10} (NDPhA-d_{10}), isotope-labeled internal standard
N-Nitrosodipropylamine-d_{14} (NDPA-d_{14}), 1000 μg/mL in dichloromethane, isotope-labeled internal standard;

2.3. Apparatus

2.3.1. Volumetric flask: 10 mL, amber glass.

2.3.2. Centrifuge tube: 15 mL, PP.

2.3.3. Membrane filter: 0.22 μm, PVDF.

2.3.4. Mortar and pestle.

2.4. Internal standard solution preparation

2.4.1. Transfer about 10 mg of NDEA-d_4 and NDPhA-d_{10} isotope-labeled internal standards accurately weighed to each 10-mL volumetric flask, dissolve and dilute to volume with methanol as the internal standard stock solutions. Store in a freezer and protect from light.

2.4.2. Accurately transfer 1 mL of NDMA-d_6 and NDPA-d_{14} isotope-labeled internal standards to each 10-mL volumetric flask, dilute to volume with methanol as the internal standard stock solutions. Store in a freezer and protect from light.

2.4.3. When to use, mix appropriate volume of each internal standard stock solution, and dilute with methanol to 200 ng/mL as the internal standard solution.

2.5. Standard solution preparation

2.5.1. Transfer about 10 mg of NDCHA, NDiBA, NDiNA, NDiPA, NMEA and NMOR reference standards accurately weighed to each 10-mL volumetric flask, dissolve and dilute to volume with methanol, and further dilute with methanol to 50 μg/mL as the standard stock solutions. Store in a freezer and protect from light.

2.5.2. Accurately transfer 1 mL of NDBA, NDEA, NDMA, NDPhA, NDPA and NPIP reference standards to each 10-mL volumetric flask, dilute to

volume with methanol, and further dilute with methanol to 50 μg/mL as the standard stock solutions. Store in a freezer and protect from light.

2.5.3. When to use, mix appropriate volume of each standard stock solution and the internal standard solution, and dilute with methanol to 1-50 ng/mL (containing 20 ng/mL the internal standard) as the standard solutions.

2.6. Standard curve establishment

Accurately inject 2 μL of the standard solutions into GC-MS/MS separately, and operate according to the following conditions. Establish the standard curve of each nitrosamine by the ratios of the peak area of each nitrosamine to that of the internal standard vs. the concentrations of each nitrosamine.

GC-MS/MS operating conditions(note 1):

Column: DB-WAX UI, 0.25 μm, 0.25 mm i.d. × 30 m.

Oven temperature gradient:

Initial temperature: 80°C, hold for 3 min;

temperature gradient rate: 20°C /min;

final temperature: 250°C, hold for 3 min.

Injector temperature: 250°C.

Injection mode(note 2): pulsed splitless, 35 psi for 1 min.

Injection volume: 2 μL.

Carrier gas and flow rate: Helium, 1 mL/min.

Interface temperature: 250°C.

Ion source temperature: 230°C.

Ionization mode: EI, 70 eV.

Detection mode: multiple reaction monitoring (MRM). Detection ions and collision energy (CE) are shown in the attached table.

Note: 1. The parameters can be adjusted depending on the instruments used if the above conditions are not applicable.

2. The vaporization volume of the sample solution should be less than the liner's volume. The injection pulse pressure and time can be adjusted depending on the instrument and the liner used.

2.7. Sample solution preparation

Grind the sample into powder, and transfer about 100 mg of the powdered sample accurately weighed to a 15-mL centrifuge tube. Add 0.5 mL of the internal standard solution and 4.5 mL of methanol, vortex-mix well, and

sonicate for 30 min. Centrifuge at 3000 xg for 5 min. Filter the supernatant with a membrane filter, and take the filtrate as the sample solution.

2.8. Identification and quantification

Accurately inject 2 µL of the sample solution and the standard solutions into GC-MS/MS separately, and operate according to the conditions in section 2.6. Identify each nitrosamine based on the retention time and the relative ion intensities(note). Calculate the amount of each nitrosamine in the sample by the following formula:

$$\text{The amount of each nitrosamine in the sample (µg/g)} = \frac{C \times V}{M} \times 10^{-3}$$

where,

C: the concentration of each nitrosamine in the sample solution calculated by the standard curve (ng/mL)

V: the volume of methanol for sample extraction (5 mL)

M: the weight of sample (g)

Note: Relative ion intensities are calculated by peak areas of qualitative ions divided by peak areas of quantitative ions. Maximum permitted tolerances for relative ion intensities by GC-MS/MS are as follows:

Relative ion intensity (%)	Tolerance (%)
> 50	± 20
> 20-50	± 25
> 10-20	± 30
≦ 10	± 50

Remark

1. Limits of quantitation (LOQs) for 12 nitrosamines are listed in the attached table.
2. Further validation should be performed when interference compounds appear in samples.

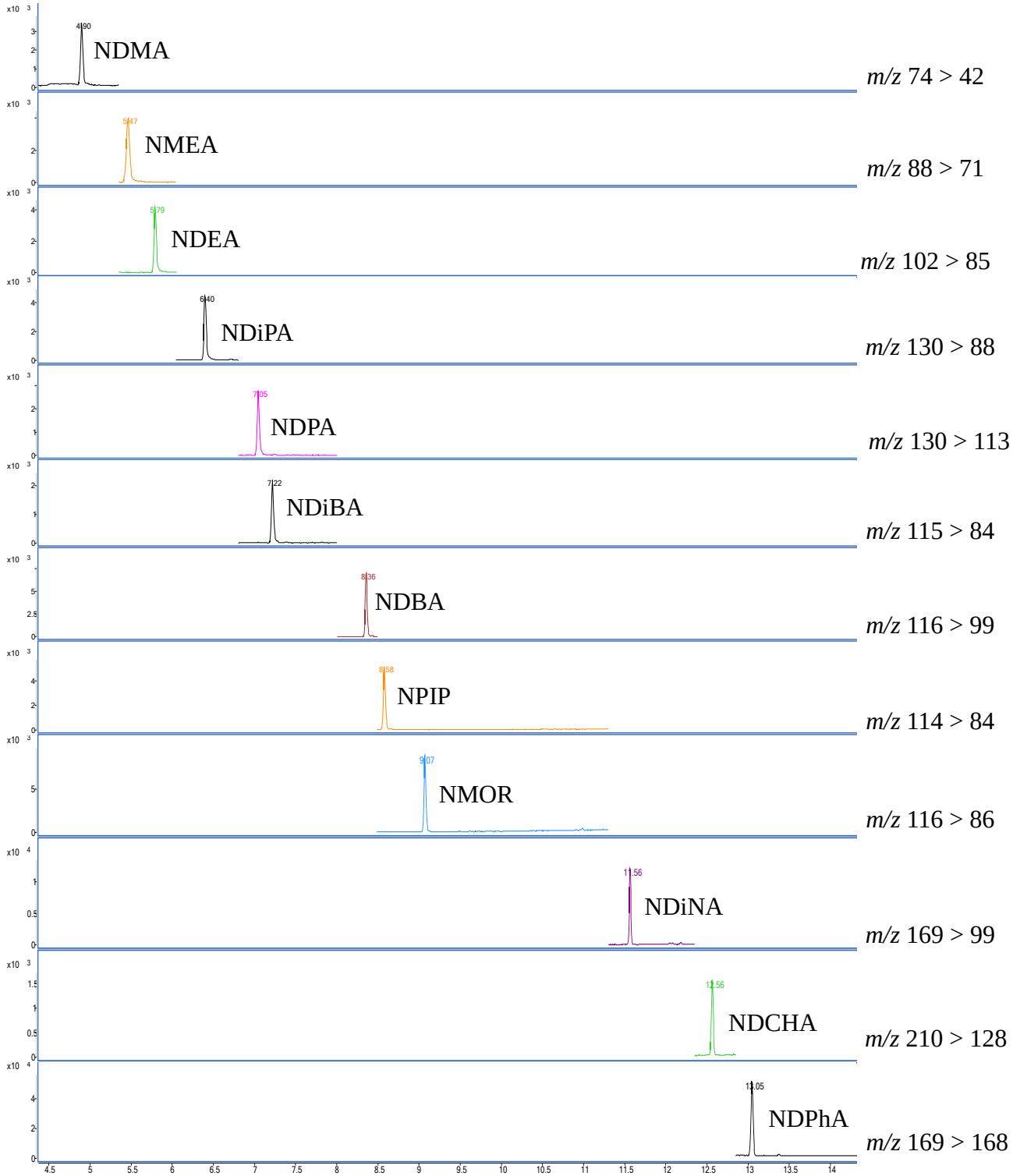
Reference

1. U.S. Food and Drug Administration. 2019. Combined direct injection *N*-nitrosodimethylamine (NDMA) and *N*-nitrosodiethylamine (NDEA) impurity assay by GC/MS. [https://www.fda.gov/media/117807/download]
2. U.S. Food and Drug Administration. 2019. Combined direct injection *N*-nitrosodimethylamine (NDMA), *N*-nitrosodiethylamine (NDEA),

N-nitrosoethylisopropylamine (NEIPA), *N*-nitrosodiisopropylamine (NDIPA), and *N*-nitrosodibutylamine (NDBA) impurity assay by GC-MS/MS. [https://www.fda.gov/media/123409/download]

3. Parr, M. K. and Joseph, J. F. 2019. NDMA impurity in valsartan and other pharmaceutical products: Analytical methods for the determination of *N*-nitrosamines. J. Pharm. Biochem. Anal. 164: 536-549.

Reference chromatogram



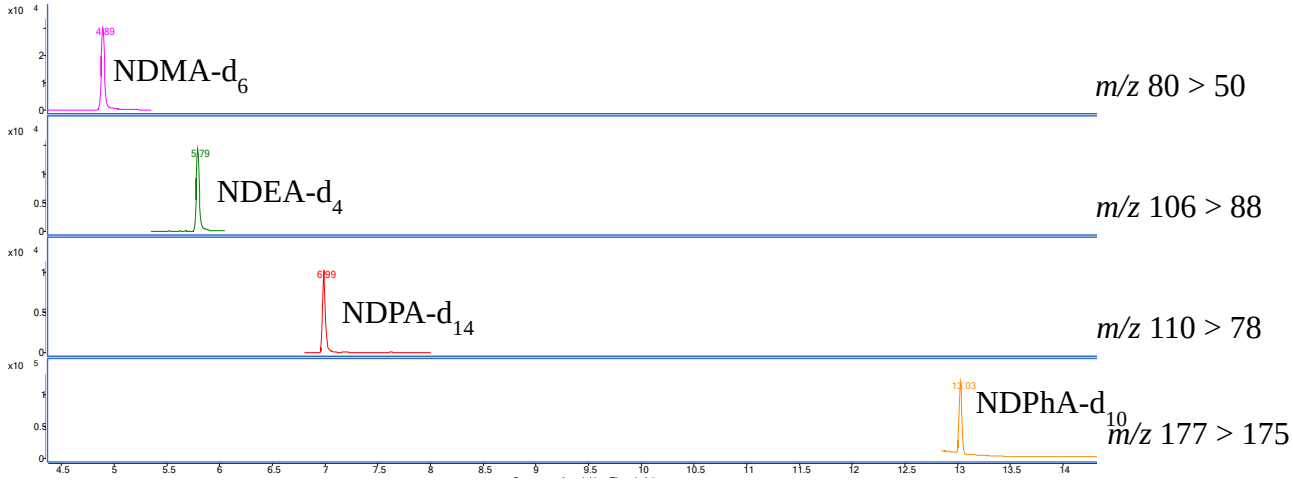


Figure. MRM chromatograms of 12 nitrosamine standards and 4 isotope-labeled internal standards analyzed by GC-MS/MS.

Table. MRM parameters and LOQs of 12 nitrosamines and 4 isotope-labeled internal standards

No.	Analyte	Quantitative ion pair		Qualitative ion pair		Internal standard	LOQ (μg/g)
		Precursor ion (*m/z*) > product ion (*m/z*)	CE (eV)	Precursor ion (*m/z*) > product ion (*m/z*)	CE (eV)		
1	*N*-Nitrosodibutylamine (NDBA)	116 > 9d	2	158 > 99	5	NDPA-d_{14}	0.05
2	*N*-Nitrosodicyclohexylamine (NDCHA)	210 > 128	8	210 > 111	20	NDPA-d_{14}	0.05
3	*N*-Nitrosodiethylamine (NDEA)	102 > 85	2	102 > 56	15	NDEA-d_4	0.05
4	*N*-Nitrosodiisobutylamine (NDiBA)	115 > 84	2	103 > 57	8	NDPA-d_{14}	0.10
5	*N*-Nitrosodiisononylamine (NDiNA)	169 > 99	12	281 > 225	12	NDPA-d_{14}	0.05
6	*N*-Nitrosodiisopropylamine (NDiPA)	130 > 88	2	130 > 42	10	NDPA-d_{14}	0.05
7	*N*-Nitrosodimethylamine (NDMA)	74 > 42	20	74 > 44	13	NDMA-d_6	0.05
8	*N*-Nitrosodiphenylamine (NDPhA)	169 > 168	18	169 > 167	30	NDPhA-d_{10}	0.05
9	*N*-Nitrosodipropylamine (NDPA)	130 > 113	2	130 > 43	13	NDPA-d_{14}	0.05
10	*N*-Nitrosomethylethylamine (NMEA)	88 > 71	2	88 > 42	15	NDEA-d_4	0.05
11	*N*-Nitrosomorpholine (NMOR)	116 > 86	2	116 > 56	13	NDPA-d_{14}	0.05
12	*N*-Nitrosopiperidine (NPIP)	114 > 84	10	114 > 97	5	NDPA-d_{14}	0.05
I.S.	*N*-Nitrosodiethylamine-d_4 (NDEA-d_4)	106 > 88	2	—	—	—	—
I.S.	*N*-Nitrosodimethylamine-d_6 (NDMA-d_6)	80 > 50	5	—	—	—	—
I.S.	*N*-Nitrosodiphenylamine-d_{10} (NDPhA-d_{10})	177 > 175	18	—	—	—	—
I.S.	*N*-Nitrosodipropylamine-d_{14} (NDPA-d_{14})	110 > 78	2	—	—	—	—