

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

### 1. Scope

This method is applicable to the determination of 12 nitrosamines such as *N*-nitrosodiethanolamine (NDELA) 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 liquid chromatography/tandem mass spectrometry (LC-MS/MS).

#### 2.1. Equipment

2.1.1. Liquid chromatograph/tandem mass spectrometer.

2.1.1.1. Ion source: atmospheric pressure chemical ionization, APCI.

2.1.1.2. Column: XSelect HSS T3, 3.5  $\mu\text{m}$ , 3 mm i.d.  $\times$  15 cm, or an equivalent product.

2.1.2. Ultrasonicator.

2.1.3. Centrifuge: centrifugal force  $\geq$  3000  $\times g$ .

#### 2.2. Chemicals

Methanol, HPLC grade;

Acetonitrile, HPLC grade;

Formic acid, HPLC grade;

Deionized water, resistivity  $\geq$  18  $\text{M}\Omega \cdot \text{cm}$  (at 25°C);

*N*-Nitrosodiethanolamine (NDELA), reference standard;

*N*-Nitrosodiethylamine (NDEA), reference standard;

*N*-Nitrosodiisopropanolamine (NDiPLA), reference standard;

*N*-Nitrosodiisopropylamine (NDiPA), reference standard;

*N*-Nitrosodimethylamine (NDMA), reference standard;

*N*-Nitrosodipropylamine (NDPA), reference standard;

*N*-Nitrosoethylisopropylamine (NEiPA), reference standard;

*N*-Nitroso-*N*-methyl-4-aminobutyric acid (NMBA), reference standard;

*N*-Nitrosomethylethylamine (NMEA), reference standard;

*N*-Nitrosomorpholine (NMOR), reference standard;

*N*-Nitrosopiperidine (NPIP), reference standard;

*N*-Nitrosopyrrolidine (NPYR), reference standard;

*N*-Nitrosodiethanolamine- $\text{d}_8$  (NDELA- $\text{d}_8$ ), isotope-labeled internal standard;

*N*-Nitrosodiethylamine- $d_4$  (NDEA- $d_4$ ), isotope-labeled internal standard;  
*N*-Nitrosodimethylamine- $d_6$  (NDMA- $d_6$ ), isotope-labeled internal standard;  
*N*-Nitrosodipropylamine- $d_{14}$  (NDPA- $d_{14}$ ), isotope-labeled internal standard;  
*N*-Nitroso-*N*-methyl-4-aminobutyric acid- $d_3$  (NMBA- $d_3$ ), 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  $\mu\text{m}$ , PVDF.

2.3.4. Mortar and pestle.

## 2.4. 10% methanol

Mix methanol and deionized water at the ratio of 1:9 (v/v).

## 2.5. Mobile phase

### 2.5.1. Solvent A

Dilute 1 mL of formic acid with deionized water to 1000 mL, and filter with a membrane filter.

### 2.5.2. Solvent B

Dilute 1 mL of formic acid and 200 mL of acetonitrile with methanol to 1000 mL, and mix well.

## 2.6. Internal standard solution preparation

Transfer appropriate amount of 5 isotope-labeled internal standards accurately weighed into each 10-mL volumetric flask, dissolve and dilute with methanol to 50  $\mu\text{g/mL}$  as the internal standard stock solutions. Store in a freezer and protect from light. When to use, mix appropriate volume of each internal standard stock solution, and dilute with methanol to 0.4  $\mu\text{g/mL}$  as the internal standard solution.

## 2.7. Standard solution preparation

Transfer appropriate amount of 12 reference standards into each 10-mL volumetric flask, dissolve and dilute with methanol to 50  $\mu\text{g/mL}$  as the standard stock solutions. Store in a freezer and protect from light. When to use, mix appropriate volume of each standard stock solution and the internal standard solution, and dilute with 10% methanol to 2.5-50 ng/mL (containing 20 ng/mL the internal standard) as the standard solutions.

## 2.8. Standard curve establishment

Accurately inject 10  $\mu$ L of the standard solutions into LC-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.

LC-MS/MS operating conditions<sup>(note)</sup>:

Column: XSelect HSS T3, 3.5  $\mu$ m, 3 mm i.d.  $\times$  15 cm.

Column temperature: 40°C.

Mobile phase: a gradient program of solvent A and solvent B is as follows.

| Time (min)              | Solvent A (%)       | Solvent B (%)         |
|-------------------------|---------------------|-----------------------|
| 0.0 $\rightarrow$ 1.0   | 95 $\rightarrow$ 95 | 5 $\rightarrow$ 5     |
| 1.0 $\rightarrow$ 5.0   | 95 $\rightarrow$ 50 | 5 $\rightarrow$ 50    |
| 5.0 $\rightarrow$ 6.5   | 50 $\rightarrow$ 50 | 50 $\rightarrow$ 50   |
| 6.5 $\rightarrow$ 7.5   | 50 $\rightarrow$ 35 | 50 $\rightarrow$ 65   |
| 7.5 $\rightarrow$ 8.5   | 35 $\rightarrow$ 35 | 65 $\rightarrow$ 65   |
| 8.5 $\rightarrow$ 9.5   | 35 $\rightarrow$ 0  | 65 $\rightarrow$ 100  |
| 9.5 $\rightarrow$ 12.0  | 0 $\rightarrow$ 0   | 100 $\rightarrow$ 100 |
| 12.0 $\rightarrow$ 12.1 | 0 $\rightarrow$ 95  | 100 $\rightarrow$ 5   |
| 12.1 $\rightarrow$ 15.0 | 95 $\rightarrow$ 95 | 5 $\rightarrow$ 5     |

Flow rate: 0.6 mL/min.

Injection volume: 10  $\mu$ L.

Ionization mode: APCI<sup>+</sup>.

Nebulizer current: 5  $\mu$ A.

Curtain gas: 25 psi.

Gas 1: 50 psi.

Collision gas: medium.

Temperature: 400°C.

Detection mode: multiple reaction monitoring (MRM). Detection ion pair, declustering potential (DP) and collision energy (CE) are shown in the attached table.

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

## 2.9. Sample solution preparation

Grind the sample into powder, and transfer about 250 mg of the powdered sample accurately weighed to a 15-mL centrifuge tube. Add 0.25 mL of the internal standard solution and 0.25 mL of methanol, vortex-mix well, and

sonicate for 5 min. Add 4.5 mL of water, vortex-mix well, and sonicate for 5 min. Centrifuge at 3000 xg for 5 min. Filter the supernatant with a membrane filter, and take the filtrate as the sample solution.

## 2.10. Identification and quantification

Accurately inject 10 µL of the sample solution and the standard solutions into LC-MS/MS separately, and operate according to the conditions in section 2.8. Identify each nitrosamine based on the retention time and the relative ion intensities<sup>(note)</sup>. Calculate the amount of each nitrosamine in the sample by the following formula:

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

where,

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

V: the volume of solvent 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 LC-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 listed in the attached table are all 0.05 µg/g.
2. Further validation should be performed when interference compounds appear in samples.

### Reference

1. Ripollés, C., Pitarch, E., Sancho, J. V., López, F. J. and Hernández, F. 2011. Determination of eight nitrosamines in water at the ng L<sup>-1</sup> levels by liquid chromatography coupled to atmospheric pressure chemical ionization tandem mass spectrometry. *Anal. Chim. Acta* 702: 62-71.

2. Chemisches und Veterinäruntersuchungsamt (CVUA) Karlsruhe. 2019. Method for the determination of NDMA and NDEA by LC-MS/MS in Sartan containing film coated tablets. CVUA Karlsruhe, Germany (OMCL BW).
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

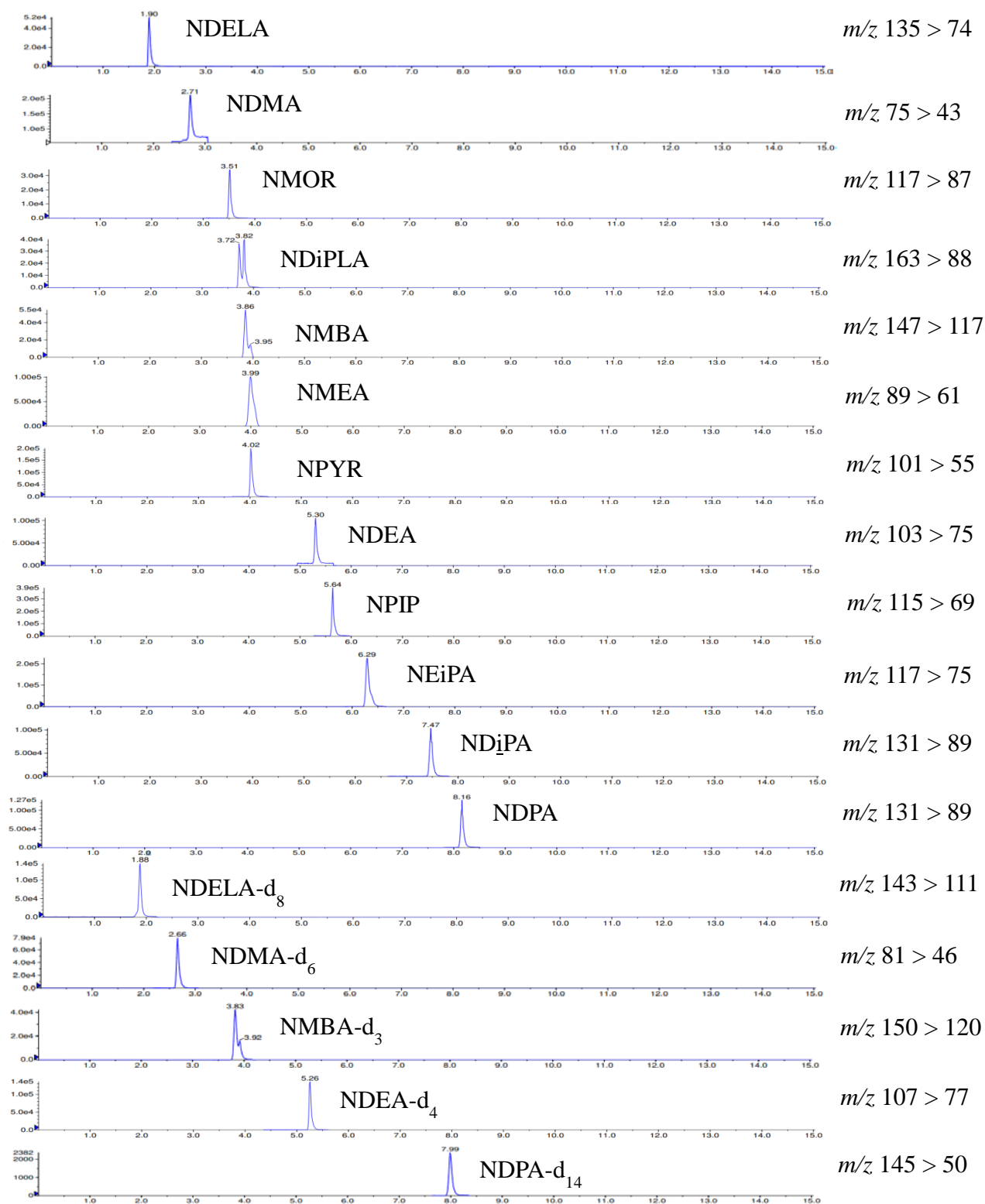


Figure. MRM chromatograms of 12 nitrosamine standards and 5 isotope-labeled internal standards analyzed by LC-MS/MS.

Table. MRM parameters of 12 nitrosamines and 5 isotope-labeled internal standards

| No.  | Analyte   | Quantitative ion pair                                     |        |         | Qualitative ion pair                                      |        |         | Internal standard    |
|------|---|---|--------|---------|---|--------|---------|----------------------|
|      |   | Precursor ion ( <i>m/z</i> ) > product ion ( <i>m/z</i> ) | DP (V) | CE (eV) | Precursor ion ( <i>m/z</i> ) > product ion ( <i>m/z</i> ) | DP (V) | CE (eV) |                      |
| 1    | <i>N</i> -Nitrosodiethanolamine (NDELA)   | 135 > 74  | 29     | 17      | 135 > 104   | 29     | 9       | NDELA-d <sub>8</sub> |
| 2    | <i>N</i> -Nitrosodiethylamine (NDEA)  | 103 > 75  | 25     | 13      | 103 > 47  | 25     | 29      | NDEA-d <sub>4</sub>  |
| 3    | <i>N</i> -Nitrosodiisopropanolamine (NDiPLA)  | 163 > 88  | 44     | 17      | 163 > 70  | 44     | 28      | NMBA-d <sub>3</sub>  |
| 4    | <i>N</i> -Nitrosodiisopropylamine (NDiPA)   | 131 > 89  | 50     | 13      | 131 > 43  | 50     | 22      | NDPA-d <sub>14</sub> |
| 5    | <i>N</i> -Nitrosodimethylamine (NDMA)   | 75 > 43   | 28     | 21      | 75 > 58   | 28     | 15      | NDMA-d <sub>6</sub>  |
| 6    | <i>N</i> -Nitrosodipropylamine (NDPA)   | 131 > 89  | 59     | 14      | 131 > 43  | 59     | 21      | NDPA-d <sub>14</sub> |
| 7    | <i>N</i> -Nitrosoethylisopropylamine (NEiPA)  | 117 > 75  | 16     | 14      | 117 > 47  | 16     | 22      | NDEA-d <sub>4</sub>  |
| 8    | <i>N</i> -Nitroso- <i>N</i> -methyl-4-aminobutyric acid (NMBA)                                | 147 > 117   | 10     | 8       | 147 > 87  | 10     | 16      | NMBA-d <sub>3</sub>  |
| 9    | <i>N</i> -Nitrosomethylethylamine (NMEA)  | 89 > 61   | 7      | 16      | 89 > 29   | 7      | 27      | NMBA-d <sub>3</sub>  |
| 10   | <i>N</i> -Nitrosomorpholine (NMOR)  | 117 > 87  | 38     | 17      | 117 > 86  | 38     | 20      | NMBA-d <sub>3</sub>  |
| 11   | <i>N</i> -Nitrosopiperidine (NPIP)  | 115 > 69  | 42     | 21      | 115 > 41  | 42     | 33      | NDEA-d <sub>4</sub>  |
| 12   | <i>N</i> -Nitrosopyrrolidine (NPYR)   | 101 > 55  | 58     | 22      | 101 > 41  | 58     | 37      | NMBA-d <sub>3</sub>  |
| I.S. | <i>N</i> -Nitrosodiethanolamine-d <sub>8</sub> (NDELA-d <sub>8</sub> )                        | 143 > 111   | 23     | 8       | —   | —      | —       | —                    |
| I.S. | <i>N</i> -Nitrosodiethylamine-d <sub>4</sub> (NDEA-d <sub>4</sub> )                           | 107 > 77  | 48     | 16      | —   | —      | —       | —                    |
| I.S. | <i>N</i> -Nitrosodimethylamine-d <sub>6</sub> (NDMA-d <sub>6</sub> )                          | 81 > 46   | 106    | 25      | —   | —      | —       | —                    |
| I.S. | <i>N</i> -Nitrosodipropylamine-d <sub>14</sub> (NDPA-d <sub>14</sub> )                        | 145 > 50  | 130    | 46      | —   | —      | —       | —                    |
| I.S. | <i>N</i> -Nitroso- <i>N</i> -methyl-4-aminobutyric acid-d <sub>3</sub> (NMBA-d <sub>3</sub> ) | 150 > 120   | 36     | 9       | —   | —      | —       | —                    |