Genotoxic Impurities in Drug Products: Review of Nitrosamine Assays

In the past couple of years, the prevalence of nitrosamines in drug substances has made them a hot topic of conversation in the medical industry

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Nitrosamines are organic compounds that we are exposed to in our everyday lives. They are common in water and foods, including cured and grilled meats, dairy products, and vegetables.

Nitrosamines are classified by the ICH M7(R1) Guideline as class one impurities, 'known mutagenic carcinogens,' based on both rodent carcinogenicity and mutagenicity data (1). They are categorised by the International Agency for Cancer Research as 2A – probable carcinogens based on data on a number of species studied (2).

Nitrosamines became a news focus for medicine authorities in mid-2018, when a Chinese drug substance (DS) manufacturer detected the presence of N-nitrosodimathylamine (NDMA), in batches of valsartan (3). Meanwhile, different nitrosamines were detected in almost every drug product which contains a 'sartan' derivate as a DS.

Recently, the FDA requested manufacturers withdraw all remaining prescription and over-the-counter ranitidine on the US market. These products are also currently being reviewed by the EMA, which has urged manufacturers to test drug products (DP) containing metformin, diabetes medicines.

The agency's notification establishes a two-step process for marketing authorisation holders (MAHs) where pharmaceutical companies were advised to avoid nitrosamines in human medicines (4).

First, a marketing authorisation holder must carry out a risk-based assessment of each product containing chemically synthesised DPs, which should be submitted before October 2020 to the European agencies.

Confirmatory Testing

Confirmatory testing should, therefore, be carried out using appropriately validated and sensitive methods and MAHs should inform the competent authorities immediately if tests confirm the presence of a nitrosamine impurity irrespective of the amount detected.

After that, MAHs should apply for a variation in a timely manner to introduce any required changes, such as an amendment of the manufacturing process or changes to product specifications.

What are the Currently Identified Root Causes for Presence of Nitrosamines?

In September 2019, the EMA published a question and answers document that quoted various potential sources of contamination: nitrosating agents, contaminated raw materials, use of recovered materials (e.g., solvents, reagents and catalysts), contaminated starting materials, cross-contaminations, degradation during finished product formulation or storage, and packaging materials.

How Should Tests Be Conducted?

Although CGMSD is the first method introduced by the FDA, this method is not recommended because of sensitivity and future requested limits and interferences. The solution to fulfil actual and future regulation is to use Tandem mass spectrometry (MS/MS) or high-resolution accurate mass systems in order to overcome interferences in the identification of the specific peak of nitrosamine (e.g., dimethylformamide [DMF] co-eluting with NDMA).

Screening Methods

The EMA is now requiring risk assessments of nitrosamine exposure risk to be completed by 1 October 2020. Pharma companies were also asked to experimentally screen products for nitrosamine content in the case of the risk assessment anticipates their possible presence.

Although the screening was initially focused on NDMA and N-nitrosodiethylamine (NDEA), the list that has to be tested for this testing can be extended to other common or specific nitrosamines according to the risk assessment.

This screening testing can be performed either by liquid chromatography-mass spectrometry (LC-MS/MS) or by gas chromatography-tandem mass spectrometry (GC-MS/MS), using a generic sample preparation and analysis method. These standardised procedures can be slightly adapted according to the galenic form (powder, tabs, pills, liquid, cream etc.), DP or DP properties (polarity, solubility). The use of LC or GC technique will depend on the nitrosamines that have to be screened, and the thermal stability of DP and excipient. Advantages and inconvenient of each technic will be further discussed.

N-nitrosamines listed in **Table 1** can be screened by LC-MS/MS at ppb level. The same compounds can be analysed by GC-MS/MS method, except NMBA and NDELA.

The generic method is based on:

- Addition of labelled internal standard (e.g., NDMA-d6 or NDEA-d10)
- Dissolution in water or other solvent, such as methyl chloride or 1-methyl-2-pyrrolidinone (depending on the analysis method)
- Agitation, centrifugation and/or filtration, to obtain a sample that can be analysed by LC-MS/MS or GC-MS/MS
- Analysis by:
  - LC method using reverse phase analytical columns and standard MS friendly solvents and additives (UV detector can be added to the configuration, to monitor the elution of drug product DP and excipients)
  - GC method, using direct injection or headspace

MS/MS detection using a multiple-reaction monitoring (quantitative) transition per N-nitrosamine (confirmatory transitions can be also recorded in order to avoid false positive results).

The screening approach is to apply this generic method to several batches of the pharmaceutical product (at least three or four batches, representative of the life cycle). The validity of the screening will be assessed by checking for each product:

- Sensitivity of the method at limit of detection (LOD) and limit of quantification (LOQ) levels
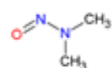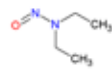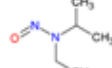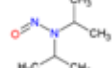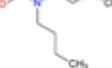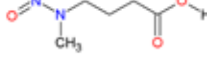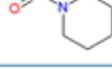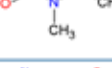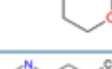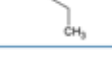
Nitrosamines	Chemical structure
N-nitrosodimethylamine (NDMA)	
N-nitrosodiethylamine (NDEA)	
N-nitrosoethylisopropylamine (NEIPA)	
N-nitrosodiisopropylamine (NDIPA)	
N-nitrosodibutylamine (NDBA)	
4-(Methyl)(nitroso)amino) butanoic acid (NMBA)	
N-nitrosopiperidine (NPIP)	
N-nitrosomethylethylamine (NMEA)	
N-nitrosodiethanolamine (NDELA)	
N-nitrosomorpholine (NMOR)	
N-nitrosodipropylamine (NDPA)	
N-nitrosopyrrolidine (NPYR)	

Table 1: List of nitrosamines (not exhaustive)

- Selectivity in the matrix
- Recovery (accuracy) of the generic method using spiked samples at two concentration levels

The approach for this limit assay can either target one nitrosamine, or screen the full list. A default specification at 30ppb was set for all listed N-nitrosamines, based on the future limit established by the health authorities.

In case of a positive result for the screening of one or more nitrosamines, a specific quantitative method has to be developed and validated for a confirmatory testing. This confirmatory method can be either an update of the generic method used for the screening, or a method developed specifically to the intended use (sample preparation, analysis technique, and detection).

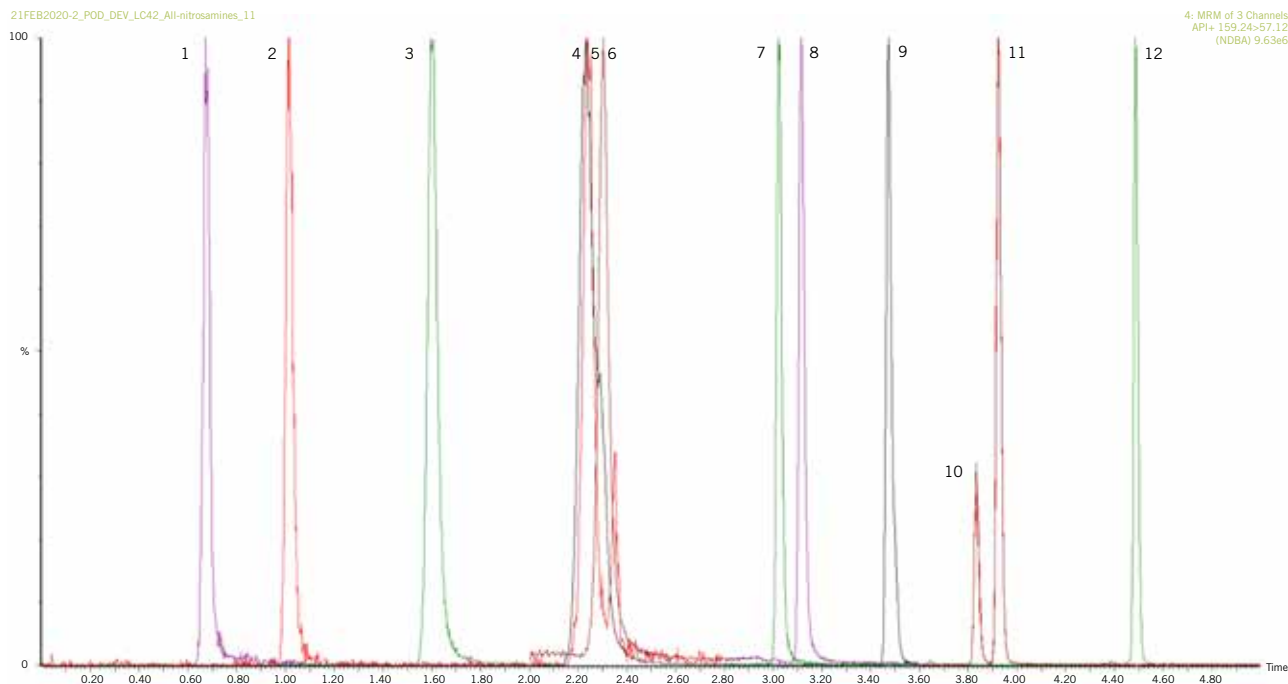Figure 1: Chromatogram obtained from a standard of 12 nitrosamines at a concentration equivalent to 30 ppb, with
(1) NDELA   (2) NDMA   (3) NMOR   (4) NMEA   (5) NMBA   (6) NPYR   (7) NDEA   (8) NPIP   (9) NEIPA   (10) NDPA   (11) NDIPA   (12) NDBA

LC-MSMS Method

One of the main advantages of LC techniques is that samples are analysed at room temperature. For this reason, LC should be preferred when issues related to thermal stability are identified. This is the case of NMBA, a nitrosamine firstly identified in losartan products; the high temperatures reached during sample injection caused thermal degradation of this nitrosamine, not allowing the correct quantification of the compound. Consequently, the existing GC methods are considered not suitable for the detection of this specific nitrosamine.

Similar issue has also been observed during ranitidine analysis. In this case, the GC high temperatures promote degradation of the ranitidine molecule itself. The breakage of the molecule is actually the main cause of NMDA formation during the product shelf-life. A subsequent *in situ* formation of NDMA was observed and results could not be considered reliable due to this artefact. Based on this experience, the FDA issued LC-MS based methods for the detection of nitrosamine in ranitidine drug substance and drug products.

Alternatively, sample preparation for high-performance liquid chromatography may be challenging due to the fact that matrix extraction with hydrophobic solvents, which usually have the best extraction yield with these kinds of compounds, has to be avoided. As a result, for complex matrices such as drug products, a liquid-solid phase extraction (SPE) step for sample preparation should be required.

In laboratories, LC-MSMS methods have been successfully developed for the detection of nitrosamine in sartans, while for ranitidine and metformin, the existing methods are all focused on NMDA.

The published methods are performed with both triple-quadrupole MS platform and high-resolution MS platform. The need for high resolution, accurate mass has been debated since the FDA first issued a method using this equipment.

High resolution could be especially useful in case of possible interferences. Residuals of DMF, for example, could eluate with NDMA as experienced in the lab during the method development and validation of a metformin drug product. Although NMDA and DMF have different weights, DMF ^{13}C and ^{15}N isotopes have a very similar molecular mass compared to NDMA (see **Figure 2**, **page 38**).

Considering a triple-quadrupole detector, the DMF-isotope ion was picked up in the MS Q1. This is due to the mass-accuracy of a triple-quadrupole that can set a mass window not able to exclude very similar molecular mass ions, such as DMF isotopes.

With a high-resolution mass detector, it is possible to narrow the mass window excluding ions with similar mass like DMF isotopes, avoiding the risk of false-positive results.
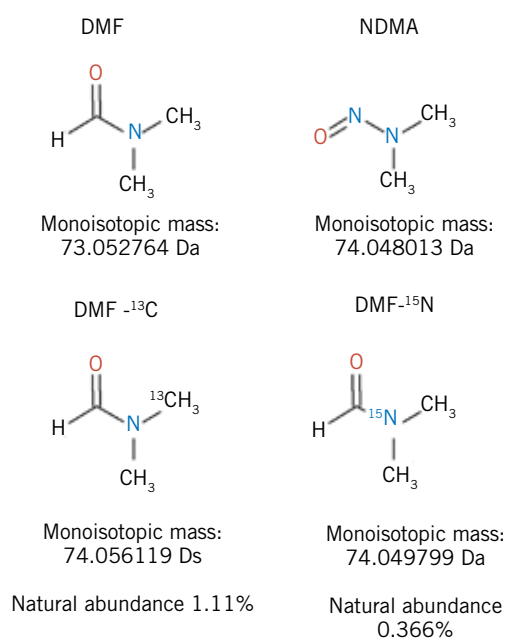
DMF

Monoisotopic mass:
73.052764 Da

NDMA

Monoisotopic mass:
74.048013 Da

DMF -^{13}C

Monoisotopic mass:
74.056119 Ds

Natural abundance 1.11%

DMF-^{15}N

Monoisotopic mass:
74.049799 Da

Natural abundance
0.366%

Figure 2: NMDA DMF, DMF 13C and 15N isotopes' molecular mass

The only way to overtake this problem without using high-resolution mass spectrometry would be to analyse DMF for specificity purpose during the method set up in order to exclude it could coelute with NDMA.

The EMA in its latest Q&A on 'Information on nitrosamines for marketing authorisation holders' highlighted the need of using accurate mass techniques (MS/MS or high-resolution accurate mass systems) in order to overcome interferences in the identification of the specific peak of a certain nitrosamine.

GC-MSMS Method

The main advantages of using GC-MSMS are good sensitivity and specificity, which enable detection of nitrosamines at very low concentrations. On the other hand, main disadvantages of this technique are the possible formation of nitrosamines *in situ* for thermosensitive compounds and matrix interference for compounds that are highly soluble in DCM.

In the lab, an analytical method has been set up, based on FDA published methods, and validated for simultaneous detection of NDMA, NDEA,N-nitrosodipropylamine (NDIPA), N-nitroso-isopropylethylamine (NIPEA), and N-nitrosodibutylamine (NDBA) in Valsartan (5-6). The first FDA methods were initially developed for HS-GC-MS with quantification and detection limits at relatively high concentrations. These methods were adapted to a direct injection method, and peak separation was improved using a DB-1701 column with adapted temperature gradient, which enables the exposure of the analytes at lower temperatures.

The sensibility of this method is very similar to the method for nitrosamine detection released by the FDA in April 2019 (7).

The method uses the internal standard NDMA-d6 for quantification, as foreseen also by the latest FDA method. During experimentation, it was observed that the addition of NDEA-d10 as second internal standard, increases the performance of the method for quantification of nitrosamines NDEA, NDIPA, NEIPA, and NDBA. Therefore, this second internal standard has also been included in our validated method.

The method was validated on Valsartan DS in order to confirm its specificity, linearity, precision (instrumental precision, method repeatability, and intermediate precision), accuracy, LOQ, and LOD. The acceptance criteria were based on ICH guidelines (8-9). The results of this validation method are summarised in **Table 2** (**page 40**).

The validated method demonstrated good specificity for five analysed nitrosamines, as the peaks were well separated and no significant interference from the blank was observed.

The linearity was established within LOQ and 0.096 ppm for NDMA, NDEA, NDIPA, and NEIPA, whereas for NDBA the concentration range was from LOQ to 0.192 ppm, which enables quantification from the lowest concentrations up to the concentrations above the specification limits.

The instrumental precision demonstrated low variations of relative area and relative retention time for all nitrosamines. Furthermore, the method and intermediate precision were within the established acceptance criteria. The accuracy was performed using spiked samples at three different concentration levels, ranging from LOQ to 0.05 ppm for NDMA, NDEA, NDIPA, and NEIPA, whereas, from LOQ to 0.10 ppm for NDBA. The obtained mean recoveries were within 60% and 140% for all nitrosamines.

The LOQ was tested at the concentrations established for DS by FDA (7). The determinations of signal to noise ratio were higher than 10 for all nitrosamines and the recovery of spiked samples at LOQ was within the acceptance criteria.

The LOD was proven at the concentrations 0.005 ppm for NDMA and NDBA, whereas at 0.0010 ppm for NDEA, NDIPA and NDBA. The applied adaptations of the initial FDA methods for determination of nitrosamines resulted in considerably improved sensitivity of the method (5-6).

The validated method has been largely used for routine testing of valsartan and other sartan drugs. Additionally, the same method has been successfully applied for screening of nitrosamines on more than 80 different DSs, which confirms the method robustness for various sample matrices.

Parameter	Criteria	NDMA	NDEA	NDIPA	NIPEA	NDBA
Specificity	No interferences from the blank should be observed in the obtained chromatograms	Meets requirements	Meets requirements	Meets requirements	Meets requirements	Meets requirements
Linearity	Regression co-efficient (r) NLT: 0.99 The % Y-intercept should be NMT ± 20%	LOQ – 0.096 ppm r=0.9995 %Y-intercept=2.49	LOQ – 0.098 ppm r=0.9994 %Y-intercept=-2.23	LOQ – 0.096 ppm r=0.9986 %Y-intercept=-3.08	LOQ – 0.096 ppm r=0.9993 %Y-intercept=-1.02	LOQ – 0.192 ppm R=0.9983 %Y-intercept=1.79
Instrumental precision	The relative standard deviation (% RSD) of the relative area must be NMT 15% for NDMA, and NMT, and 30% for the NDEA, NDIPA, NIPEA, and NDBA The relative retention time: NMT 5%	0.03 ppm RSD rel. area: 2.4% RSD RRT: 0.04%	0.03 ppm RSD rel. area: 2.1% RSD RRT: 0.00%	0.03 ppm RSD rel. area: 3.1% RSD RRT: 0.01%	0.03 ppm RSD rel. area: 3.6% RSD RRT: 0.01%	0.03 ppm RSD rel. area: 5.3% RSD RRT: 0.02%
Method precision	RSD must be NMT 30%	0.03 ppm RSD: 2.3%	0.03 ppm RSD: 5.8%	0.03 ppm RSD: 4.4%	0.03 ppm RSD: 2.7%	0.06 ppm RSD: 7.9%
Intermediate precision	The %RSD for 12 values must be NMT 40% for all nitrosamines	0.03 ppm RSD: 4.7%	0.03 ppm RSD: 8.9%	0.03 ppm RSD: 18.6%	0.03 ppm RSD: 10.7%	0.03 ppm 25.3%
Accuracy	The mean recovery for all nitrosamines must be 60-140%	LOQ – 0.05 ppm Mean recovery: 109.0%	LOQ – 0.05 ppm Mean recovery: 101.6%	LOQ – 0.05 ppm Mean recovery: 139.5%	LOQ – 0.05 ppm Mean recovery: 110.4%	LOQ – 0.10 ppm Mean recovery: 136.7%
LOQ	The %RSD of the areas in the standard LOQ must be NMT 30% The signal to noise ration (S/N) must be NLT 10 The mean recovery of the nitrosamines in the spiked samples at LOQ must be 60-140% for all nitrosamines and the %RSD of results must be NMT 30%	0.008 ppm RSD: 3.8% Mean recovery: 120.8%	0.005 ppm RSD: 7.5% Mean recovery: 107.1%	0.005 ppm RSD: 7.5% Mean recovery: 131.6%	0.005 ppm RSD: 5.1% Mean recovery: 102.8%	0.025 ppm RSD 15.2% Mean recovery: 103.2%
LOD	The S/N on the detection limit was evaluated according to ICHQ2B to confirm a value of NLT 3	0.005 ppm	0.001 ppm	0.001 ppm	0.001 ppm	0.005 ppm

Table 2: Results of method validation of nitrosamines in Valsartan

The validated method for simultaneous detection of NDMA, NDEA, NDIPA, NIPEA, and NDBA in valsartan by direct injection on GC-MS/MS, using DCM as solvent, was successfully applied in our lab for screening of nitrosamines for more than 80 DSs, which confirmed the robustness of the method for various sample matrices.

Conclusion

Appropriately sensitive screening or quantitative analytical methods for determination of the specific nitrosamines medicinal products could be developed and validated accordingly before testing either on GC or LC MSMS systems.

When developing analytical methods, the two following technical aspects may require particular attention:

1. Interferences caused by presence of trace amounts of nitrosamines in testing materials used (e.g., water, airborne sources, plastics products, rubber/elastomeric products)
2. *In situ* formation of nitrosamines

References
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9. Visit: www.eoma.aoac.org/app_f.pdf

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