Nitrosamines Analysis: Solutions for Risk Management and Analytical Testing from Experienced Industry Leaders

N-nitrosamines (referred throughout as "nitrosamines") are a class of highly potent genotoxins containing a nitroso functional group (NO^+) bound to an amine (see example in Figure 1), are listed as potential human carcinogens.[1,2] Sources of human exposure to nitrosamines varies widely, however the most common routes include food and beverage intake, air and water pollution, cosmetics, rubber/latex, and cigarettes.[3]

While lifestyle choices can have a significant impact on overall exposure, the FDA recommends a daily intake limit of no more than (NMT) 0.03 ppb for total nitrosamines, equivalent to 26.5 ng/day for a 100 mg daily dose, making control of these analytes a top priority for manufacturers of APIs and drug products.

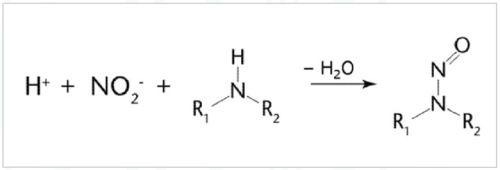


Figure 1. Representative chemical formation of nitrosamines from secondary amines.[4]

However, with a growing number of drugs recalled from 2018 onwards for the presence of nitrosamines such as *N*-nitrosodimethylamine (NDMA) in certain receptor antagonist/sartan drugs, ranitidine, and metformin drug products, the Food and Drug Administration (FDA) has continued to release updated Guidance for Industry regarding acceptable nitrosamine levels.[3, 4] While lifestyle choices can have a significant impact on overall exposure, the FDA recommends a daily intake limit of no more than (NMT) 0.03 ppb for total nitrosamines, equivalent to 26.5 ng/day for a 100 mg daily dose, making control of these analytes a top priority for manufacturers of APIs and drug products. For perspective, the FDA limit for ICH M7 Class 2 mutagenic compounds is several orders of magnitude higher at NMT 1.5 µg/day, corresponding to 15 ppm of a 100 mg daily dose. Other examples include the FDA limit for benzene at 2 ppm, and the WHO limit for p,p'-dichlorodiphenyltrichloroethane (DDT) in drinking water at 1 ppb (1 µg/L) versus the limit for NDMA, which is 10-fold lower (0.1 ppb / 0.1 µg/L).[5, 6]

About Cambrex

Cambrex is a leading global contract development and manufacturing organization (CDMO) that provides comprehensive analytical and IND enabling services, as well as drug substance development and manufacturing across the entire drug lifecycle.

With over 40 years of experience and a team of 2,000 experts servicing global clients from North America and Europe, Cambrex is a trusted partner in branded and generic markets for API development and manufacturing.

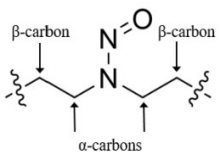
Control of nitrosamines is not limited to the API chemical process, as other sources of nitrosamines include contaminants in starting materials, aqueous solvent and common organic solvents (DMF, DMAc, and NMP), catalysts, organic bases (TEA, DIPEA / Hünig's Base), acids (nitrous acid anhydride) nitrite salts and esters, nitroso halides, nitrosonium salts, nitrogen oxides, nitro alkanes, nitroso sulfonamides, and also redox products of hydrazines, hydrazides, and hydrazones. Nitrosamines may also form on stability either as a nitrosatable degradant of the API or from the drug product formulation interacting with the packaging components (i.e. rubber, amine-containing inks / labels, nitrocellulose blister packs), forming nitrosamines as a result. Even select excipients commonly used in drug product formulations may contain nitrosamines or reactive nitrites at trace levels for additional sources to be controlled. Such examples include sodium starch glycolate, croscarmellose sodium, pre-gelatinized starch, polyvinylpyrrolidone (PVP) and lactose.[6]

Cambrex Approach – A Strategic Partnership for CMC Solutions to Your Product

Risk Assessment – Potential to form nitrosamines in the API and Drug Product manufacturing process

Often times, drug innovators and manufacturers are waiting until the final stages of their NDA filing to begin tackling the challenge of controlling nitrosamines in their finished product. When you partner with Cambrex we can perform the risk assessment for you if not already performed, to identify potential sources of nitrosamines in your chemical process, the storage conditions of the API and/or the container/closure configuration of the drug product. In recent updates from FDA and the European Medicines Agence (EMA) guidance on control of nitrosamines, a scoring system was provided for establishment of allowable intake (AI) levels using a structure-based Carcinogenic Potency Characterization Approach (CPCA) when carcinogenic limits are not already available for a specific compound (See Figures 2 and 3).[4,7,8]

1. Evaluate structure of nitrosamine to determine AI – CPCA



2. Rating nitrosamine on a scale of 1 to 5
   - 1 – most potent, lowest AI = 18 ng/day
   - 5 – Least potent AI = 1500 ng/day

3. Calculate **Potency Score:**

   = α-**Hydrogen Score**

   + **Deactivating Feature Score**
   (sum all scores for features present in the *N*-nitrosamine)

   + **Activating Feature Score**
   (sum all scores for present in the *N*-nitrosamine)

Deactivating Feature	Score
Carboxylic acid anywhere	+3
N-nitroso group in a pyrrolidine ring	+3
N-nitroso group in a 6-membered ring containing at least one sulfur atom	+3
N-nitroso group in a 5- or 6-membered ring*	+2
N-nitroso group in a morpholine ring	+1
N-nitroso group in a 7-membered ring	+1
Chain of ≥ 5 non-hydrogen atoms	+1
Electron withdrawing group on α-Carbon on one side or two sides	+1 / +2
Hydroxyl group on β-Carbon on one side or two sides	+1 / +2
Activating Feature	**Score**
Aryl group bonded to α-carbon (i.e., benzylic or pseudo-benzylic substituent on *N*-nitroso group)	-1
Methyl group bonded to β-carbon (cyclic or acyclic)	-1

Potency Score	5	4	3	2	1
Limit (ng/day)	1500	1500	400	100	18

Figure 2. Structure-based Carcinogenic Potency Characterization Approach (CPCA) to Evaluate Acceptable Intake (AI) Levels of Nitrosamines.[7, 8]

Our analytical services offer confirmatory testing to determine whether specific nitrosamine impurities are present in your materials and, if detected, we can investigate to control the root case. As part of the assessment, or following confirmatory testing, we may recommend additional studies (if not already performed) depending on the current stage of clinical development and may include extractables and leachables studies or fate and purge experiments if in a Phase III trial, preparing for an NDA filing, or for commercial registration. With these additional considerations in mind, there are significant potential savings in development costs when you partner with an experienced team of industry leaders that deliver efficient solutions to prevent delays and "ensure you achieve the regulatory milestones according to your desired timelines.

Common mitigation approaches include seeking alternatives to the reaction conditions that form nitrosamines (or complete avoidance if possible), which could be the order of addition, optimization of pH, temperature, and reaction times. We recommend development of a process that encourages purge of nitrosamine impurities and then establish controls for repackaged/recovered materials, reagents, starting materials, solvents, and excipients. As part of the mitigation strategy, the FDA has recommended the addition of antioxidants to the formulation, such as ascorbic acid and alpha-tocopherol, as a means to inhibit nitrosamine formation where applicable. Based on the information gathered for the risk assessment and the strategies selected for mitigation, it may also be necessary to add new or adjust existing specifications for the API and finished drug product.

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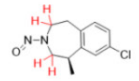
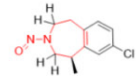
Calculate **Potency Score:**

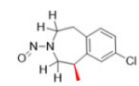
= α-**Hydrogen Score**

+ **Deactivating Feature Score**
(sum all scores for features present in the *N*-nitrosamine)

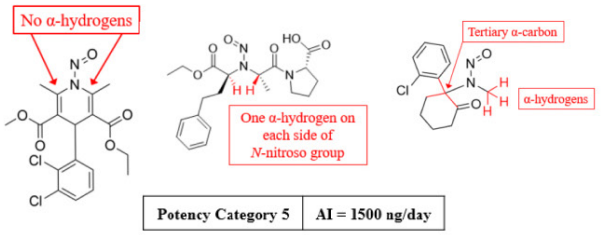
+ **Activating Feature Score**
(sum all scores for present in the *N*-nitrosamine)

Count of α-Hydrogens	Score	Feature Highlighted in Red
2,2	1	

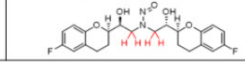
Deactivating Features	Score	Feature Highlighted in Red
N-nitroso group in a 7-membered ring	+1	

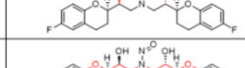
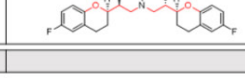
Activating Features	Score	Feature Highlighted in Red
Methyl group bonded to β-carbon (cyclic or acyclic)	-1	

Potency Score = 1 + 1 - 1 = 1	Potency Category 1	AI = 18 ng/day



Potency Category 5	AI = 1500 ng/day

Count of α-Hydrogens	Score	Feature Highlighted in Red
2,2	1	

Deactivating Features	Score	Feature Highlighted in Red
Hydroxyl group bonded to β-carbons*** on both sides of *N*-nitroso group (cyclic or acyclic)	+2	
Chains of ≥5 consecutive non-hydrogen atoms (cyclic or acyclic) on both side of acyclic *N*-nitroso group. Not more than 4 atoms in each chain may be in the same ring.	+1	
No Activating Features Present		

Potency Score = 1 + 2 + 1 = 4	Potency Category 4	AI = 1500 ng/day

Figure 3. Example Nitrosamine Scoring System provided in revised EMA and FDA guidance.[7, 8]

Available MS Detection Platforms	Nitrosamine Chemical Name	Acronym	Limit ng/day
• UPLC triple quadrupole (QQQ) MS/MS with ESI or APCI sources • UPLC Q-ToF (ESI) • HS/DI-GC with EI source and single quadrupole or QQQ MS/MS	*N*-nitroso-dimethylamine	NDMA	96.0
	N-nitroso-diethylamine	NDEA	26.5
	N-nitroso-ethylisopropylamine	EIPNA	26.5
	N-nitrosodiisopropylamine	DIPNA	26.5
	N-nitroso-n-methyl-4-aminobutyric acid	NMBA	96.0
	N-nitroso-di-n-butylamine	NDBA	26.5
	N-nitroso-methylphenylamine	NMPA	34.3
	N-nitroso-morpholine	NMOR	127
	N-nitroso-di-n-propylamine	NDPA	26.5
	N-nitroso-piperidine	NPip	1300
	N-nitroso-1,2,3,6-tetrahydropyridine	NTHP	37
	N-methyl-N-nitrosophenethylamine	NMPEA	8
	N-nitroso-pyrrolidine	NPYR	1700

Table 1: List of Most Common Nitrosamine Analytes Analyzed by Cambrex Analytical Services

Development and Validation of the Analytical Methodology

The low-level limits set by the FDA for individual and total nitrosamine analytes in the ppb range requires highly specialized analytical instrumentation and experienced industry professionals to develop and validate quality methods suitable to test reliably and consistently with high confidence in the results. Our teams of analytical scientists have extensive experience with trace impurity methods including nitrosamines, that require mass spectrometry (MS) technologies for quantitative analysis (Table 1). We have worked with several pharmaceutical companies to develop, validate, and batch test under cGMP for the most common nitrosamines listed in the FDA guidance, in addition to several more provided in the EMA guidance (included in Table 1) that have been identified in pharmaceutical products, including derivatives of the API or as leachables in the drug product container/closure system. We commonly receive requests for analysis and synthesis of custom nitrosamines derived from the API if not readily available from commercial sources. Our synthetic chemistry teams offer this service and work closely with our analytical teams to achieve the desired quantity and purity needed to qualify custom nitrosamines as reference standards for quantitative studies under cGMP.

Analytical Considerations

The ppb sensitivity requirement also necessitates high degrees of specificity/selectivity and therefore the MS detection platform must be maintained in optimal operating conditions free of contaminants that may impact the analysis. Additionally, methods will require specificity from potential interferents such as residual solvents, impurities, or matrix components present in the material. One common example of this is the ubiquitous organic solvent, dimethylformamide (DMF; molecular weight 73.09 g/mol), which has a similar m/z profile to NDMA (molecular weight 74.08 g/mol). If the analytical method utilized exhibits a co-elution of DMF and NDMA, the target nitrosamine, the MS detector must be capable of high-resolution mass accuracy to distinguish between the two species, otherwise the method may overestimate the abundance of NDMA inadvertently due to the interference of DMF.

Sample preparation also must be carefully controlled as it is often necessary to perform a liquid-liquid extraction and/or evaporative concentration step to enhance method sensitivity, however these steps have the potential to lose volatile nitrosamines in the process and contribute to false negative results. Cambrex offers solutions to this problem and recommends using headspace GC-MS/MS (triple quadrupole/QQQ) to increase sensitivity and avoid loss of the analyte. Another precaution to take is the artificial formation of nitrosamines not only during the sample preparation steps, but also during the analysis. For example, acidic work ups or high temperature incubations (i.e. GC analysis) may contribute to artifact nitrosamine formation if not properly controlled. Nitrosamines are also light-sensitive compounds and therefore should be protected from photolysis for risks of inaccurate quantitation from degraded sample solutions or the quantitative reference standards themselves.

The instrumental setup utilized depends on a variety of factors, primarily which nitrosamine analytes are pursued and the specific composition of the sample matrix and limits of detection and quantitation needed. Headspace and direct-inject GC-MS approaches may appear to offer advantages over LC-MS based on the ability to prepare samples sometimes at significantly higher concentrations than what is typically used for LC-MS ESI-based approaches as a means to enhance method sensitivity. However individual nitrosamines will exhibit variable response factors in GC-MS and in LC-MS approaches (LC-MS/MS example provided in Figure 4), making it often necessary to apply multiple methods to fully characterize the nitrosamines impurity profile at the desired quantitation limits for the sample test article. In practice, Cambrex analytical scientists have observed that APCI ionization sources may offer superior signal response over ESI for select low molecular weight nitrosamines using by LC-MS triple quadrupole MS/MS (QQQ), as shown in Figure 5 and Table 2.

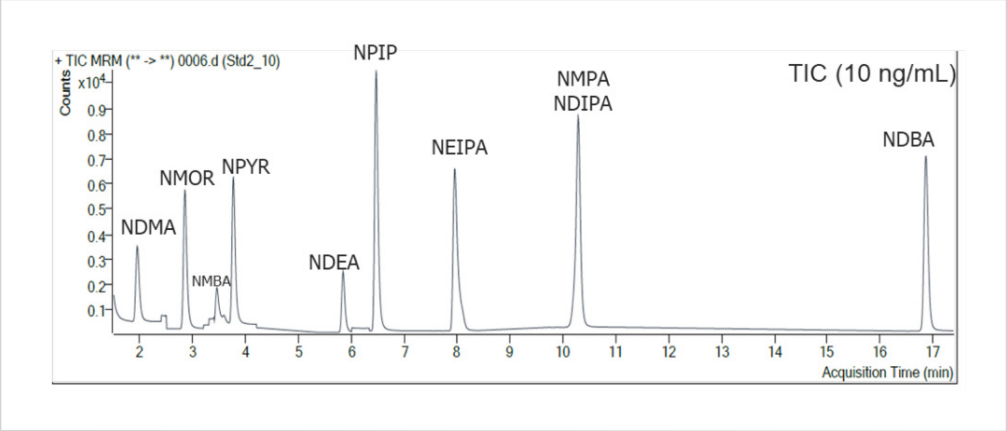


Figure 4. LC-MS/MS Total Ion Chromatogram from Multiple-Reaction Monitoring (MRM) for Selected Nitrosamines premixed at 10 ng/mL

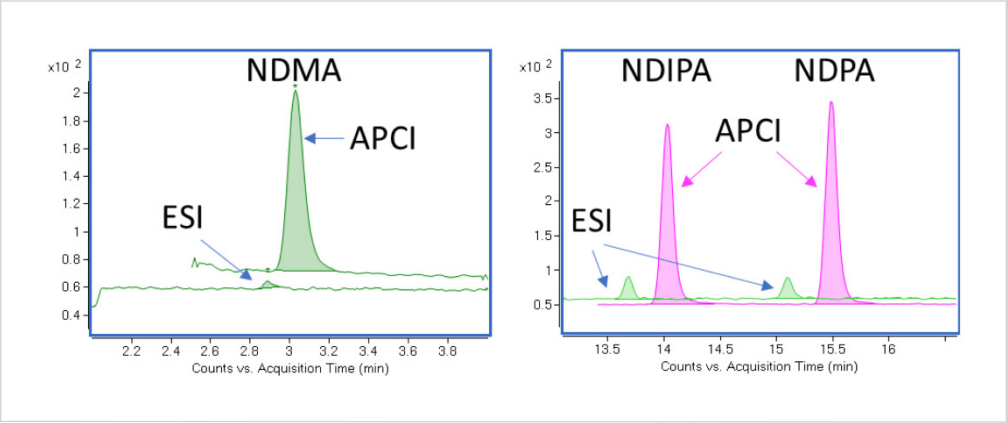


Figure 5. Comparison of LC-MS/MS Chromatograms for Selected Nitrosamines using APCI vs. ESI Sources

Nitrosamine	S/N at ~1 ng/mL	
	APCI	ESI
NDMA	59	2
NMEA	197	4
NMBA	325	1275
NIPEA	285	40
NDIPA	179	9
NMPA	58	50
NDPA	229	10
NDBA	369	320

Table 2: Comparison of Individual Nitrosamine Signal to Noise (S/N) Levels from LC-MS/MS using APCI vs. ESI Sources

It takes an experienced industry partner to develop and validate suitable methods specific for your API and drug product matrices.

We also will mention that high throughput, high resolution methods capable of separating the most common nitrosamines are widely available for nitrosamine reference standard resolution mixtures, it takes an experienced industry partner to develop and validate suitable methods specific for your API and drug product matrices. In addition, our customers are now asking us for quantitative methods to analyze trace levels of nitrite as a nitrosamine precursor, for which we offer analysis using Ion Chromatography (IC) approaches for this analyte as part of our Analytical Services.

When you partner with us, the Cambrex team provides our clients with access to state-of-the art technology, veteran industry experience, and creative approaches to problem solving while maintaining the highest degree of quality in the process to ensure your products meet your desired specifications and ultimately advance to your next regulatory and commercial milestones.

References

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7. EMA/409815/2020 Rev. 17: European Medicines Agency – Questions and answers for marketing authorization holders/applicants on the HMP Opinion for the Article 5(3) of Regulation (EC) No 726/2004 referral on nitrosamine impurities in human medicinal products
8. EMA/307633/2024 Rev .5 – Appendix 1 Acceptable Intakes established for N-nitrosamines

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