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REVIEW

The clinical and regulatory status of NDSRI: A global imperative

Ritu Tiwari[1*] **Gaurav Sanjay Mahalpure**[1] **Sakshi Mahalpure**[2] **Anuanshika Tiwari**[3]

[1] Indian Pharmacopoeia Commission, Ministry of Health & Family Welfare, Government of India, Ghaziabad, Uttar Pradesh, India
[2] Shri Vile Parle Kelavani Mandal's Institute of Pharmacy, Dhule, Maharashtra, India
[3] Shri Ramswroop Memorial University, Lucknow, Uttar Pradesh, India

Correspondence to: Ritu Tiwari, Indian Pharmacopoeia Commission, Ministry of Health & Family Welfare, Government of India, Ghaziabad, Uttar Pradesh, India; Email: ritutiwari.ipc@gov.in

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Abstract: Detecting N-nitrosamine impurities in medicines has been a significant challenge for drug manufacturers and regulators, especially with the recent emergence of nitrosamine drug substance-related impurities (NDSRIs). The formation of NDSRIs is complex and primarily associated with reactions in the drug product. This paper explores the current technical knowledge on forming these impurities, including the risk factors, reaction conditions, and possible mitigation strategies. While significant scientific progress has been made in these areas, substantial gaps in mechanistic knowledge still make accurate predictions of NDSRI formation very difficult. The pharmaceutical industry's continued work on potential mitigation strategies and the generation of additional scientific data to address these knowledge gaps are crucial. Regulatory guidance and policy will continue to evolve in response to further changes in scientific understanding. In this article, we will delve into the detection methods, the mechanism of action, sample preparation techniques, and regulatory limits for nitrosamine impurities. We also discuss various reported nitrosamine impurities, their chemical structures, and their detection using methods like LC-MS/MS, GC-MS-HS, and HPLC. Additionally, we discuss different sample preparation techniques, such as solid-phase extraction, liquid-liquid extraction, and rapid-fire techniques. This review is intended to provide detailed information to analytical personnel working in various quality control laboratories and research organizations.

Keywords: nitrosamine impurities, NDSRI, regulatory status, NDMA, AI limit

1 Introduction

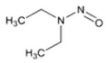
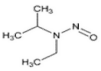
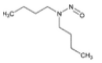
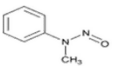
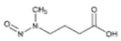
N-nitrosamines are a group of compounds known for their potent carcinogenic effects, and they are widely present in our environment, including air, water, food, and drugs. Scientists have dedicated significant effort towards understanding these compounds as contaminants and developing strategies for their prevention, detection, and remediation. To achieve this, it is essential to comprehend N-nitrosamines' chemistry, especially for alkyl N-nitrosamines. With this in mind, our Perspective focuses on the structure, reactivity, and synthetic applications of N-nitrosamines. Recently, nitrosamine impurities have been discovered in various pharmaceutical products, resulting in the recall of several drugs like sartans, ranitidine, nizatidine, and metformin from the markets. Nitrosamine impurities are a cause for concern, and this review aims to provide a brief yet comprehensive overview of these impurities. We also discuss N-nitrosamines' role as water contaminants and the techniques used for their detection. Nitrosamine impurities have been found in various drug substances and products. In 2018, two nitrosamines, N-nitroso dimethylamine (NDMA) and N-nitroso diethylamine (NDEA), were found in valsartan drug substances and products manufactured with specific synthetic routes. This discovery led to the assessment of synthetic routes and the development of analytical procedures to measure these nitrosamines. As more pharmaceuticals were tested, other nitrosamines beyond NDMA and NDEA were identified as impurities of concern. Due to the potential harm caused by these carcinogenic chemicals, a science- and risk-based approach has been developed in this chapter to control nitrosamine impurities. This approach ensures that the presence of nitrosamines in drug substances and products is identified, assessed, and controlled. Recommendations are provided for:

a) Controlling nitrosamine levels to eliminate or reduce their presence, and
b) Establishing performance characteristics for analytical procedures used to monitor nitrosamine levels.

1.1 Nitrosamine impurities

Nitrosamines are known as high-potency mutagenic carcinogens in several animal species. Table 1 enlists the common and chemical names of nitrosamines. This list is a compilation of information shared by multiple global health authorities. As new nitrosamines are identified as potential concerns, the principles described herein should be applied for their assessment. If a manufacturer discovers a nitrosamine not listed in Table 1, the appropriate regulatory authority should be contacted to determine appropriate AI limits.

Table 1 Nitrosamines found as contaminants in drug substances and drug products

Common Name and Chemical Name	Acronym	CAS #	Structure	Chemical Formula	Molecular Weight (g/mol)
Nitrosodimethylamine *N*-Methyl-*N*-nitrosomethanamine	NDMA	62-75-9		$C_2H_6N_2O$	74.08
Nitrosodiethylamine *N*-Ethyl-*N*-nitrosoe- thanamine	NDEA	55-18-5		$C_4H_{10}N_2O$	102.14
Nitrosodiisopropylamine *N*-Isopropyl-*N*-nitrosoi- sopropylamine	NDIPA	601-77-4		$C_6H_{14}N_2O$	130.19
Nitrosoethylisopropyla- mine *N*-Ethyl-*N*-nitroso- 2-propanamine	NEIPA	16339-04-1		$C_5H_{12}N_2O$	116.16
Nitrosodibutylamine *N*-Butyl-*N*-nitroso-1-bu- tanamine	NDBA	924-16-3		$C_8H_{18}N_2O$	158.25
Nitrosomethylphenyla- mine *N*-Methyl-*N*-nitroso- phenylamine	NMPA	14-00-6		$C_7H_8N_2O$	136.15
Nitrosomethylaminobu- tyric acid 4-[Methyl(nitroso)ami- no] butanoic acid	NMBA	61445-55-4		$C_5H_{10}N_2O_3$	146.15
Nitrosomorpholine N-nitrosomorpholine [4]	NMor	59-89-2		$C_4H_8N_2O_2$	116.12
1-Nitrosopyrrolidine N-nitrosopyrrolidine	NPyr	930-55-2		$C_4H_8N_2O$	100.12
N-nitrosodiphenylamine N-nitroso-N-phenyl- Nitrosodiphenylamine	NDPhA	86-30-6		$C_{12}H_{10}N_2O$	198.22
1-nitrosopiperidine N-nitrosopiperidine	NPip	100-75-4		$C_5H_{10}N_2O$	114.15
N-nitrosomethylethylamine	NMEA	10595-95-6		$C_3H_8N_2O$	88.11

The presence of these impurities depends on the reaction chemistries and processes used. The list of nitrosamines is not intended to be exhaustive, but it represents those that regulators and manufacturers have observed as potentially present or observed. Some N-nitroso compounds are classified as probable or possible human carcinogens referred to as the “cohort of concern” in ICH M7: Assessment and Control of DNA Reactive (Mutagenic) Impurities in Pharmaceuticals to Limit Potential Carcinogenic Risk [1]. This designation carries with it a recommendation to control the impurities at or below the acceptable cancer risk. Due to the potential toxicity associated with these impurities, it is recommended that steps be taken to control and limit their presence in pharmaceutical materials.

1.2 Formation of nitrosamines

Nitrosamines are a group of compounds with a nitroso group bonded to an amine (R1N(-R2)—N=0) in their chemical structure. These compounds are formed through a nitrosating

reaction that occurs when secondary, tertiary, or quaternary amines react with nitrous acid (nitrite salts under acidic conditions). (Figure 1)

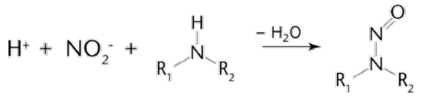


Figure 1 Nitrosamine Formation Reaction

1.3 Sources of nitrosamine

Nitrosamines can find their way into pharmaceutical drug products through several pathways. Specifically, nitrosamines are formed when nitrites react with secondary or tertiary amines under acidic conditions (1.2 Nitrosamine Formation Reaction). There are several ways nitrosamines can be generated or introduced into drug products, including but not limited to the following sources or pathways identified in empirical studies and literature [2, 3].

During the processing of drug substances, specific reagents, solvents, raw materials, and processing aids may be used under particular conditions. Despite undergoing purification and processing steps, evidence points to the presence of reactive species in subsequent stages. These species can either form during the reaction or be intentionally added. For instance, nitrites and secondary amines may form under acidic conditions and carry over to the next steps. Particular attention should be given to the formation of nitrogen-containing heterocycles. This can be achieved by using azide and quenching it with nitrous acid to remove excess azide.

The drug substance itself may degrade under some conditions, resulting in the formation of nitrosamines (e.g., ranitidine). Degradation of solvents (e.g., dimethylformamide [DMF]) leads to the formation of dialkyl amines. Impurities in raw materials, solvents (including recycled solvents), reagents, or catalysts. Impurities in materials and intermediates, reagents, and solvents are used to prepare the starting materials or intermediates. Impurities in water, excipients, or processing aids are used to produce the finished drug product. During drug product manufacture, it is under certain reaction conditions and in the presence of requisite precursors necessary for forming nitrosamines.

It is essential to consider impurities in the container-closure system of the finished drug product. These impurities may contain nitrosamines, which can form when materials containing amines come into contact with nitrosating agents, such as nitrite and nitrocellulose. A thorough risk assessment should be conducted to identify the materials contributing to the potential for nitrosamine inclusion in the drug product. This assessment should include all potential sources, such as the drug substance, excipients, water, solvents, manufacturing process, packaging components, and stability. Please refer to Figure 2 for a diagram of some potential sources.

Figure 2 Potential sources of nitrosamine impurities in drug products. [3]

Some examples of the reported sources or pathways leading to the generation of nitrosamines identified empirically or reported include (but are not limited to) the following: [4, 5]

+NO	Nitrosonium Ion	ONO-NO (N2O3)	Nitrous Anhydride
Phenol-NO	Nitrosyl Phenol	CNS-NO	Nitrosyl Thiocyanate
Halide-NO	Nitrosyl Halide	O2.NO-NO (N2O4)	Dinitrogen Tetraoxide
HNO2	Nitrous Acid	Y.NO(SO4-ClO4-)	Nitrosyl Sulfate/Perchlorate

1.3.1 Sources of secondary, tertiary, quaternary amines and nitrite salts that can form nitrosamines

It's possible that the API (or its degradants), intermediates, or starting materials can have secondary or tertiary amine functional groups. Also, tertiary and quaternary amines used to synthesise APIs may contain other amine impurities. For example, tertiary amines like triethylamine contain low levels of other secondary amines (dipropylamine and isopropyl ethylamine). Secondary and tertiary amines may be present as impurities or degradants generated by dealkylation of quaternary amines.

The drug substance itself can degrade in certain conditions, leading to the formation of nitrosamines (for instance, ranitidine).

Impurities in the finished drug product's container-closure system may also exist. These impurities may form nitrosamines, mainly related to materials containing amines and potential sources of a nitrosating agent (such as nitrite or nitrocellulose).

1.3.2 Contamination in vendor-sourced raw materials

Nitrosamine impurities can be formed in contaminated vendor-sourced materials such as starting and raw materials. Nitrosamine contamination occurs when solvents such as o-xylene, toluene, and methylene chloride are contaminated during shipment from vendors, such as transfer between storage vessels.

Starting materials may cross-contaminate if manufactured at sites where nitrosamine impurities are produced in other processes. Sodium nitrite in starting materials reacts with amines under acidic conditions to form nitrosamines. To prevent the formation of nitrogen-containing heterocycles, special attention should be given to employing azide followed by quenching with nitrous acid to remove excess azide.

Some raw materials and fresh solvents like toluene have reported impurities such as secondary or tertiary amines.

1.3.3 Recovered solvents, catalysts, and reagents as sources of contamination

It is important to note that residual amines, such as trimethylamine or diisopropylethylamine, present in recovered solvents, catalysts, and reagents, can pose a risk of nitrosamine impurities.

Raw materials can also be contaminated if proper equipment cleaning between customers or different materials is not carried out or validated to remove each impurity of concern.

1.3.4 Lack of process optimization and control

Nitrosamine impurities can form when the manufacturing process for APIs is not optimised. This can happen when reaction conditions like temperature, pH, or the sequence of adding reagents, intermediates, or solvents are not appropriately controlled. To avoid this, a risk assessment should be conducted to determine the materials that contribute to the potential for inclusion of nitrosamines in drug products.

The risk assessment should consider all possible sources for introducing nitrosamines, such as the drug substance, excipients, water, solvents, and the manufacturing process. It should also take into account packaging components and formation on stability.

2 Results

2.1 Recommended AI limit for nitrosamine drug substance-related impurities (NDSRIs)

On August 4th, 2023, the FDA issued its final guidance on Recommended Acceptable Intake Limits for Nitrosamine Drug Substance-Related Impurities (NDSRIs) in the NDSRI Guidance document. Due to the constantly changing and highly technical nature of the information, the FDA will provide updated NDSRI-specific details related to the guidance on this website.

The updated information on this website will include:
(1) Recommended acceptable intake limits for certain NDSRIs based on their predicted carcinogenic potency categorisation listed by APIs that could hypothetically be at risk of forming such NDSRIs.
(2) Recommended acceptable intake limits for certain NDSRIs based on compound-specific data or read-across analysis from a surrogate.

(3) Recommended interim acceptable intake limits for certain NDSRIs.
(4) Recommended testing methods for confirmatory testing of certain NDSRIs.
(5) Recommended safety testing methods for NDSRIs.

Based on predicted carcinogenic potency categorisation, the API's hypothetical risk of forming such NDSRIs was evaluated. It's important to note that APIs containing secondary amine or dimethyl tertiary amine centers may form NDSRIs through nitrosation of the amine center. This can happen due to formulation and manufacturing process conditions, such as reaction with residual nitrites in the excipients used for drug product formulation. Table 1 presents a list of hypothetical NDSRIs from various amine-containing APIs that can form through this process. Additionally, recommended AI limits are offered for these NDSRIs based on the predicted carcinogenic potency categorisation approach. If any of the NDSRIs listed in Table 1 are formed, then the corresponding recommended AI limit in Table 1 would apply based on the predicted carcinogenic potency categorisation approach. If compound-specific data or read-across analysis from a surrogate becomes available and an updated recommended AI limit applies, the FDA may move an NDSRI to Table 2. It's important to note that the recommended AI limits based on the predicted carcinogenic potency characterisation approach should not be applied to NDSRIs in circumstances where the FDA otherwise recommends an AI limit (e.g., based on compound-specific assessments or read-across analysis from a surrogate) in Table 4.

Table 2 Some of the identified risks associated with several of the potential sources of nitrosamines are as follows [3]

Potential Source of Nitrosamines	Observed Risk
Solvents	• several factors can contribute to the degradation of solvents. These include the presence of residual dialkyl amines or tri-substituted amines that can break down to form intermediates, which can further react with nitrosating agents. Additionally, the presence of nitrites or other nitrosating agents, as well as acid, can also contribute to the degradation of solvents. Moreover, the lack of proper controls or specification limits for recycled solvents and the poor quality of solvents can also lead to the degradation of solvents.
Water	• Residual dialkyl amines or impurities may degrade into dialkyl amines. Acid and nitrosating agents may also be present.
Excipients	• Presence of nitrites, other nitrosating agents and nitrosamine impurities (if applicable).
Drug substance	The following are the methods that can lead to nitrosation reactions: • The use of sodium azide in the synthesis, followed by nitrites in an acidic medium (nitrous acid) to quench excess azides. • The use of di- or tri-alkylamines and amides (such as dimethylformamide [DMF], dimethylamine [DMA], triethylamine [TEA], N-methylpyrrolidone [NMP]) in the presence of nitrites and acidic media. • The use of recycled solvents that may contain nitrosamines or their precursors. • The use of sanitized water (such as chloramines). • Insufficient purification. • Degradation of drug substances containing functional groups that can then participate in nitrosation reactions.
Manufacturing process	• Contamination can occur using low-quality or recycled solvents that may contain nitrosamines or their precursors. Another possible cause is the presence of nitrous oxides in the air used to dry the drug substance or drug product. Additionally, relevant reactive species may carry over into subsequent steps, leading to contamination.
Drug product (including stability)	The following factors are essential to consider when evaluating a drug substance: • Whether the molecule of the drug substance has a secondary, tertiary, or quaternary amine group. • Whether nitrate counter ions are present, which may contain nitrite impurities. • Whether any potential reactions, such as acidic conditions, moisture, or heat, could occur within the formulation matrix over time.
Container–Closures	• Packaging materials may contain amines that react with nitrosating agents in the packaging, such as amines in inks reacting with nitrocellulose print base.

Table 3 provides the predicted carcinogenic potency category for each unique NDSRI formed by mono-nitrosation of APIs with multiple nitrosatable amine centers. The NDSRI Name column differentiates each NDSRI from the same API using suffixes like "-1," "-2," and so

on. You can access chemical structure images, * for NDSRIs in Table 1 by clicking on their hyperlinked names. Please note that this table will be periodically updated based on new information.

Table 3 FDA recommended AI limits for certain NDSRIs based on compound-Specific data or read-across analysis from a surrogate

NDSRI Name	Source	Recommended AI Limit (ng/day)	Surrogate	Date
7-Nitroso-3-(trifluoromethyl)-5,6,7,8-tetrahydro[1,2,4]triazolo-[4,3-a]pyrazine (NTTP)	Sitagliptin	37	N-nitroso-1,2,3,6-tetrahydropyridine (NTHP)	8/4/2023*
N-nitroso-varenicline	Varenicline	37	NTHP	8/4/2023**
N-nitroso-ciprofloxacin	Ciprofloxacin	Pending	Pending	8/4/2023
N-nitroso-duloxetine	Duloxetine	100	4-(methylnitrosoamino)-1-(3-pyridinyl)-1-butanone (NNK)	8/4/2023
N-nitroso-fluoxetine	Fluoxetine	100	4-(methylnitrosoamino)-1-(3-pyridinyl)-1-butanone (NNK)	10/11/23

Note: * This limit was previously communicated on August 9, 2022; ** This limit was previously communicated on July 16, 2021.

Please note that the APIs listed on this website are not the only ones that can potentially form NDSRIs. There are other possible ways in which NDSRIs can be formed. It is important to understand that the recommended AI limits for NDSRIs on this website may not correspond to the observed level of NDSRI(s) present in a specific drug product. Additionally, the inclusion of an API in the list does not necessarily confirm the presence of an NDSRI in a drug product containing that particular drug substance. The recommended AI limit is based on a safety assessment that evaluates the mutagenic and carcinogenic potential of the impurity. The recommended AI limit represents the level at or below which the FDA has determined that the impurity or impurities would not pose a safety concern for patients taking the drug product.

2.1.1 Based on compound-specific data or read-across analysis from a surrogate

According to the NDSRI guidance, the recommended limit for an impurity in a drug is determined by assessing its safety based on its mutagenic and carcinogenic potential. This limit is set at a level where the impurity would not pose any patient safety risks. To calculate a specific limit for a compound, its rodent carcinogenic potency data, such as TD50 values, is evaluated from published scientific literature. Suppose the mutagenic potential of an NDSRI is not well-characterized. In that case, the FDA uses a similar surrogate compound that has been thoroughly tested to estimate the carcinogenic potency and determine an AI limit. The surrogate's structure and reactivity must be identical to the NDSRI to ensure reliable results. This estimate is then used for compounds that lack robust mutagenicity and carcinogenicity data, commonly known as a read-across analysis.

Table 4 provides the FDA's recommended AI Limits for specific NDSRIs based on compound-specific data or read-across analysis from a surrogate. If a particular NDSRI is listed in Table 2, the recommended AI limit should be used instead of a limit based on the predicted carcinogenic potency categorization approach.

2.1.2 Recommended interim AI limits for certain NDSRIs

According to the NDSRI Guidance, if drug product batches that are already in distribution contain NDSRIs at levels higher than the FDA's recommended AI limit, and if making changes in the manufacturing process or recalling the batches is likely to disrupt the drug supply, then manufacturers and applicants should immediately get in touch with CDER's Drug Shortage Staff at drugshortages@fda.hhs.gov. Upon being contacted about a potential disruption in the drug supply, the FDA will evaluate each circumstance on a case-by-case basis. If necessary, the FDA may work directly with a specific manufacturer or applicant of the marketed drug and consider whether it is appropriate to temporarily recommend an interim AI limit. Suppose the FDA recommends an interim AI limit. In that case, it generally won't object to the distribution of drug product batches that contain NDSRI levels at or below the recommended interim AI limit during the specified period, provided there are no underlying CGMP violations. FDA also intends to post these interim AI limits on its website in some instances where it doesn't intend

Table 4 The Five Predicted Potency Categories and Associated AI Limits for *N*-Nitrosamines

Potency Category	Recommended AI Limit (ng/day)	Comments
1	18	The recommended limit for AI intake is 18 ng daily, equivalent to the class-specific TTC for N-nitrosamine impurities. N-nitrosamines classified as Category 1 are predicted to have a high carcinogenic potency; however, the class-specific TTC for N-nitrosamine impurities is considered to be sufficiently protective for patients.
2	100	The recommended limit for daily AI intake of 100 ng is based on two thoroughly tested and potent N-nitrosamines, namely N-nitrosodimethylamine (NDMA) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-(butanone) (NNK), each having recommended AI limits of 96 ng/day and 100 ng/day, respectively. Carcinogenic potency of N-nitrosamines classified as Category 2 is expected to be no higher than that of NDMA and NNK.
3	400	Compared to Potency Category 2, N-nitrosamines in Potency Category 3 have a lower carcinogenic potency due to the presence of a weakly deactivating structural feature, for example. The recommended acceptable intake limit was set to reflect a 4-fold decrease in carcinogenic potency from Category 2.
4	1500	N-Nitrosamines that are classified under Category 4 can undergo metabolic activation through the α-hydroxylation pathway. However, they are believed to have low carcinogenic potential due to factors such as steric hindrance or electronic influences that disfavor this pathway, or because clearance pathways are favored. The recommended Acceptable Intake (AI) limit of 1500 ng/day is set based on the Threshold of Toxicological Concern (TTC) per the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) M7 guidelines.**
5	1500	N-Nitrosamines that fall under Category 5 are not expected to undergo metabolic activation through an α-hydroxylation pathway as a result of steric hindrance or the absence of α-hydrogens. Alternatively, they may create unstable species that do not react with DNA. The acceptable daily intake (ADI) limit of 1500 ng/day is established based on the threshold of toxicological concern (TTC) as defined by the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) M7 *guideline.***

Note: * Assessment report Procedure under Article 5(3) of Regulation EC (No) 726/2004 Nitrosamine impurities in human medicinal products Procedure number: EMEA/H/A-5(3)/1490; ** See the International Council for Harmonisation guidance for industry *M7 Assessment and Control of DNA Reactive (Mutagenic) Impurities in Pharmaceuticals To Limit Potential Carcinogenic Risk*. The threshold of Toxicological Concern (TTC) of 1.5 μg/day (1500 ng/day), as explained in ICH M7, represents an AI for any unstudied chemical with a negligible risk of carcinogenicity or other toxic effect. [2]

to object to the distribution of drug products from multiple drug manufacturers that contain NDSRI levels at or below the recommended interim AI limit.

2.1.3 Recommended testing methods for confirmatory testing of certain NDSRIs

As per the NDSRI Guidance, if manufacturers and applicants identify a risk of NDSRI formation in a drug product, they must conduct confirmatory testing of batches using sensitive and appropriately validated methods. The FDA plans to provide information on FDA-generated testing methods to enable regulators and industry to detect NDSRI impurities in specific drug substances and products. These methods must be validated by the user before the resulting data is used to support a required quality assessment of the drug substance or drug product or in a regulatory submission.

2.1.4 Recommended safety testing methods for NDSRIs

Per the NDSRI Guidance, a manufacturer or applicant can present a scientifically sound explanation to justify an AI limit higher than the FDA's recommended limit for the predicted carcinogenic potency category for that NDSRI. Alternative methods, such as obtaining compound-specific data or using read-across assessment to a suitable surrogate, can back up a higher limit.

(1) If in vitro mutagenicity testing is being considered, the FDA advises that an enhanced Ames assay be used to determine if an NDSRI poses a mutagenic risk. A negative result in a valid enhanced Ames assay may be used to justify a higher limit for an NDSRI. However, the FDA may require additional safety data beyond the enhanced Ames assay to support alternative AI limits. These recommendations reflect the FDA's current thinking. Future research will need to address data gaps in the available information to support the safety of NDSRIs.

(2) The OECD's Test Guideline No. 471, "Bacterial Reverse Mutation Test", offers standard recommendations for conducting the bacterial reverse mutation test (also known as the Ames assay) to assess the mutagenic potential of a test compound. The FDA recommends enhanced testing conditions for the Ames assay for N-nitrosamines due to the reported reduced sensitivity under standard conditions for some N-nitrosamines, such as N-nitroso-dimethylamine (NDMA).

(3) FDA recommends using an enhanced Ames assay for NDSRIs because very little is known about the sensitivity of the Ames assay to NDSRIs. Moreover, NDSRIs generally have a wider variety of functional groups present than typically found in low molecular weight N-nitrosamines (such as NDMA) historically studied, and the additional testing conditions

described in the enhanced Ames assay have been shown to help assess mutagenic risk for NDSRIs.

(4) If a standard Ames assay produces a positive result on an NDSRI, the FDA recommends that there is no need to conduct an additional assay using enhanced testing conditions.

(5) The enhanced Ames assay test conditions recommended by the FDA for NDSRIs have been formulated based on the work done by the FDA's National Center for Toxicological Research (NCTR) (Li X et al., 2023). These conditions have been evaluated for a variety of N-nitrosamines, including NDSRIs. Work on assessing Ames assay test conditions for N-nitrosamines is ongoing to identify the most robust Ames testing conditions. The enhanced Ames assay test conditions for NDSRIs described below will be updated as necessary. Deviations from the recommended conditions should be scientifically justified.

(6) Tester strains: *S. typhimurium* TA98, TA100, TA1535, TA1537, and E. coli WP2 uvrA (pKM101) tester strains should be included.

(7) Type of assay and preincubation time: The recommended method is pre-incubation for 30 minutes, not plate incorporation.

(8) Species and concentration of S9The FDA recommends conducting Ames assays without the presence of a post-mitochondrial fraction (S9), and with the presence of 30% rat liver S9 and 30% hamster liver S9. Additionally, the rat and hamster post-mitochondrial fractions (S9s) should be prepared from rodents treated with inducers of cytochrome P450 enzymes, such as a combination of phenobarbital and β-naphthoflavone.

(9) Negative (solvent/vehicle) control: The FDA recommends that solvents be compatible with the Ames assay per the OECD 471 guideline. Solvents may include, but are not limited to water and organic solvents such as acetone, methanol and DMSO.

(10) When utilizing an organic solvent, it is important to use the smallest volume possible in order to prevent any interference with the metabolic activation of the N-nitrosamine or NDSR. Positive controls: Concurrent strain-specific positive controls should be included per the OECD 471 guideline.

Including two N-nitrosamines as positive controls in the presence of S9 is important, one of which is NDSRI. These nitrosamines are known to be mutagenic, and their selection should be based on the expected metabolism and the cytochrome P450 enzymes involved. Moreover, suppose an organic solvent is used to dissolve the test compound. In that case, the volume of organic solvent employed to dissolve the N-nitrosamine positive controls should be such that the concentration is similar to that of the test compound in the pre-incubation mix, if possible. *N*-Nitrosamine positive controls to consider include: 1) NDMA (CAS # 62-75-9); 2) 1-Cyclopentyl-4-nitrosopiperazine (CAS # 61379-66-6); 3) An NDSRI. All other recommendations for the Ames assay should follow the OECD 471 guideline. [1]

Figure 3 contains a decision tree advising Market Authorization Holders about regulatory filing requirements related to nitrosamines. This Appendix also describes some changes that may impact the possibility of nitrosamine formation when a product is modified.

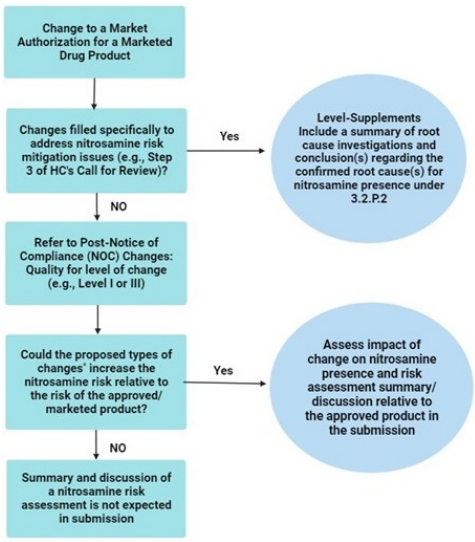


Figure 3 Guidance concerning nitrosamine impurities and risk assessments for Post NOC Changes of new drug products containing chemically synthesised and semi-synthetic APIs (updated) [2]

Figure 4 shows a step-by-step process for assigning a potency category to an N-nitrosamine. Each step has two options, and the steps are followed until a potency category of 1, 2, 3, 4, or 5 is reached. The potency category determines the acceptable intake limit for controlling the N-nitrosamine impurity.

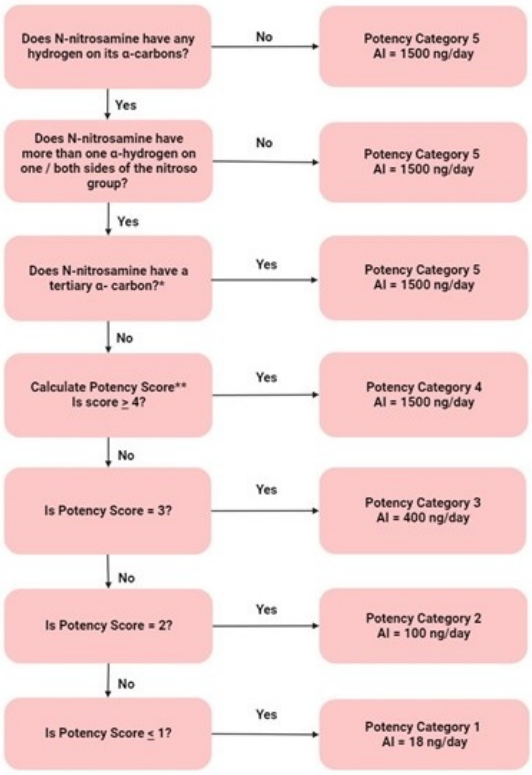


Figure 4 Flowchart to Predict the Potency Category of an *N*-nitrosamine

*A tertiary α-Carbon is an α-carbon atom in an sp^3 hybridisation state, bonded to three other carbon atoms; **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 features present in the *N*-nitrosamine)

2.2 Nitrosamine risk assessments—Development of a control strategy

The manufacturer must control nitrosamines' potential formation or contamination to ensure that the level of nitrosamine impurities in drug products is within acceptable limits. For this purpose, the ICH guidance for industry Q9 Quality Risk Management can be referred to. In addition, the risk assessment should also consider various sources with a high potential for nitrosamines. These include the drug substance's synthetic route, manufacturing process, manufacturing route, excipients, and raw materials.

If the risk assessment predicts or confirms the presence of nitrosamines through testing the drug substance, drug product, or other materials, a control strategy should be defined to ensure that the nitrosamine levels comply with the established acceptable intake levels (Als). This control approach should be in line with the current regulatory requirements. Please refer to Figure 5 for more information.

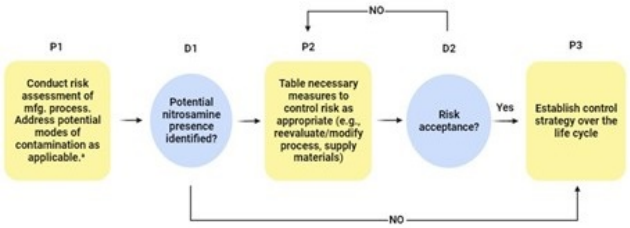


Figure 5 High-level process for developing a nitrosamine impurity control strategy ([a] Refer to Table 2; P1, P2, P3 = Process1, 2, 3; D1, D2 = Decision 1, 2)

2.3 Limits of nitrosamines

The Acceptable Intake (AI) limit is the maximum amount of a compound consumed daily without posing a significant health risk. For compounds like N-DMA, N-DEA, NMBA, NMPA, NIPEA, and NDIPA, the AI limit is set to result in a 1 in 100,000 cancer risk after 70 years of exposure. The term AI is used in ICH M7 (R1) to indicate the threshold of toxicological concern (TTC) considered for the impurity to be associated with negligible risk of carcinogenicity or other toxic effects. However, this limit does not apply to nitrosamines because they are known to be highly potent carcinogens. For nitrosamines, the available safety data should be used to establish a material-specific intake on a case-by-case basis.

Toxicologists use different methodologies to establish AI limits. In the case of NDMA, NDEA, and other nitrosamines, the median tumorigenic dose (TD50) is used as representative data in a linear extrapolation to establish an acceptable risk level. The AI can be calculated based on rodent carcinogenicity potency data such as TDc0 values (doses giving a 50% tumour incidence equivalent to a cancer risk probability level of 1 in 2) identified in the public literature. (Table 5)

Table 5 AI Limits for guidance of industry

Nitrosamine	Al Limit (ng per day)
NDMA	96
NDEA	26.5
NMBA	96
NMPA	26.5
NIPEA	26.5
NDIPA	26.5

It is important to note that exposure to nitrosamines is directly linked to the maximum daily dose (MDD) of the drug substance. Therefore, different concentrations of nitrosamines (ng per g) may be considered acceptable for each material under evaluation. The permissible concentration of nitrosamines in the material varies depending on the product. It can be calculated in ppm, based on a drug's MDD, using the formula Al (ng)/MDD (mg). (Table 6)

Table 6 Example Using Al Limits 96 and 26.5 ng per day for the Target Nitrosamines

Active substance (Maximum daily dose)	NDMA		NDEA		NMBA [4]	
	Al (ng/ day)	Limit (ppm)	AI (ng/ day)	Limit (ppm)	AI (ng/ day)	Limit (ppm)
Candesartan (32 mg)	96	3.00	26.5	0.83	96	3.00
Irbesartan (300 mg)		0.32		0.09		0.32
Losartan (100 mg)		0.96		0.27		0.96
Olmesartan (40 mg)		2.40		0.66		2.40
Valsartan (320 mg)		0.30		0.08		0.30

Please note that the limits mentioned below are applicable only if a drug substance and drug product contain a single nitrosamine. Suppose more than one of the nitrosamine impurities listed in Table 1 is detected, and the total quantity exceeds 26.5 ng per day (the Acceptable Limit for the most potent nitrosamines) based on the maximum daily dose (MDD). In that case, it is recommended that the manufacturer contact and consult with the relevant health authority or the regulatory authorities for evaluation to determine the specific path forward.

For drug products with an MDD of less than 880 mg per day, a recommended limit for total nitrosamines of 0.03 ppm is not more than 26.5 ng per day, which is considered acceptable. For drug products with an MDD above 880 mg per day, the limit for total nitrosamines should be adjusted so as not to exceed the recommended limit of 26.5 ng per day. Suppose the nitrosamine Acceptable Limit is not found for a drug product. In that case, manufacturers should use the approach outlined in ICH M7 (R1) to determine the risk associated with the nitrosamine and contact the regulatory authorities about the acceptability of any proposed limit.

2.4 Testing for the presence of nitrosamines

After conducting a risk assessment, it may be necessary to perform exploratory testing to validate the findings and proposed risk mitigation measures. If a drug product is found to have a risk of nitrosamines, the batches should be subjected to confirmatory testing using sensitive and

validated methods. If nitrosamine impurities are detected, the manufacturing process should be investigated to identify the root cause and implement necessary changes to mitigate or reduce the impurities.

The current published guidelines for AI limits only consider the presence of a single nitrosamine. However, suppose multiple nitrosamines are detected in a drug substance or product, and their levels exceed the maximum limit set by the regulatory authority. In that case, consulting the relevant health authority for guidance is recommended. In such cases, manufacturers should contact the FDA to determine the appropriate AI limits, especially if the total nitrosamine level exceeds 26.5 ng/day based on MDD.

2.5 Test method performance characteristics of nitrosamine

The detection of nitrosamines in AI requires susceptible analytical procedures. The most reliable methods involve chromatographic separation techniques combined with quantitation using mass spectrometry, such as HPLC-MS/MS and GC-MS/MS.

2.5.1 Considerations for sample preparation

When performing trace impurity analyses to evaluate the levels of nitrosamines in drug substances and products, it is crucial to prepare samples appropriately. This is especially important to prevent the loss or generation of nitrosamines as artefacts of the analytical procedure itself. Nitrosamines can be formed as an artefact in GC analyses due to the presence of dialkyl amine (dimethylamine) as a process impurity or the counter ion of the salt form of the active ingredient in the presence of nitrite and acid. In such circumstances, sample extractions should be modified to avoid solubilisation of the active ingredient while maintaining the extraction efficiency of nitrosamines in the material.

When performing quantitative analysis of nitrosamines, evaluating the recommended method performance characteristics, including range, accuracy, repeatability, intermediate precision, and limit of quantitation, is essential. If a limit test is intended for use, it is recommended to assess specificity, recovery, detectability, and solution stability. The performance criteria for these parameters should be set appropriately and confirmed through validation to ensure that the method is suitable for its intended use based on the specific analytes, matrices, and required precision and accuracy of the analytical procedures. Precision and recovery depend highly on concentration and matrix complexity. Therefore, the procedure validation documentation should justify the final proposed acceptance criteria. It is acceptable to tolerate higher variability at concentrations approaching the limit of quantitation of the procedure, while lower variability (higher precision) should be expected at higher concentrations. Analytical procedures [5] (See IP 2022, Nitrosamine Impurities, Vol-I, Page no. 1215-1223). (Table 7)

2.6 Clinical trials

As clinical development progresses, it is expected that the number of structures assessed for mutagenicity and the collection of analytical data will increase. Here are some guidelines for different phases of clinical development:

(1) For Phase 1 studies that last for 14 days or less, efforts to mitigate risks of mutagenic impurities should be focused on Class 1, Class 2, and those in the cohort of concern (as outlined in Section 7). For Phase 1 clinical trials that are longer than 14 days and for Phase 2a clinical trials, Class 3 impurities that require analytical controls should also be included.

(2) For Phase 2b and Phase 3 clinical development trials, a list of the impurities assessed by (Q)SAR should be included. Actual and potential Class 1, 2, or 3 impurities should be described, along with control plans. The in silico (Q)SAR systems used to perform the assessments should also be described. It is also necessary to report the results of bacterial mutagenicity tests of actual impurities.

(3) A risk-based approach based on process chemistry fundamentals is encouraged to prioritise analytical efforts on those impurities with the highest likelihood of being present in the drug substance or product. Analytical data may not be expected to support early clinical development when the possibility of an impurity being present is low. However, analytical data may be appropriate to support the control approach for the marketing application. The commercial formulation design occurs later in clinical development, so efforts associated with drug product degradation products will be limited in the earlier phases.

2.7 NMR assignment

Asymmetrical N-nitrosamines are typically analysed using HPLC, GC or NMR. Such analysis results in two separate signals attributed to the asymmetric N-nitrosamines' isomers. However,

Table 7 Regulatory status of nitrosamine

National Regulatory Authority (NRA)	Approved Category	Name of Medicines(Brand Name, Dosage Form & Dose)	Nitrosamine Impurity present in the drug/medicine	FONI (Month/Year)
USFDA- USA [6]	Prescription drug Drug Class: Angiotensin-II Receptor Blockers (ARB's) (used to treat high blood pressure and heart failure) [7]	"-sartan" drugs like as, Valsartan, Losartan, Irbesartan Tablets	-N-Nitrosodimethylamine (NDMA) & N-Nitrosodiethylamine (NDEA), are probable human carcinogens (a substance that could cause cancer), and -N-Nitroso-N-methyl-4-aminobutyric acid (NMBA) is a potential human carcinogen.	2018
-	-OTC [8]	-Ranitidine (all dosage form & dose) -Brand name: *Zantac*	N-Nitrosodimethylamine (NDMA)	-
-	Prescription Drug (used to control high blood sugar in patients with type 2 diabetes) [9]	Metformin Tablets	N-Nitrosodimethylamine (NDMA)	-
TGA Australia [10]	Prescription drug Drug Class: Angiotensin Receptor Blockers (ARB's) (issued to treat high blood pressure) [11]	'sartan' Blood Pressure medicines example: -Candesartan 32 mg, -Sandoz Irbesartan/HCT 300/12.5 mg Tablet, -Sandoz Irbesartan 150 mg Tablet , -Losartan Film-Coated 100 mg, -Olmesartan Tablets 40 mg, -Valsartan Film-Coated Tablets 320 mg	*N*-nitroso compounds	July 2018
-	Prescription Medicine (used to treat Type-2 diabetes.) [12]	Metformin Tablet	*N*-nitrosodimethylamine (NDMA)	2019
-	Prescription/Non-prescription Drug (to treat and prevent heartburn, reflux and ulcers) [13]	-Ranitidine Tablet -Aspen Ranitidine (Brand Name- *Zantac*), Sandoz Ranitidine	*N*nitrosodimethylamine (NDMA)	2019
-	Prescription Medicine (that assists adults to stop smoking) [14]	-Varenicline Tablet -Brand Name '*Champix*', Pfizer Australia	N-nitrosovarenicline	2021
-	Prescription Medicine (Antibiotic, first-line medicine for the treatment of TB, serious infections including blood infections and leprosy) [15]	Rifampicin Tablets Brand Names: - *Rifadin* and - *Rimycin*	1-methyl-4-nitrosopiperazine (MeNP or MNP)	2021
-	Prescription medicine (used to treat Type-2 diabetes) [16]	Sitagliptin Tablets	7-Nitroso-3-(trifluoromethyl)-5,6,7,8-tetrahydro[1,2,4]triazolo-[4,3- a]pyrazine (NTTP)	2022
-	Prescription medicine Drug Class: Angiotensin Converting Enzyme (ACE) Inhibitors (used to treat high blood pressure/ hypertension) [17]	Quinapril & Quinapril Hydroclorthiazide Tablets	*N*-nitroso-quinapril	2022
European Medicines Agency (EMA)-Europe [18]	Prescription medicine (Angiotensin-II Receptor Blockers)	'sartan' medicines; Example: Candesarta 32mg, Irbesartan 300 mg, Losartan 150 mg, Olmesartan 40 mg, Valsartan 320 mg	N-nitrosodimethylamine (NDMA) and Nnitrosodiethylamine (NDEA)	2018
-	Prescription Medicine (It's a first-line treatment for Tuberculosis, also used for management of other serious infections, including blood infections and leprosy) [19]	Rifampicin	1-nitroso-4-methyl piperazine	Feb 2021
-	OTC Medicine [20]	Ranitidine	N-nitrosodimethylamine (NDMA)	Sep 2020
-	Prescription Medicine (used for the treatment of diabetes) [18]	Metformin Tablets	N-nitrosodimethylamine (NDMA)	2019
-	Prescription Medicine (smoking cessation medicine)	Varenicline; Brand Name- *Champix*	N nitroso-varenicline	Jun 2021
Pharmaceuticals and Medical Devices Agency (PMDA)-Japan [21,22]	Prescription Medicine Drug Class: Angiotensin Receptor Blockers ARB's (used to treat high blood pressure)	'sartan' medicines Valsartan Tablets 20 mg, 40 mg, 80 mg, 160 mg ; Brand Name 'AA' *AMVALO* Combination Tablets "Pfizer"	N-nitrosodimethylamine (NDMA) Nnitrosodiethylamine (NDEA)	Jul 2018 Feb 2019
-	-	Irbesartan Tablets	Nnitrosodiethylamine (NDEA)	Sep 2018
-	-	Losartan Potassium Tablets	N-nitroso-N-methyl-4-aminobutyric acid (NMBA)	Mar 2019
-	OTC (to treat and prevent heartburn, reflux and ulcers)	Ranitidine Hydrochloride Tablets 75 mg, 150 mg (9 companies) & Ranitidine Hydrochloride Injection 50 mg, 100 mg (3 companies)	N-nitrosodimethylamine (NDMA)	Sep 2019
-	-	Nizatidine Capsules Brand Name: OHARA	N-nitrosodimethylamine (NDMA)	Sep 2019
-	Prescription Medicine (used to treat Type-2 diabetes)	Metformin Metformin HCL Tablets 500 mg METGLUCO Tablets 250 mg, 500 mg MT "JG"	N-nitrosodimethylamine (NDMA)	Apr 2020
-	-	Metformin HCL Tablets 500 mg MT "TOWA" / "NICHIIKO"	N-nitrosodimethylamine (NDMA)	Sep 2020
Medicines and Healthcare Products Regulatory Agency (MHRA)-UK [23]	Prescription Medicine (used to treat blood pressure and heart attacks and heart failures)	"sartans" Valsartan Tablets	N-nitrosodimethylamine (NDMA), N-nitrosodiethylamine (NDEA)	2018
-	-	Irbesartan Tablets	N-nitrosodiethylamine (NDEA)	2019
-	-	Losartan Tablets	N-nitroso-N-methylamino butyric acid (NMBA)	2018
-	Prescription Medicine (used to treat conditions such as heartburn and stomach ulcers) [24]	Ranitidine Brand name: Zantac 150mg/10ml Syrup Zantac 50mg/2ml Injection Zantac 150mg Tablets and Zantac 300mg Tablets Company: GlaxoSmithKline	N-nitrosodimethylamine (NDMA)	2019
-	Prescription Medicine (used to treat Type-2 diabetes) [25]	Metformin Oral Solution	N-nitrosodimethylamine (NDMA)	2020
-	Prescription Medicine (used to treat vertigo, nausea vomiting, and may also be used for schizophrenia) [26]	Prochlorperazine Maleate Brand name: Stemetil 5mg/5ml Syrup Company: Aventis Pharma Limited (t/a Sanofi)	N-nitrosomethylphenylamine (NMPA)	2022
Health Canada [27]	Prescription Medicine (used for muscle relaxant to relieve muscle spasm)	Orphenadrine citrate Brand name: OrfenAce 100 mg Tablets Company: SteriMax Inc.	N-methyl-N-nitroso-2-[(2 methylphenyl) phenylmethoxy]ethanamine (NMOA)	2021
-	Prescription Medicine (used as an antidepressant mecicine)	Amitriptyline Brand name: Elavil (Amitriptyline Hydrochloride Tablets USP 10 mg) Company: AA Pharma Inc.	N-nitrosodimethylamine (NDMA)	2022
-	Prescription Medicine Drug Class- Beta Blockers (used in adults to treat high blood pressure and prevent angina pectoris)	Propranolol hydrochloride Brand name: Inderal-LA Extended Release Capsules 60 mg, 80 mg, 120 mg and 160 mg Company: Pfizer	*N*-nitroso-propranolol (NNP)	2022
-	Prescription Medicine Drug Class: Angiotensin-Converting Enzyme (ACE) inhibitors (used to treat high blood pressure)	Quinapril Brand name: 1. Accupril (quinapril hydrochloride) 10 mg, 20 mg & 40 mg 2. Accuretic (quinapril hydrochloride and hydrochlorothiazide) 10/12.5 mg, 20/12.5 mg & 20/ 25 mg Company: Pfizer Canada ULC	*N*-nitroso-quinapril	2022
-	Prescription Medicine Drug Class: Synthetic Nucleoside Analogues (it is antiviral drug used to treat shingles, and to treat or reduce the recurrence of genital herpes)	Acyclovir Brand Name: Apo-Acyclovir 200 mg & 800 mg Company: Apotex Inc.	*N*-nitrosodimethylamine (NDMA)	2022

there aren't many reports on NMR assignments of asymmetrical N-nitrosamine isomers. This study employed density functional theory (DFT) calculations to investigate the NMR assignments of the Z/E isomers of six asymmetrical N-nitrosamines. The Z-configuration was found to be the significant isomer of asymmetrical N-nitrosamine. Furthermore, the E-configuration was found to be the major isomer of the remaining asymmetrical N-nitrosamines. We then determined the Z/E ratios of these asymmetrical N-nitrosamines using variable temperature (VT) and room temperature (RT) 1H-NMR experiments. Our results showed that the ratios of the Z/E isomer quickly increased beyond 100% in the VT 1H NMR experiments. The ratios of Z/E isomers were increased in the range of 10–60% in the VT 1H NMR experiments. This study has significant implications for the quality control of N-nitrosamines used as active pharmaceutical ingredients (APIs). It is necessary to identify the isomers of asymmetrical N-nitrosamine to ensure their quality.

3 Conclusion

Over the past two years, the focus has shifted from highly potent small-molecule N-nitrosamine impurities formed during the manufacture of drug substances to NDSRIs predominantly formed during formulation or storage of the drug product. Effective strategies to avoid or mitigate the presence of N-nitrosamine impurities originating during drug substance manufacturing processes are well-known and documented, but controlling NDSRIs in drug products is presently a significant challenge. Vulnerable amines (or their precursors) are common and critical structural elements of many drug substances. They are essential for in vivo activity and important in modulating pharmacokinetic and physicochemical properties. Thus, their elimination from medicines is unlikely, and alternative strategies must be employed to avoid generating NDSRIs at unacceptable levels.

Although much progress has been made in understanding the various risk factors associated with NDSRI formation in drug products, there are significant gaps in mechanical knowledge. This makes it almost impossible to predict accurately whether and to what extent an API bearing a vulnerable amine will result in an NDSRI, considering the formulation, manufacturing process, and storage conditions. MAHs and API manufacturers must consider whether the API's structure risks forming NDSRIs and whether nitrosatable impurities or degradants may pose a risk of forming N-nitrosamine during drug product manufacture. The mitigating measures discussed in this article should be considered where an NDSRI is identified in a medicinal product above the acceptable intake. MAHs, drug product and substance manufacturers, and excipient and packaging manufacturers are encouraged to collaborate further to expand the understanding of risk factors for N-nitrosamine formation in medicines. Further collaboration and research will hopefully enable appropriate reductions in NDSRI levels when required, allow for more accurate risk assessments, and reduce the need for specific testing in the future.

Regulatory agencies such as CDSCO, US-FDA, and the European Medicines Agency (EMA) have made continuous efforts for quantitative determination of amine impurities present in foodstuffs and various intermediates in organic synthesis because nitrosamines are genotoxic impurities and pose a significant threat due to their carcinogenic behaviour. However, it is challenging for researchers and industrialists to explore innovative techniques and methods for precisely estimating nitrosamine impurities from various pharmaceutical APIs because of their low molecular weight and high hydrophilicity.

Various modern analytical tools, methods, and sampling procedures have been reported for the smooth detection and quantification of nitrosamines from complex mixtures. This review has revealed broad analytical quantification and detection of nitrosamine from a series of Valsartan drugs and Ranitidine drugs. Although the EMA and US FDA recognise the presence of amine impurity in Nizatidine and Metformin drugs, their quantification method is still a matter of concern. The USFDA and EMA provide the permissible limit of NA impurity in APIs, and it should be below the threshold toxicological concern of 1.5 g/day.

4 Final remarks and outstanding challenges

In conclusion, this study discusses the chemistry of nitrosamines, their impact on water pollution, and the methods used to detect them. Although most recent research has focused on NDMA, it is essential to note that nitrosamines are a diverse group of chemicals that all share a common feature: the N-N=O group. This diversity makes detecting and removing nitrosamines challenging since different nitrosamines can have significant variations in size, shape, and hydrophilicity. However, given its relative stability under mild conditions, the

shared Nitroso moiety is a challenging functional handle for practical applications. This core N-N=O structure can be formed from a wide range of precursors under numerous different conditions, making nitrosamine prevention a multifaceted problem with no single solution. Understanding the chemistry of nitrosamines is crucial to developing effective processes to mitigate the environmental and public health risks posed by these chemicals. By leveraging the chemical behaviour of these carcinogens, better sensors and extraction materials can be developed.

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Conflicts of interest

The authors declare that they have no conflict of interest.

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