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N-nitrosamine risk assessment in pharmaceuticals: Where are we from a regulatory point of view in 2025?

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ABSTRACT

N-nitrosamines have been a concern for decades due to their potential mutagenicity and widespread occurrence across various matrices. While evidence suggests carcinogenicity in animals, their potential carcinogenicity in humans has prompted their initial inclusion in the "cohort of concern" since in ICH M7(R1), and the current ICH M7(R2) guideline is now in effect. Intensified control of *N*-nitrosamines began in 2018 following the detection of *N*-nitrosodimethylamine in valsartan-containing products. Subsequent investigations revealed *N*-nitrosamine contamination across multiple drug classes, triggering widespread recalls, withdrawals, and regulatory actions. Recently, the emergence of *N*-nitrosamine drug substance-related impurities and drug linker-related impurities has drawn additional regulatory attention. This review presents the methodologies used to determine the acceptable daily intake of *N*-nitrosamines and traces the evolution of regulatory guidelines, offering a comparative analysis of the 3-step investigation approaches adopted by the European Medicines Agency and Food and Drug Administration. It provides a comprehensive examination of potential root causes for *N*-nitrosamine contamination, outlines the analytical requirements for confirmatory testing, as well as mitigation strategies to prevent or minimize contamination. Additionally, the review summarizes risk assessment tools used to predict *N*-nitrosamine formation. By presenting a comprehensive workflow for impurity investigations, this review aims to assist industrial stakeholders in managing *N*-nitrosamine risks, ensuring regulatory compliance, and safeguarding public health.

1. Introduction

N-nitrosamines are a class of organic compounds sharing the common chemical structure of a nitroso functional group bonded to an amine, represented by the general structure $R^1N(-R^2)-N{=}O$, as illustrated in Fig. 1 [1]. These compounds can naturally occur in the environment, found in air, drinking water, and soil [2], and also form in various matrices, including food, beverages, tobacco, and cosmetics [3]. *N*-nitrosamines have raised concerns for decades due to their potential mutagenicity in humans, most are classified by the International Agency for Research on Cancer into groups 2A or 2B [4]. While these classifications suggest evidence of carcinogenicity in animals, further research is necessary to confirm the risk in humans. The impurities are also included in the "cohort of concern" as specified in the ICH M7(R2) guideline [5].

The pharmaceutical spotlight on *N*-nitrosamine impurities began in June 2018 with the detection of harmful levels of *N*-nitrosodimethylamine (NDMA) in batches of valsartan-containing medicinal products. Valsartan is an angiotensin II receptor blocker (ARB) prescribed to treat chronic heart failure and hypertension [6]. The unexpected NDMA contamination was reported to the European Union authorities and the United States Food and Drug Administration (FDA). It was identified as originating from the valsartan active pharmaceutical ingredient (API) manufacturer, Zhejiang Huahai Pharmaceutical Co., Ltd., China [7,8]. The API manufacturer attributed the undesirable NDMA formation to process-related impurities introduced after chemical synthesis modifications during the tetrazole ring-forming step. Subsequent investigations revealed additional *N*-nitrosamines, such as *N*-nitrosodiethylamine (NDEA) and *N*-nitroso-*N*-methyl-4-aminobutyric acid (NMBA), in ARBs containing a tetrazole moiety, including losartan and irbesartan, as shown in Fig. 2 [9]. The scope of contamination expanded to include other drug classes, such as antacids (*e.g.*, ranitidine and nizatidine [10,11]), antidiabetics (*e.g.*, metformin [12] and pioglitazone [13]), antibiotics (*e.g.*, rifampin, rifapentine [14]). Despite their trace presence, these genotoxic impurities often exceeded acceptable limits, posing significant health risks [15–18]. Numerous decisions of

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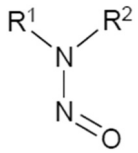

Fig. 1. General chemical structure of nitroso functional group.

recalls, suspensions, and withdrawals of affected pharmaceuticals have been taken to protect patient safety [9,11].

A new class of impurities, *N*-nitrosamine drug substance-related impurities (NDSRIs), emerged in 2021, beginning with the discovery of *N*-nitroso-varenicline in varenicline-containing drugs [19]. Unlike small-molecule *N*-nitrosamines (*e.g.*, NDMA, NDEA, NDMA, *etc.*), which can be generated from multiple sources, NDSRIs share structural similarities with APIs. In general, they are larger molecules and unique to specific drugs, generally forming through the nitrosation of drug substances in the presence of nitrite [1], as illustrated in Fig. 3.

Recent regulatory attention has also shifted towards biologicals, particularly antibody-drug conjugates (ADCs). ADCs, which combine monoclonal antibodies with synthetic drugs *via* drug linkers, present a risk for *N*-nitrosamine drug linker-related impurities (NDLRIs) due to their synthetic components [20,21]. These major events, which have challenged the entire pharmaceutical industry and global health regulators, are illustrated in Fig. 4.

International regulatory bodies have responded promptly to concerns about *N*-nitrosamine contamination by forming a global network. Regulatory bodies, industrial stakeholders, and official medicines control laboratories (OMCLs) worldwide have collaborated to centralize information and develop strategies to address and control *N*-nitrosamine impurities. One key initiative led by the United States Pharmacopeia (USP) is the "Nitrosamine Exchange" platform [22], an interactive, knowledge-based online forum that brings together pharmaceutical companies, API manufacturers, and excipients/packaging suppliers actively engaging, discussing, and sharing best practices related to *N*-nitrosamines [22,23]. Moreover, this platform hosts the "Nitrosamine Analytical Hub", a publicly accessible online repository of analytical methods for the detection of *N*-nitrosamine impurities in pharmaceuticals [23,24].

In February 2021, key regulatory authorities, including the European Medicines Agency (EMA) and the U.S. FDA, released guidance documents Revision 1 [25]. Subsequently, in April 2024, the World Health Organization (WHO) introduced a draft working document titled *WHO Good Manufacturing Practices: Considerations for the Prevention and Control of Nitrosamine Contamination in Pharmaceutical Products*, soliciting public feedback [26]. These documents outline detailed procedures and mandate API manufacturers, marketing authorization holders (MAHs), and applicants to undertake a systematic 3-step investigation to assess the presence of *N*-nitrosamine impurities in their products [1,27].

In late 2020, both the USP and the European Pharmacopoeia (Ph. Eur.) introduced general chapters addressing *N*-nitrosamine impurities. These chapters were officially implemented in December 2021 for the USP and January 2022 for the Ph. Eur. The pharmacopeias provide a wide range of quantitative and limit test analytical procedures [28,29]. Furthermore, a large selection of reference standard materials has been specifically developed to facilitate analytical testing for these impurities. Key publication dates for these documents are summarized in the event timeline shown in Fig. 4.

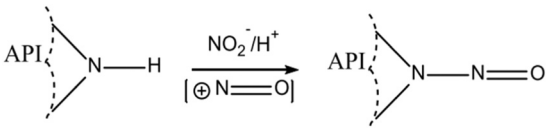

Fig. 3. Nitrosation reaction of NDSRI formation.

Numerous reviews in the field of *N*-nitrosamines have been published, focusing on various aspects such as analytical procedures [30,31], formation pathways in pharmaceutical products [32,33], or documented cases of detection [34]. This review first presents the mechanisms underlying the genotoxicity of *N*-nitrosamines and methodologies used to determine the acceptable intake (AI) limit of *N*-nitrosamines. It also traces the evolution of regulatory guidelines, offering a comparative analysis of the 3-step investigation approaches adopted by the EMA and FDA, highlighting their similarities and differences. A comprehensive workflow is proposed to guide the investigation process. Finally, the review summarizes risk assessment tools used to predict *N*-nitrosamine formation.

2. Carcinogenicity and acceptable intake limit determination

2.1. Carcinogenicity mechanisms

N-nitrosamines can be ingested or form endogenously within the organism *via* nitrosating reactions in the acidic environment of the stomach. In this reaction, nitric oxide, derived from nitrite or nitrate, reacts with amines present in food [3]. Once ingested, *N*-nitrosamines are metabolically activated *in vivo*, primarily by cytochrome P450 enzymes. While CYP2E1 is known to metabolize smaller *N*-nitrosamines, a broader range of cytochrome isoforms can contribute to their activation. Regardless of the cytochrome involved, the metabolic activation pathway is generally consistent across different classes of *N*-nitrosamines: hydroxylation at the α-carbon adjacent to the *N*-nitroso group. In the case of aliphatic *N*-nitrosamines, the α-hydroxylation generates an unstable dealkylated *N*-nitrosamine that decomposes rapidly into a reactive alkyldiazonium ion. Alkyldiazonium ions can alkylate DNA material *via* either S_N1 or S_N2 mechanisms, forming adducts that lead to DNA damage and potentially contribute to cancer development [2,35]. The metabolic biotransformation pathways and subsequent DNA adduct formation are represented in Fig. 5.

In contrast to aliphatic *N*-nitrosamines, aromatic *N*-nitrosamines ultimately yield aryldiazonium ions, which are incapable of undergoing S_N1 or S_N2 reactions and instead form diazo adducts. Consequently, aromatic *N*-nitrosamines tend to exhibit lower carcinogenic potential than their aliphatic counterparts. This distinction is reflected in the AI

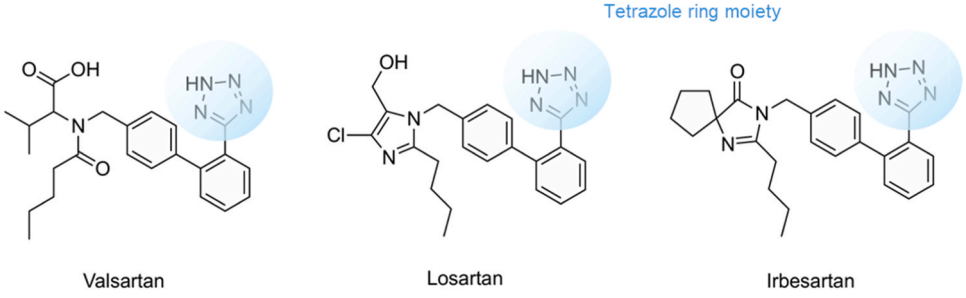

Fig. 2. Chemical structures of valsartan, losartan, and irbesartan.

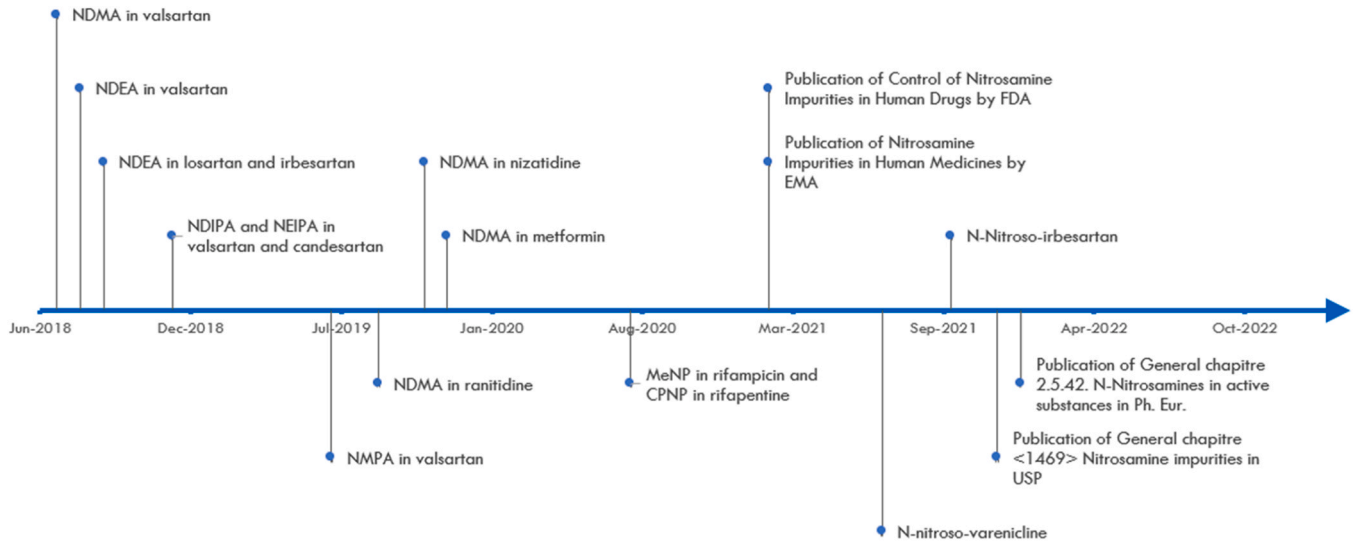

Fig. 4. Timeline of key N-nitrosamine events for the pharmaceutical industry.

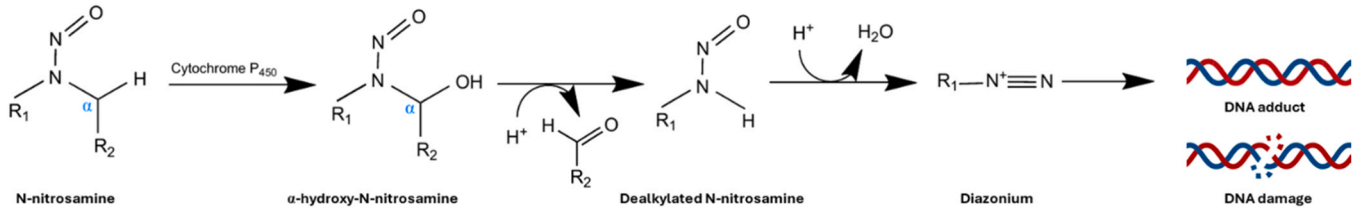

Fig. 5. Metabolic biotransformation of N-nitrosamines and formation of NDA adducts.

Table 1
CAS number, source, CPCA category, and recommended AI limit of *N*-nitrosamines according to EMA and FDA procedures.

Small-molecule *N*-nitrosamines	CAS No.	Source [27]	CPCA category [27,38]	AI limit (ng/day)	
				EMA [27]	FDA [37]
N-nitroso-dimethylamine (NDMA)	62-75-9	Multiple	2	96	96
N-nitroso-diethylamine (NDEA)	55-18-5	Multiple	1	26.5	26.5
N-nitroso-*N*-methyl-4-aminobutyric acid (NMBA)	61445-55-4	Multiple	4	1500	1500
N-nitroso-ethylisopropylamine (NEIPA)	16339-04-1	Multiple	3	400	400
N-nitroso-diisopropylamine (NDIPA)	601-77-4	Multiple	5	1500	1500
N-nitroso-methylphenylamine (NMPA)	614-00-6	Multiple	2	100	100
N-nitrosodi-*N*-propylamine (NDPA)	621-64-7	Multiple	1	26.5	UA
N-nitrosodi-*N*-butylamine (NDBA)	924-16-3	Multiple	1	26.5	UA
4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)	64091-91-4	Multiple	2	100	UA
N-nitrosomorpholine (NMOR)	59-89-2	Multiple	N/A	127	UA
N-nitrosodiphenylamine (NDPhA)	86-30-6	Multiple	N/A	78,000	UA
N-nitrosopyrrolidine (NPYR)	930-55-2	Multiple	N/A	1700	UA
N-nitrosopiperidine (NPIP)	100-75-4	Multiple	N/A	1300	UA
NDSRIs					
1-methyl-4-nitrosopiperazine (MeNP)	16339-07-4	Rifampin	3	400	400 3000#
1-cyclopentyl-4-nitroso-piperazine (CPNP)	61379-66-6	Rifapentine	3	UA	400
N-nitroso-desmethyl-tramadol	UA	Tramadol	1	18	26.5
N-nitroso-STG-19 (NTTP)	2892260-32-9	Sitagliptin	2	100	100
N-nitroso-varenicline	2755871-02-2	Varenicline	3	400	400
N-nitroso-betaxolol	UA	Betaxolol	4	1500	1500
N-nitroso-enalapril	2994317-06-3	Enalapril	5	1500	1500
N-nitroso-duloxetine	2680527-91-5	Duloxetine	2	100*	100*

CAS No.: Chemical abstracts service number; UA: Unavailable; N/A: Not applicable.
Data from the EMA was last updated in February 2025, and from the FDA in March 2025.
* Recommended AI limit based on read-across analysis from a surrogate *N*-nitrosamine (NNK).
# Recommended interim AI limit for *N*-nitrosamines for approved or currently marketed products.

limits: for instance, *N*-nitroso-diphenylamine (NDPhA) has a considerably higher AI limit of 78,000 ng/day, compared to 96 ng/day for NDMA. Additionally, cyclic *N*-nitrosamines may form cyclic diazonium intermediates that can rearrange into oxonium ions, which serve as alternative alkylating agents [35].

2.2. Acceptable intake limit

The EMA and FDA have elaborated, periodically reviewed, and updated comprehensive lists of *N*-nitrosamines, including both small-molecule *N*-nitrosamines and NDSRIs, along with their respective AI limit. These AI limits are established based on the carcinogenic risk associated with the impurities. Given the large number of small-molecules impurities and NDSRIs identified to date and the ongoing discovery of new ones, some of these are summarized in Table 1, along with the source and CAS number. Notably, 1-methyl-4-nitrosopiperazine (MeNP) and 1-cyclopentyl-4-nitrosopiperazine (CPNP) represent particular cases: although they have low molecular weights, they are classified as NDSRIs because they are specifically associated with rifampin and rifapentine, respectively [33,36]. These examples will be discussed in detail in Section 4.4.2. Furthermore, the EMA and FDA lists provide complementary information, with each including different *N*-nitrosamines [27,37].

2.3. Acceptable intake limit determination

The ICH M7(R2) guideline defines the AI limit for mutagenic impurities as a threshold representing an increased cancer risk of one case per 100,000 people, based on lifetime daily exposure (70 years) [5]. For most unstudied substances, this corresponds to a threshold of toxicological concern (TTC) of 1.5 μg/day, which is associated with a negligible cancer risk. However, highly potent mutagenic compounds classified as part of the "cohort of concern", such as *N*-nitrosamines, pose a significant cancer risk even at doses below this threshold. Consequently, the TTC approach is inappropriate for determining the AI limit for *N*-nitrosamines, necessitating a compound-specific assessment instead [5].

2.3.1. Carcinogenic potency categorization approach

The EMA and FDA have suggested a novel predictive carcinogenic potency categorization approach (CPCA) for determining AI limits for both small-molecule *N*-nitrosamines and NDSRIs [27,38]. This approach offers a significant advantage in its applicability even when robust carcinogenicity data are lacking for the identified *N*-nitrosamines in pharmaceutical products. This is particularly relevant for NDSRIs, as their carcinogenicity data are rarely available due to their recent identification and ongoing detection in drugs [39].

The CPCA is a conservative approach and user-friendly prediction tool that leverages SAR principles and databases [39,40], such as the Carcinogenic Potency Database [41,42] and Lhasa Carcinogenicity Database [43]. This methodology assumes that enzymatic α-hydroxylation by cytochrome P450 is the primary mechanism responsible for the mutagenic and genotoxic effects observed for *N*-nitrosamines [40].

The structural features of molecules can either enhance or reduce the enzymatic activation of metabolization mechanisms or facilitate alternative biological clearance pathways [40]. Using SAR-assisted predicted carcinogenic potency, the CPCA classified molecules into five potency categories, assigning four discrete AI levels: 18 or 26.5, 100, 400, and 1500 ng/day, as summarized in Table 2.

Notably, the EMA and FDA have adopted different AI values for category 1, using distinct strategies. Specifically, the EMA sets the AI limit for category 1 at 18 ng/day, while the FDA assigns a slightly higher limit of 26.5 ng/day, as detailed in Table 2. This classification system enables AI limits to be derived solely from the evaluation of structural features [27,38,40]. The potency categories for selected *N*-nitrosamines are listed in Table 1.

Table 2
Five predicted CPCA categories and associated recommended AI limits for *N*-nitrosamine impurities.

CPCA category	Recommended AI limit (ng/day)		Comments [27,38]
	EMA [27]	FDA [37]	
1	18	26.5	*N*-nitrosamines assigned to category 1 are predicted to have high carcinogenic potency but no higher than the class-specific threshold. The class-specific TTC for *N*-nitrosamine impurities is considered sufficiently protective for patients. EMA: The recommended AI limit of 18 ng/day is set, equivalent to the class-specific TTC for *N*-nitrosamines, as outlined in the *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*. FDA: The recommended AI limit of 26.5 ng/day is set based on the carcinogenic potency of NDEA, the most potent and robustly studied *N*-nitrosamine.
2	100	100	*N*-nitrosamines assigned to category 2 are predicted to have carcinogenic potency no higher than NDMA and NNK, which have recommended AI limits of 96 ng/day and 100 ng/day, respectively. The recommended AI limit of 100 ng/day is set and aligns with the limits for NDMA and NNK.
3	400	400	*N*-nitrosamines assigned to category 3 are predicted to have lower carcinogenic potency than potency category 2 due to certain structural features. The recommended AI limit of 400 ng/day is set to reflect a 4-fold reduction in carcinogenicity potency compared to category 2.
4	1500	1500	*N*-nitrosamines assigned to category 4 are predicted to be probably metabolically activated through an enzymatic α-hydroxylation pathway but with low carcinogenic potency. The recommended AI limit of 1500 ng/day (or 1.5 μg/day) is set at the TTC defined in the ICH M7(R2) guideline.
5	1500	1500	*N*-nitrosamines assigned to category 5 are not predicted to be metabolically activated through an enzymatic α-hydroxylation pathway due to steric hindrance or the absence of α-hydrogens or are predicted to form unstable species that will not react with DNA. The recommended AI limit of 1500 ng/day (or 1.5 μg/day) is set at the TTC defined in the ICH M7(R2) guideline.

2.3.2. Read-across analysis

An alternative to the CPCA is a read-across analysis. This method consists of using a structurally similar *N*-nitrosamine, referred to as a "surrogate", which has sufficient and robust carcinogenicity data to predict the carcinogenic potency of the compound of interest [1,27]. The application of this strategy must be scientifically justified and can be used to determine AI limits for both small-molecule *N*-nitrosamine impurities and NDSRIs [1]. An example of this approach is provided in Table 1. For instance, the recommended AI limit for *N*-nitroso-duloxetine is set at 100 ng/day based on read-across analysis from NNK, which serves as the surrogate *N*-nitrosamine [37].

2.3.3. Temporary approaches

If drug product batches in distribution contain *N*-nitrosamine impurities at levels exceeding the recommended AI limits, both the FDA and EMA have proposed interim measures to mitigate supply disruptions [1,27]. The FDA's approach includes updated recommended interim AI limits for certain *N*-nitrosamine impurities, applicable for a temporary

period under specific conditions. At the end of this period, the FDA intends to reassess these interim limits [1].

In contrast, the EMA has adopted a less-than-lifetime (LTL) approach to calculate interim limits for *N*-nitrosamine impurities above AI limits. This approach is applied only after consultation with competent authorities and serves as a temporary measure until further steps can be taken to reduce the contaminant levels to or below the established AI limits. The recommended interim limits under the LTL approach depend on the treatment duration of the drug product, with two adjustment factors: 13.3 × for treatments lasting up to 12 months and 6.7 × for treatments exceeding 12 months [27].

Both the EMA's and FDA's temporary approaches represent conservative measures to prevent disruptions in drug supply and ensure patients' continued access to essential treatments.

2.3.4. Other approaches

For any *N*-nitrosamines with sufficient and robust compound-specific carcinogenicity data from animal studies, the TD_{50} (the dose giving a 50 % tumor incidence) should be determined and used to derive a compound-specific AI limit for lifetime exposure as recommended in the ICH M7(R2) guideline [27].

A negative result in a Good Laboratory Practice-compliant enhanced Ames test (EAT) permits the control of the *N*-nitrosamine at the TTC of 1.5 μg/day. Furthermore, a negative result in a relevant well-conducted *in vivo* mutagenicity study allows the classification of the *N*-nitrosamine as a non-mutagenic impurity, enabling its control under less stringent criteria [27]. For instance, transgenic rodent mutation assays study and measure gene mutations *in vivo* for carcinogenic risk and potential assessment. The mutation assay procedure is outline in the Organisation for Economic Co-operation and Development's (OECD) test guideline [44].

3. A 3-step *N*-nitrosamine investigation guidance

Regulatory agencies have extended the requirement for *N*-nitrosamine investigations to all medicines, including herbal and homeopathic medicinal products, as well as those containing chemically synthesized APIs and biologicals containing chemically synthesized fragments. Investigations apply to marketed drugs and drugs under marketing authorization applications [1,27]. The FDA has further recommended extending this investigation to semi-synthetic and fermentation products at risk of *N*-nitrosamine contamination [1]. The investigation guidance follows a standardized 3-step approach [1,27]:

- Step 1: Risk assessment,
- Step 2: Confirmatory testing,
- Step 3: Implementation of risk mitigation measures.

The EMA refers to this procedure as a "call for review". Deadlines for completing these steps depend on the regulatory agency and the product nature or *N*-nitrosamine class. While the EMA categorizes deadlines based on the nature of chemically synthesized APIs or biologicals, the FDA differentiates deadlines by *N*-nitrosamine structure (*e.g.*, small-molecule *N*-nitrosamines *versus* NDSRIs) [1,27]. The inclusion of NDSRIs is a new requirement introduced in Revision 2 of the FDA's *Control of Nitrosamine Impurities in Human Drugs Guidance for Industry* [1]. Notably, the FDA's timeline shows a two-year difference between the deadlines for small-molecule *N*-nitrosamines and NDSRIs. This gap reflects that the discovery and recognition of NDSRIs as a distinct risk emerged approximately three years after the initial detection of NDMA in valsartan [1].

3.1. Theoretical risk assessment

The initial step in investigating *N*-nitrosamine contamination involves a theoretical, risk-based assessment using prior knowledge and industry experience. This approach evaluates the likelihood of *N*-nitrosamine formation during the manufacturing of APIs, as well as throughout the drug product manufacturing and storage. Collaboration with API manufacturers is essential to ensure a complete and accurate assessment.

The risk assessments should clearly outline the impact of potential *N*-nitrosamine formation on the product's benefit-risk balance [1,27]. The evaluation should consider all possible root causes of *N*-nitrosamine contamination, including any pathways that could contribute to *N*-nitrosamine formation, as detailed in 4.

If the risk assessment (Step 1) indicates a potential *N*-nitrosamine presence, confirmatory testing (Step 2) becomes mandatory to confirm their presence or absence in the product. The analytical methods used for Step 2 must be sufficiently sensitive, capable of accurately detecting trace levels of *N*-nitrosamines [1,27]. Conversely, if a robust risk assessment concludes that *N*-nitrosamine formation is not possible, no further action is required.

3.2. Analytical confirmatory testing

3.2.1. Regulatory requirements for analytical methods

During the development of analytical procedures, several technical aspects must be carefully considered. In the context of trace analysis, it is essential to prevent both contamination and *in situ* formation of *N*-nitrosamines during sample preparation and analysis, as these can result in false-positive results [27]. Given the relatively large quantities of APIs and excipients compared to the trace levels of *N*-nitrosamines, sample preparation protocols must be meticulously designed. Effective sample preparation enables the removal of interfering substances, minimizes matrix effects, and lowers the detection limit.

Beside sample preparation, an appropriately sensitive analytical method needs to be developed. Regulatory agencies recommend the use of mass spectrometers due to their high specificity and sensitivity in trace-level, multi-analyte analysis [27]. Tandem mass spectrometry (MS/MS) is considered the gold standard for the quantification of trace impurities, owing to its dual mass-to-charge ratio selection, which enhances specificity and sensitivity. When a method is intended for the simultaneous determination of multiple *N*-nitrosamines, its selectivity/specificity must be demonstrated. Method sensitivity is typically characterized by lower limits like the detection and quantification limits [45]. The required quantification limit depends on the acceptable limit (or specification limit) of a *N*-nitrosamine in a given product. The acceptable limit in ppm is calculated by dividing the AI limit of a specific *N*-nitrosamine in ng/day (see Tables 1 and 2) by the maximum daily dose of the product expressed in mg/day [27]. Certain high-dose products, such as metformin or paracetamol formulations ($\geq$ 3 g/day), may face challenges in meeting these sensitivity requirements due to technical limitations. In such cases, regulators have suggested that scientifically justified alternatives may be considered [27].

To assist pharmaceutical companies, organizations such as the FDA, OMCLs, USP, and Eur. Ph. have developed and published validated analytical procedures for analyzing both small-molecule *N*-nitrosamines and NDSRIs. Additionally, several highly sensitive LC-MS/MS methods have been published in the literature, with detection limits reported as low as 0.75 pg/mL in a synthetic drug product [46] and 5 pg/mL in a biological medicine [47].

3.2.2. Possible future testing scenarios

Before performing confirmatory studies, analytical procedures must undergo rigorous validation to ensure the compliance with regulatory standards [1,27]. The use of validated analytical procedures guarantees the quality and reliability of confirmatory testing results, providing confidence to health authorities and safeguarding public health.

Both the EMA and FDA require that API manufacturers, MAHs and applicants test a representative number of API or finished product batches. The number of batches to be tested should be commensurate

with the annual production volume and should include both newly manufactured and retained samples that remain within the expiry dates [1,27].

3.2.2.1. EMA investigation procedure. Following confirmatory testing (Step 2), the EMA outlines 3 possible testing strategies depending on the test results, as illustrated in Fig. 6 [27]:

- Specification omission: No further testing is required.
- Periodic or skip testing: Future testing is performed at release on pre-selected batches or predetermined intervals. This requires assurance that untested batches still must meet all acceptance criteria [48].
- Routine analysis: Future testing is performed on a batch-to-batch basis [48].

Testing scenarios depend on both the sensitivity of the analytical methods and *N*-nitrosamine levels detected in the product [27]. Even if theoretical levels of *N*-nitrosamine remain consistently below 10 % of the acceptable limit, inadequate method sensitivity may disqualify or exclude a product from specification omission. Conversely, if *N*-nitrosamine levels exceed the acceptable limit, regulatory actions such as recalls or withdrawals may be necessary following a benefit-risk assessment. In certain circumstances, interim limits may be temporarily established to prevent supply disruptions while risk mitigation measures are being implemented to reduce impurity levels below the acceptable limit. For products still in development, further formulation or process optimization is required to ensure that *N*-nitrosamine levels remain within safe limits prior to submission of a marketing authorization or new drug application.

3.2.2.2. FDA investigation procedure. In contrast to the EMA's unified approach, the FDA distinguishes between API manufacturers and drug product manufacturers/applicants, with different expectations and testing strategies.

According to FDA guidance, if *N*-nitrosamines are consistently found above 10 % of the acceptable limit in APIs, API manufacturers should test each production batch at release as well as retained stability samples. For drug products, even when *N*-nitrosamines are detected below 10 % of the AI limit, the FDA still recommends testing. As with the EMA procedure, API and drug product batches containing *N*-nitrosamine levels above the acceptable limit should generally not be released for distribution. However, exceptions may be granted by the regulatory bodies on a case-by-case basis when the API or product is deemed essential to prevent or mitigate a drug shortage [1].

3.3. Comprehensive risk mitigation measure implementation

As illustrated in Fig. 6, under the EMA approach, if *N*-nitrosamine levels consistently remain below the acceptable limit, indicating compliance with drug safety requirements, no risk mitigation measures are required. However, if the levels exceed the acceptable limit, API manufacturers, MAHs or applicants must implement mitigation strategies based on identified root causes [27].

The FDA takes a more stringent stance, if *N*-nitrosamines are detected above the quantification limit, API, drug product manufacturers or applicants should develop mitigation strategies to ensure that the level remains within the acceptable limit. Furthermore, the FDA recommends introducing control measures when there is a significant risk of *N*-nitrosamine formation due to the API structure, the synthetic route, or the manufacturing process [1].

Risk mitigation measures aim to effectively prevent or reduce *N*-nitrosamine formation in drug substances and products [1,27]. Section 5 provides detailed source-specific mitigation strategies proposed by the regulatory agencies to guide pharmaceutical companies in managing contamination risks.

4. Sources of *N*-nitrosamine impurity formation

Pharmaceutical impurity designates any components present in the drug substance or product that are not the API or an excipient [49,50]. Impurities are unwanted compounds that remain in APIs or finished product formulations. *N*-nitrosamine impurities, including small-molecule *N*-nitrosamine impurities and NDSRIs, can form through various pathways and at different stages of drug manufacturing, namely i) during the chemical synthesis of APIs, ii) in the manufacturing processes, and iii) during the storage of the drug product formulations.

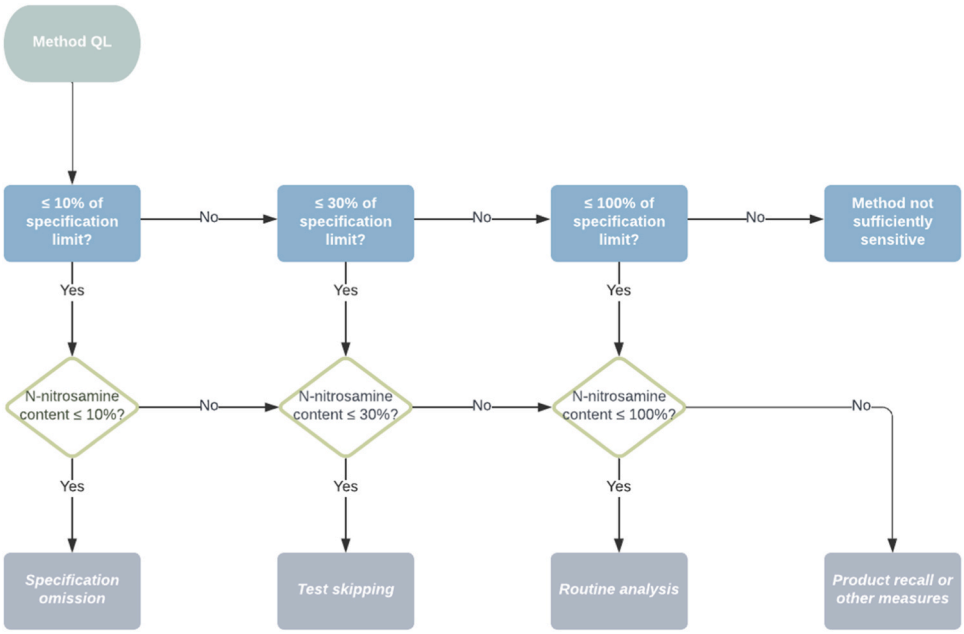

Fig. 6. Possible scenarios following analytical method performance and confirmatory testing outcomes according to the EMA investigation approach. QL: Quantification limit.

4.1. Chemical synthesis of APIs

During API synthesis, most *N*-nitrosamines formed are small-molecule *N*-nitrosamine impurities, which arise from several root causes.

4.1.1. Routes of chemical synthesis

The *N*-nitrosamine crisis in the pharmaceutical industry began with the detection of high levels of NDMA in sartan medications. The root cause was traced to poorly controlled modifications in the synthetic routes of APIs. In particular, the formation of *N*-nitrosamines was found to be intrinsically linked to the synthetic routes of certain functional groups, such as tetrazole rings in sartans. Azide compounds are commonly employed in the synthesis of nitrogen-containing heterocycles, including tetrazole rings. The tetrazole moiety is typically formed through a reaction between nitrile and azide compounds, often using tributyltin azide as a reagent [51].

The contamination of valsartan API with NDMA was reported by Zhejiang Huahai Pharmaceutical Co. as a consequence of changes to the API manufacturing process, specifically replacing tributyltin azide with sodium azide (NaN_3) in the tetrazole ring-forming step [52,53]. The use of NaN_3, a more reactive and cost-effective inorganic alternative, resulted in a higher yield of valsartan compared to the original patented method developed by Novartis, which utilized tributyltin azide [53]. However, NaN_3 has poor solubility in organic solvents and is often added in excess to compensate for its limited solubility in dimethylformamide, a preferred solvent employed by the Chinese manufacturer [32]. Sodium nitrite ($NaNO_2$) is then introduced to quench excess NaN_3 after tetrazole ring formation [52–54]. Under acidic conditions, $NaNO_2$ can convert to nitrous acid, a strong nitrosating agent [52,55]. Dimethylamine and diethylamine, which are known impurities in dimethylformamide, can undergo nitrosation in the presence of nitrous acid or nitrite under acidic conditions to form NDMA or NDEA, respectively. Such impurities were absent in the originator Novartis product Diovane® [53,56].

4.1.2. Use of nitrosating agents

Nitrosating agents, such as nitrites and nitrous acid, used in API synthesis can directly contribute to *N*-nitrosamine formation when secondary, tertiary, or quaternary amines are present [1]. Moser et al. experimentally compared the secondary and tertiary amine reactivity and highlighted that secondary amines are significantly more reactive with nitrite [57]. The nitrosation reaction is most pronounced under acidic conditions, with an optimal pH of approximately 3–4 [58]. Nitrite may also come from impurities from solvents, water, and reagents used during API manufacturing. Even trace amounts of nitrite-contaminated materials can lead to *N*-nitrosamine formation.

The formation of *N*-nitrosamines is not limited to the direct use of nitrosating agents, nitrite can also be generated indirectly during various synthetic steps, including *via* the oxidation of hydroxylamine [32,59]. Notably, Jires et al. demonstrated that nitrosation in metformin film-coated tablets can occur even in the absence of nitrosating agents. In this case, the process involves the oxidation of dimethylamine in the presence of strong oxidants such as hydrogen peroxide. The NDMA content formed through this pathway is relatively low, contributing less than 10 % of the total impurities [59].

4.1.3. Use of low-quality materials

The introduction of low-quality or contaminated materials into the production process inevitably leads to the presence of *N*-nitrosamine impurities. Sources of contamination include starting materials, intermediates, recycled solvents, reagents, and catalysts. For instance, solvents such as ethyl acetate, ortho-xylene, toluene, and methylene chloride have been reported to contain *N*-nitrosamines [1,60]. Kosuri et al. analyzed commercial solvent samples and found that toluene was contaminated with 550 ppb of NDEA, while ortho-xylene contained 120 ppb of NDEA and 3400 ppb of *N*-nitrosodi-*N*-butylamine (NDBA) [60]. Ensuring the quality of raw materials involved in the chemical synthesis is therefore essential to maintaining the quality of the finished product.

4.1.4. Reaction parameters

Reaction parameters such as pH and temperature can significantly promote or reduce the yield of API synthesis and influence the formation of *N*-nitrosamines [1]. Elevated temperatures and humidity exacerbate *N*-nitrosamine formation during synthesis [32]. Nitrosation reactions are facilitated in the pH range from 3 to 4 [58], while alkaline conditions favor nitrite formation through hydroxylamine oxidation [59]. Additionally, poor control over the sequence of reagent, intermediate, catalyst, or solvent addition may increase the risk of *N*-nitrosamine formation. Uncontrolled reaction conditions can lead to batch-to-batch inconsistencies in API quality [1].

4.2. Manufacturing of finished products

The manufacturing of finished pharmaceutical products includes two critical stages: drug product formulation and packaging. These operations are more commonly associated with the formation of NDSRIs rather than small-molecule *N*-nitrosamine impurities. NDSRIs predominantly form due to the coexistence of nitrite and nitrosatable drug substances containing amine moieties. Over the past two years, NDSRIs have attracted increasing regulatory attention, their reported numbers have grown substantially. As of the most recent update in February 2025, the EMA has reported 194 *N*-nitrosamines, of which 176 are classified as NDSRIs, representing over 90 % of the total, while small-molecule *N*-nitrosamines account for less than 10 % [27].

Processes involving nitrite or generating it in the presence of amines should be considered high-risk for *N*-nitrosamine formation [61]. The reaction between secondary amines and nitrite under acidic conditions is the most likely pathway for *N*-nitrosamine generation during the manufacturing and storage stages of APIs and drug products [62]. Furthermore, starting materials and intermediates may be susceptible to carry-over or cross-contamination if manufacturing is conducted in shared facilities without sufficient cleaning controls [1,63].

4.2.1. Drug product formulation

4.2.1.1. Nitrite-contaminated excipients. Excipients play an essential role in the formulation of dosage forms and the selection of delivery routes [64,65]. Although they are pharmacologically inert, excipients contribute various functional roles, such as enhancing the volume or size of dosage forms, promoting disintegration, binding particulates, taste masking, and modifying drug release [64]. However, nitrite contamination in excipients has emerged as a major source of NDSRI formation due to unintended interactions with nitrosatable APIs. Jires et al. used ultra-high-performance liquid chromatography coupled with a simple quadrupole mass detector (UHPLC-MS) to evaluate nitrite content in excipients, finding nitrite levels below the QL (0.2 ppm) in most excipients, although some excipients showed nitrite levels up to 3.3 ppm [61]. Schlingemann et al. reported nitrite levels of a similar magnitude, with a maximum of 6.1 ppm nitrite detected in excipients [66]. Although small-molecule *N*-nitrosamines are formed less frequently, they can still occur during manufacturing or storage. For instance, dimethylamine is an impurity in metformin hydrochloride and reacts with nitrite to form NDMA [67].

Interestingly, Moser et al. highlighted that secondary amines originating from excipients present a considerably lower risk of *N*-nitrosamine formation compared to secondary amines that are integrated into the drug substance structures [57]. Nevertheless, higher nitrite levels are generally associated with an increased risk of *N*-nitrosamine formation in pharmaceuticals [61].

These data highlight the crucial need for rigorous quality control of

excipients, particularly those used in solid oral dosage forms, given their prevalence in the pharmaceutical industry, their simplicity and high acceptability by patients [68].

4.2.1.2. Manufacturing processes. Purified water, one of the most widely used utilities in the pharmaceutical industry, plays an indispensable role in various steps of API synthesis and manufacturing. It represents a potential source of trace *N*-nitrosamines and nitrite contamination. The quality of purified water is directly influenced by the quality of the input water [32]. Regarding *N*-nitrosamine presence, although disinfection with chloramine can generate trace amounts of NDMA and other *N*-nitrosamines in input water [69], the EMA has stated that water used in pharmaceutical manufacturing is unlikely to be a significant source of *N*-nitrosamine contamination in APIs. Nevertheless, the potential risk for degradation processes in drug substances due to disinfected water cannot be entirely excluded [70]. Among existing techniques, UV direct photolysis has been identified as the most effective for removing several *N*-nitrosamines from water [71,72]. In addition, despite ongoing rigorous purification processes, purified water may still contain residual nitrite at very low levels. Suresh Kumar et al. developed an ion chromatography (IC) method to quantify nitrite content in manufacturing water. Their study revealed that 21 of 22 purified water samples contained nitrite levels below the quantification limit (QL) of 0.1 ppb, indicating that the risk of *N*-nitrosamine formation from this source is negligible [73].

Nitrogen oxides (NO_x) in the air may act as nitrosation precursors and react with at-risk APIs [32]. For example, trace levels of nitrite can result from oxidation processes involving NO_x species during drying operations [61,74].

Certain manufacturing processes characterized by excessive energy, temperature, forced air, or high moisture can create favorable conditions for *N*-nitrosamine formation [1]. Solid oral dosage forms, such as tablets and capsules, remain the most popular pharmaceutical products [68]. Among common manufacturing processes, wet granulation often involves the use of solvents, introducing moisture into the process [75], whereas processes such as jet milling and fluidized bed drying typically operate under high temperatures and pressures [76].

Therefore, manufacturing process parameters must be carefully controlled to ensure batch-to-batch consistency. Poorly optimized process parameters could inadvertently facilitate *N*-nitrosamine formation. The lack of process control may also lead to significant variability in product quality and inconsistent *N*-nitrosamine levels across batches [1].

4.2.2. Packaging and printing

Packaging materials, integral to pharmaceutical products, are classified as primary, secondary, or tertiary. Primary packaging, which comes into direct contact with the formulation, logically poses the highest risk of interacting with the drug product [77]. Certain polymers, elastomers, and adhesives used in packaging can either contain *N*-nitrosamines or contribute to their formation under specific conditions, particularly during storage [78,79]. Among these, nitrocellulose-based blisters have been identified as a major concern regarding *N*-nitrosamine contamination [27]. Blister packaging, widely used for solid dosage forms, often incorporate lidding foils containing nitrocellulose in the print primer and/or lacquer layers [80]. The classical structure of a multi-layer aluminum lidding foil is illustrated in Fig. 7 [81].

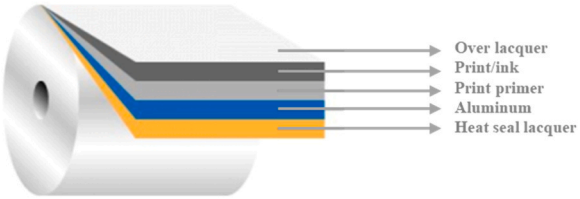

Fig. 7. Structure of a multi-layer lidding foil.

Golob et al. demonstrated that nitrocellulose can act as a nitrosating agent, capable of reacting with vulnerable amines in printing inks, *e.g.*, dimethylamine or diethylamine, to form NDMA or NDEA. *N*-nitrosamines or their precursors may migrate from the packaging materials into the product, particularly when there is direct contact. High-temperature heat-sealing processes can volatilize *N*-nitrosamines from the lidding foil, allowing their transfer and deposition onto tablets in adjacent blister cavities [81]. The possibility of nitroso-compounds migrating into the blister cavity and subsequently interacting with the bulk product to form NDSRIs cannot be excluded [32].

Additionally, elastomers used as vial stoppers in container closure systems are a well-established potential source of *N*-nitrosamine contamination in the primary packaging of sterile injectable or parenteral dosage forms [78,82].

While secondary packaging and manufacturing equipment pose a smaller risk, they may still contribute to *N*-nitrosamine contamination through extractable and leachable substances. However, Regulatory agencies now mandate extractable, leachable, and ink migration studies during Step 1 of the investigation procedure to assess the risks of substance migration from packaging materials to drug formulations [1,77]. For example, the USP has identified *N*-nitrosamines as “special case” compounds, and 6 *N*-nitrosamines (NDMA, NDEA, NDBA, *N*-nitrosomorpholine, *N*-nitrosopiperidine, and *N*-nitrosopyrrolidine) are typically investigated as extractables and leachables in orally inhaled and nasal drug products [79]. The assessment of extractables and leachables associated with pharmaceutical packaging and delivery systems will be discussed in Section 6.7.

4.3. Storage of drug substances and products

Inappropriate storage conditions, such as high temperatures, humidity, or exposure to light, can lead to the degradation of APIs. This degradation compromises the efficacy, quality, and safety of medicines. Under such conditions, *N*-nitrosamines can form as degradation impurities through chemical reactions such as oxidation, hydrolysis, and photolysis. Additionally, they can arise as byproducts, for instance, following the nitrosation of APIs or API-related substances in the presence of precursors.

For example, Jires et al. studied the stability of sitagliptin during storage and identified that the hydrolysis of its amide bond results in the formation of degradation products such as amino acid compounds and 3-(trifluoromethyl)-5,6,7,8-tetrahydro-(1,2,4)triazolo(4,3-a)pyrazine, the latter can react with nitrosating precursors to form *N*-nitroso-STG-19 (*N*-nitroso-3-(trifluoromethyl)-5,6,7,8-tetrahydro-(1,2,4)triazolo(4,3-a) pyrazine) [83].

Furthermore, chemical reactions or degradation processes occurring within the packaging material itself during storage can also contribute to *N*-nitrosamine formation.

4.4. Combined root causes

Although *N*-nitrosamine contamination is sometimes attributed to a single source, it is often the result of multiple interacting factors. Understanding these combined root causes is central for implementing effective risk mitigation strategies. This section explores the topic through selected illustrative examples.

4.4.1. Metformin

In metformin, dimethylamine, designated as Impurity F in the Ph. Eur. monograph, is limited to a maximum of 0.05 % in metformin hydrochloride [84]. Dimethylamine is a well-documented impurity in metformin raw materials and forms NDMA when exposed to nitrite [67]. However, forced degradation studies by Hao et al. revealed that NDMA formation is not limited to dimethylamine contamination. These studies

demonstrated that metformin degrades into dimethylamine and NDMA under stress conditions such as elevated temperatures, strong alkaline and oxidative conditions. NDMA can also form during the storage of metformin APIs or finished products under inappropriate conditions [85]. It is important to note that NDMA is not the only *N*-nitrosamine that can form through the nitrosation of synthesis byproducts or degradation impurities.

4.4.2. Rifampin and rifapentine

Rifampin and rifapentine are associated with two impurities, MeNP in rifampin, and CPNP in rifapentine [33,36]. Although both MeNP and CPNP have low molecular weights, they are considered NDSRIs due to their structural similarities with rifampin and rifapentine, as illustrated in Fig. 8.

MeNP and CPNP can be formed from the synthesis of 1-methyl-4-aminopiperazine (MAP) and 1-cyclopentyl-4-aminopiperazine (CPAP), respectively, using $NaNO_2$ [86]. These intermediates are key precursors in the synthesis of rifampin and rifapentine [33,36,86]. Tian et al. confirmed that MeNP contamination could originate from MAP supplied by manufacturers [86]. Furthermore, higher levels of MeNP and CPNP have been generally observed in drug products compared to their corresponding API batches [36]. This observation may be attributed to the oxidation of MAP or CPAP, or the hydrolysis of APIs during storage, leading to the formation of MeNP and CPNP as degradation products [36,86]. Additionally, the polymorphic form of rifampin API affects the generation of MeNP. Tian et al. found that the crystal form I of rifampin is chemically more stable than form II, resulting in significantly lower MeNP content when form I is used [86].

4.4.3. Particular case of biopharmaceuticals

This section addresses distinct considerations related to biopharmaceuticals. Although biological drugs generally pose a lower risk of *N*-nitrosamine formation compared to APIs derived from chemical synthesis, the risk cannot be entirely dismissed. Several studies have identified specific risks associated with biopharmaceuticals, particularly regarding NDLRIs in ADCs. ADCs represent a unique case within the biopharmaceutical category due to their hybrid nature, combining drug components with monoclonal antibodies *via* chemical drug linkers. The synthetic components within ADCs inherently increase the risk of NDLRIs. Consequently, this therapeutic class is considered higher risk compared to monoclonal antibodies and oligonucleotides, without synthetic components in their structures [20,21,87].

As already mentioned, the quality of water, reagents, and raw materials are critical factors in risk assessment. For instance, the risk of *N*-nitrosamine contamination in biological products is lower than in synthetic drugs due to the use of water for injection and high-purity reagents or excipients. Furthermore, the presence of nitrosating precursors as impurities in drug linkers is typically negligible, as these precursors can be effectively removed post-conjugation through purification techniques such as ultrafiltration or diafiltration [20,21]. Unlike oral solid dosage forms, biopharmaceuticals are usually packaged in glass containers, which are free from nitrocellulose to minimize the presence of nitrosating precursors. Glass container closure systems are specifically designed to prevent permeation of oxygen, moisture, or microorganism, thereby ensuring sterility and reducing degradation caused by oxidation or hydrolysis. However, when elastomeric materials are used as vial stoppers, the associated risk should be carefully evaluated, as previously discussed [82]. Moreover, biopharmaceuticals are typically stored under controlled conditions and low temperature to maintain product stability and prevent degradation.

5. *N*-nitrosamine risk mitigation measures

When a risk of *N*-nitrosamine contamination over the acceptable limit is identified, well-established, and confirmed, API manufacturers and (bio)pharmaceutical companies are required to implement mitigation measures. The actions are tailored to address the specific root causes of contamination, aiming to effectively prevent or reduce *N*-nitrosamine levels in APIs and drug products.

Regulatory agencies have published official documents to guide manufacturers to control *N*-nitrosamine impurities [1,27]. The mitigation plans focus on specific root causes, including synthesis pathways, manufacturing processes, packaging materials, and storage. Based on the potential sources of *N*-nitrosamine impurity formation presented in 4, a summary of possible mitigation strategies is provided in Table 3.

6. Risk-based prediction tools for *N*-nitrosamine formation

6.1. Nitrite excipient database

As developed in previous sections, *N*-nitrosamine impurities predominantly originate from nitrite in excipients and amines, whether present as impurities or as functional groups within drug substances. However, the availability of reliable information regarding nitrite levels

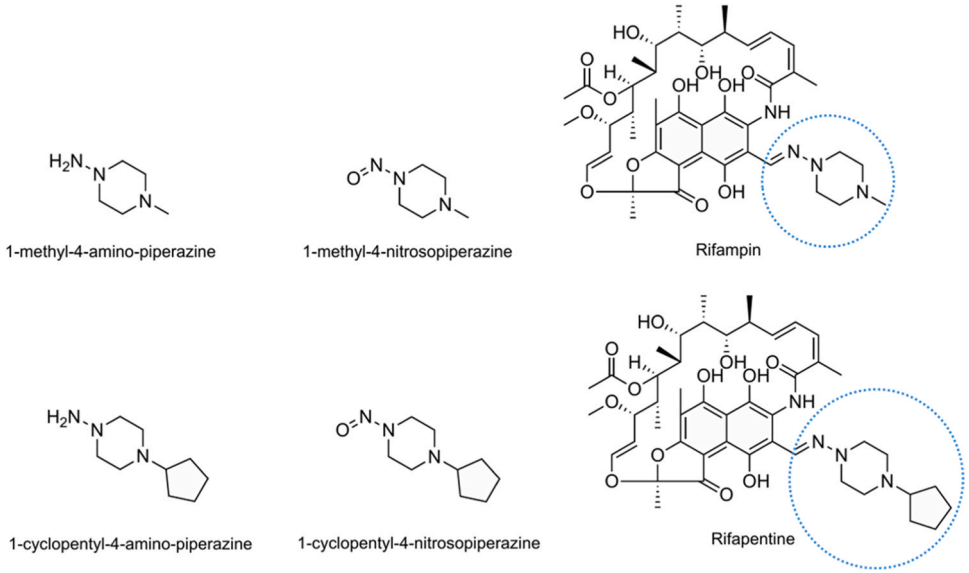

Fig. 8. Structures of MeNP, CPNP, MAP, CPAP, rifampin and rifapentine.

Table 3
Risk mitigation measures.

Stage	Root cause	Mitigation measure
Chemical synthesis of APIs	Routes of chemical synthesis	Use of alternative synthesis routes Use of alternative quenching agents other than NaN_3
	Use of nitrosating agents	Use of alternative reagents Addition of scavengers to remove the residual nitrosating precursors [35]
	Use of low-quality materials	Screening materials for pharmaceutical use [1] Supplier qualification and audits [1]
	Reaction parameters	Control and optimization of reaction parameters to maintain *N*-nitrosamine contents within acceptable limits and ensure batch-to-batch consistency [1]
Formulation of finished products	Nitrite-contaminated excipients	Screening excipients for nitrite content [61] Supplier qualification and audits [1] Use of low-nitrite excipients [35] Addition of a scavenger [35]
	Uncontrolled manufacturing processed	Control and optimization of process parameters (*e.g.*, temperature, moisture, energy) to reduce *N*-nitrosamine formation and ensure batch-to-batch consistency [1]
Packaging and printing	Use of nitrocellulose-based lidding foils	Use of nitrocellulose-free lidding foils or alternative materials [81] Use of alternative packaging such as plastic bottles
	Use of elastomeric vial stoppers	Use of alternative packaging forms
	Inappropriate packaging	Redesigning packaging systems or employing alternative packaging materials/forms to minimize the risk of *N*-nitrosamine formation or migration
Storage	API degradation - oxidation	Addition of antioxidants to inhibit NDSRI formation in drug products due to oxidation [35] Use of vapor(air)-impermeable packaging
	API degradation - hydrolysis	Addition of desiccant in packaging to prevent or control excessive moisture Use of moisture-impermeable packaging Addition of Na_2CO_3 in tablet formulation to modify the microenvironment to neutral or weak basic pH for inhibition of NDSRI formation in drug products [59,85]
	API degradation - polymorphism	Use of stable crystal forms [86] (without compromising the solubility and processability if possible)
	Inappropriate storage conditions	Avoid exposure to light, and storage under high temperature or humid conditions
	Intrinsic API instability	Shorten the expiration date

in excipients remains limited. This lack of data creates challenges for regulators and manufacturers to predict and evaluate the risks of *N*-nitrosamine formation in medicinal products [65].

To address this challenge, Lhasa Limited, a non-profit organization and educational charity, has established a data-sharing initiative to encourage and motivate pharmaceutical companies to report nitrite concentrations in excipients, measured using validated analytical procedures. Excipients covered in the database are representative of common *N*-nitrosamine formations. Generally, the nitrite contribution is dominated by major excipients such as diluents (*e.g.*, microcrystalline cellulose, mannitol), which are used in larger quantities in drug products but typically contain low nitrite levels. In contrast, excipients like magnesium stearate (a lubricant) and crospovidone (a disintegrant), although present in smaller amounts, are associated with higher nitrite levels. The assessment also takes into account factors such as the average nitrite content, batch-to-batch variability, and differences between suppliers. The database, which continues to expand, is a valuable tool for supporting the risk assessment of *N*-nitrosamine presence in drug products [65].

6.2. Nitrite testing

The determination of nitrite content in excipients plays a central role in the quality control of excipients and aids indirectly in predicting or assessing the potential *N*-nitrosamine formation. Commonly employed methods for nitrite determination include IC with conductivity detection and the Griess reaction followed by liquid chromatography coupled with UV detection [65,73]. Both techniques have demonstrated adequate sensitivity and specificity to distinguish nitrite from nitrate [73]. Table 4 summarizes various analytical procedures for nitrite determination in excipients reported in the literature.

However, Yamamoto et al. highlighted that other compounds than nitrite, such as NO_x species in excipients, could also contribute to the nitrosation of amines. Focusing solely on nitrite may underestimate the risk of *N*-nitrosamine formation during manufacturing and storage. To overcome this limitation, a novel approach based on the quantification of total reactive NO_x species in excipients was proposed to evaluate the risk of *N*-nitrosamine formation [89].

6.3. Nitrosation assay procedure

In 1978, the WHO Expert Group proposed the nitrosation assay procedure (NAP test) as a general *in vitro* test system for evaluating the nitrosatability of drug substances [90]. The conventional NAP test is conducted under standardized conditions using an API concentration of 10 mM and a nitrite ($NaNO_2$) concentration of 40 mM. The reaction is carried out at 37 °C in an aqueous acidic solution with a pH of 3–4 for 1–4 h to facilitate the nitrosation reaction, intentionally leading to the formation of NDSRIs [90].

Regulatory authorities have started recommending NAP tests to assess the risk of NDSRI formation from APIs. It is advised that the NAP test be performed on starting materials, intermediates, and APIs during manufacturing process development [70]. For instance, applicants may include NAP test results as supportive documentation in the *N*-nitrosamine risk evaluation (Step 1) of the Common Technical Document [27]. The Common Technical Document is a harmonized format designed for the submission of new marketing authorizations or new drug applications across all regions [91].

In 2022, Schmidtsdorff et al. applied the conventional NAP test to evaluate the potential formation of NDSRIs *via* direct nitrosation of drug substances. Among 67 compounds studied, 33 APIs were found to form *N*-nitroso compounds as a result of API-nitrite interactions [92].

In 2023, Nitish et al. introduced a modified version of the NAP test to address certain limitations of the conventional method. These shortcomings include the restricted aqueous solubility of drugs belonging to biopharmaceutical classification system classes II and IV, as well as the degradation of many drugs into unintended by-products rather than NDSRIs. In the modified procedure, the drug is solubilized in an organic solvent and incubated at 37 °C with tertiary butyl nitrite as the nitrosating agent in a 1:10 molar ratio. This alternative approach is more reactive and specific, enabling the generation of higher levels of NDSRIs in organic solvents within a shorter time frame [93].

6.4. Carcinogenicity prediction – Ames test

If NAP tests yield positive results, regulators recommend further investigations, including the Ames test, in accordance with the ICH M7 (R2) guideline. The Ames test, developed by Ames in the 1970s, is an *in vitro* bacterial bioassay designed to evaluate the mutagenic potential of chemical compounds [94]. This test provides direct experimental

Table 4
Summary of analytical procedures for nitrite determination in excipients.

Technique	Sample	DL	Range	Sample preparation	Column	Mobile phase/gas	Ref.
IC-Conductivity suppressor	Purified water	0.033 ppb	0.1–0.6 ppb	25-fold concentration	Dionex IonPac RFIC Analytical AS19 column, 4 × 250 mm Dionex IonPac RFIC Guard AG19, 4 × 50 mm	20 mM sodium hydroxide in water	[73]
UHPLC-(ESI+) MS	Excipients and metformin tablets	0.005 ppm	0.2–10 ppm	Liquide extraction 2,3-diamino-naphthalene Derivatization	Acquity UPLC HSS T3, 100 × 2.1 mm (1.8 μm)	0.1 % formic acid acetonitrile	[61]
GC-MS	Excipients	Not reported	Lower range: 0.05–2.5 ppm Upper range: 1.0–50.0 ppm	Stearate magnesium: liquid-liquid extraction Carbonates: CO_2 release Other samples: liquid extraction	DB-624 UI, 30 m × 0.25 mm (1.4 μm)	Helium	[62]
Two dimensional IC-MS	Hydroxypropyl methylcellulose	8.9 ppb	29.6–5005.8 ppb	Liquid extraction	^{1}D: Dionex IonPac AS-24A, 4 × 250 mm (7.0 μm, 2000 Å) and IonPac AG-24A 4 × 50 mm (11.0 μm, <10 Å) ^{2}D: IonPac AS-19, 2 × 250 mm (7.5 μm, 2000 Å) and IonPac AG-19 2 × 50 mm (11.0 μm, < 1 Å)	Potassium hydroxide in water (concentration not specified)	[88]

IC: Ion chromatography; UHPLC: Ultra-high-performance liquid chromatography; ESI: Electrospray ionization; MS: Simple quadrupole mass spectrometer; GC: Gas chromatography (by analyzing cyclohexene generated from the reaction of cyclamate and nitrite); DL: Detection limit.

evidence regarding a compound's ability to induce mutations in bacteria, which correlates with an increased risk of carcinogenicity [95]. Standardized recommendations for conducting the Ames test are outlined in the OECD's test guideline [96].

Ames test data have been incorporated into the Carcinogenic Potency Database and have played an important role in the development of structure-activity relationship (SAR) models [95]. While the Ames test has demonstrated positive results for most *N*-nitrosamines, a small number of these compounds have shown negative outcomes.

Although the Ames test data serve as highly effective qualitative tools for predicting carcinogenicity in rodent studies, it has limitations in providing a quantitative assessment of carcinogenic potency [97,98]. To establish non-mutagenicity, reliable data from a well-conducted Ames test, as outlined in the ICH M7(R2) guideline, are essential [70]. However, reduced sensitivity has been observed for certain *N*-nitrosamines under standard Ames test conditions [27,37].

To address this limitation, the FDA recommends using of the EAT, a modified version of the classic Ames test. The EAT is specifically designed to evaluate *N*-nitrosamine compounds that may exhibit limited sensitivity in the standard Ames test due to constrained metabolic activation [37]. The EAT protocol was developed based on research conducted by the FDA's National Center for Toxicological Research [99].

If the classic Ames test yields positive results, these findings are considered sufficient, and conducting an additional EAT is not required [27,37].

6.5. Forced degradation study

While NAP tests are utilized to predict NDSRI formation from drug substances, forced degradation studies serve as a complementary approach to assess the potential formation of *N*-nitrosamine impurities as degradants [100,101]. These studies involve exposing drug substances and/or products to stress conditions, such as heat, moisture, strong acidic or alkaline environments, oxidation, and photolysis to simulate extreme storage conditions that are more severe than accelerated conditions [102]. Forced degradation studies provide valuable insights, including comprehensive impurity profiling, degradation behavior, structural elucidation of degradation products, and an understanding of degradation pathways and kinetics. These studies are a regulatory requirement for assessing the intrinsic stability of drug substances and products [100,101]. Moreover, they provide insights that guide manufacturing, packaging, and storage optimizations to enhance product stability and minimize impurity formation.

For instance, Hao et al. demonstrated that metformin undergoes significant degradation into NDMA under strong oxidative conditions and elevated temperatures, highlighting the importance of controlling such conditions during manufacturing and storage to minimize impurity formation [85]. Tian et al. investigated the pathways of MeNP formation in rifampin through forced degradation studies. Their findings revealed that MeNP is not only a by-product of the oxidative degradation of rifampin but also introduced during the synthesis process involving MAP, either through oxidation or nitrosation reactions [86]. Emery Pharma's stability study revealed that elevated temperatures significantly promote and accelerate ranitidine degradation leading to NDMA formation. The NDMA content in ranitidine API increased 6-fold when stored at 70 °C compared to 25 °C after 12 days. For ranitidine formulations (Zantac®, 150 mg), NDMA levels in a tablet increased from 19 ng at the initial time to 70 ng after 14 days at 70 °C [103].

6.6. Stability testing

Unlike forced degradation studies, the purpose of stability testing is to generate evidence on how the quality of an API or finished product changes over time under the influence of temperature and humidity. Another objective is to establish appropriate storage conditions that ensure the quality of the product and to determine the retest period for APIs or the shelf life of finished products. Stability studies should be performed on packaged forms, mimicking real use conditions, provide supporting data on the suitability of the packaging and potential packaging-product interactions [100].

Stability studies should take into account zone-specific climate factors, particularly temperature and humidity. Several types of stability testing exist. Long-term testing evaluates the stability under recommended storage conditions for extended periods. Intermediate and accelerated testing are conducted under increased temperature and humidity for shorter periods to assess the impact of short-term excursions beyond recommended storage conditions [100]. In line with the guidance, confirmatory testing (Step 2) must include both newly manufactured batches and stability batches stored under long-term

conditions and still within their expiry date [27].

6.7. Extractable and leachable assessment

Management of extractables and leachables in packaging systems and final drug products has become a critical aspect of pharmaceutical development and regulatory submissions. This is due to their potential safety risks for patients and possible compatibility issues with the drug product. Scientific principles and best practices for the assessment of extractables and leachables are described in the USP general chapters <1663> [104] and <1664> [105], respectively.

Extractables and leachables refer to inorganic and organic chemical entities. Extractables are compounds that can be released from pharmaceutical packaging or delivery system into extraction solvents under controlled laboratory conditions. In contrast, leachables are substances that migrate from the packaging or delivery system into the drug product under normal storage and use conditions, or during accelerated stability studies [104,105].

Extraction studies are conducted to determine the extractable profile of packaging components. It is essential that these studies are carefully designed to ensure the extraction of substances. Key factors include the choice of solvent, extraction time, temperature, and technique. For examples, rubber stoppers from vials may be extracted using buffers at pH 5.2 or 9.5, or a 50 %/50 % (v/v) mixture of isopropanol and water; the mouthpiece of a dry powder inhaler may be extracted with water or isopropanol; the elastomer seal of a metered-dose inhaler valve may require extraction with organic solvents. Once generated under laboratory conditions, extractables must be characterized to understand their potential impact [104].

After packaging, dosage forms come into direct contact with packaging materials, and interactions can occur. To evaluate the risk of leachables, it is necessary to identify them and determine the level to which they may accumulate in the final product over its shelf life. Among various dosage forms, orally inhaled and nasal drug products are considered the most susceptible to interactions with packaging components [105]. These interactions may lead to the formation of leachables such as *N*-nitrosamines [79]. A specific case is migration, which involves the transfer of a substance across a physical barrier, for instance, ink migrating through blister packaging into the drug product.

In a recent study, Dalkılıç et al. investigated the presence of 15 *N*-nitrosamines as extractables in various pharmaceutical packaging materials, including polypropylene bags, disposable eye drop containers, low-density polyethylene bottles, and bromobutyl stoppers. Multiple extraction methods such as Soxhlet extraction, reflux, oven heating, and ultrasonic bath were used to extract *N*-nitrosamines from the packaging components. Obtained results showed that all *N*-nitrosamines were below the method's detection limits, which ranged from 0.006 to 0.909 ng/mL. In parallel, leachable studies were conducted on commercial drug formulations containing sugammadex, metformin, gliclazide, and paracetamol. These studies also confirmed that none of the formulations contained detectable levels of *N*-nitrosamines [106].

6.8. In silico purge-based approach

Potential mutagenic impurities, including *N*-nitrosamines, can be introduced or formed at any stage during the synthesis of APIs [107]. Relying solely on analytical testing of pharmaceuticals to investigate *N*-nitrosamine presence poses significant challenges to MAHs and/or applicants. To address this, Burns et al. proposed a novel purge-based approach in 2020. This conservative, risk-based strategy maintains regulatory confidence and ensures patient safety [54]. This approach also offers the benefit of minimizing costs and time associated with analytical development and testing, when potential mutagenic impurities are unlikely to be present in the final drug product [108].

In the purge-based approach, purge factors are calculated by evaluating physicochemical properties, such as reactivity, solubility, volatility, and acid-base characteristics (pKa/pKb), in relation to the reactions involved, to estimate the likelihood of impurity removal [54]. Understanding *N*-nitrosamine reactivity is essential for assessing the risk of their presence in the final product and evaluating their expected removal during existing chemical processes [109].

Mirabilis™ is an *in silico* risk assessment tool developed through collaboration between Lhasa Limited and pharmaceutical companies [54,107]. This software standardizes purge factor calculations to enhance the control and management of potential mutagenic impurities, harmonizing industry practices. In 2022, the release of Mirabilis™ 4 introduced refinements for improved predictive accuracy [108]. The tool utilizes advanced predictive technologies to evaluate PMI formation risks and identifies potential impurity structures based on the reagents and conditions used during each step of API manufacturing. By integrating the purge-based approach with *in silico* tools, the industry can perform rapid, consistent, and reproducible risk assessments for mutagenic impurities [54].

7. Conclusion

Facing the challenges posed by *N*-nitrosamine impurities, regulatory guidelines have rapidly evolved. Regulatory bodies have required API manufacturers and (bio)pharmaceutical companies to adopt a proactive, systematic 3-step approach for controlling these impurities. The approach involves evaluating contamination risks, performing analytical confirmatory testing, and implementing mitigation strategies.

The establishment of collaborative platforms such as the USP Nitrosamine Exchange, combined with advancements in the analytical chemistry field, has significantly enhanced the industry's ability to respond effectively. While the current understanding of small-molecule *N*-nitrosamines has progressed significantly, recent concerns focusing on NDSRIs and NDLRIs highlight persistent challenges. Further research is essential to refine risk assessment methodologies and establish robust carcinogenic potency data for these emerging impurities.

The aim of this review is to provide a clear overview of the current situation of *N*-nitrosamine control in pharmaceutical products. It presents methodologies used to determine acceptable daily intake limits for *N*-nitrosamines and traces the evolution of regulatory guidelines, offering a comparative analysis of the 3-step investigation approaches adopted by the EMA and FDA. The reviews examines potential root causes for *N*-nitrosamine contamination, outlines the analytical requirements for confirmatory testing, and describes mitigation strategies to prevent or minimize contamination. Additionally, it summarizes risk assessment tools used to predict *N*-nitrosamine formation.

Finally, by providing a comprehensive workflow for *N*-nitrosamine impurity investigation, this review also aims to support API manufacturers and (bio)pharmaceutical companies in managing *N*-nitrosamine risks. It emphasizes the need for continued vigilance in light of their carcinogenic potential and highlights the importance of innovation and collaboration among regulatory agencies, pharmaceutical manufacturers, and researchers to minimize the presence of *N*-nitrosamine in medicines, thereby ensuring drug safety.

CRediT authorship contribution statement

Yue Zhang: Writing – review & editing, Writing – original draft. **Joëlle Widart:** Writing – review & editing. **Eric Ziemons:** Writing – review & editing. **Philippe Hubert:** Writing – review & editing. **Cédric Hubert:** Writing – review & editing.

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Data availability

No data was used for the research described in the article.

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