Active Pharmaceutical Ingredients Committee (APIC)

Nitrosamine Risk Management: Guidance for API Manufacturers

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1. Foreword

This guidance document was first prepared in 2020 by the APIC Nitrosamines Task Force on behalf of the Active Pharmaceutical Ingredient Committee (APIC). 2nd revision was prepared by a sub-group of this taskforce in 2025, with significant input from others in their companies.

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2. Revision History

Version	Changes	Date
1	First issue. Previous title "Additional guidance on the assessment on the risk assessment for presence of N-nitrosamines in APIs".	18 February 2020
2	Revised to reflect latest guidance and recommended practice. The structure of the document is extended to follow recent regulatory requirements (mainly EMA) regarding risk assessment principles, root causes considered, analytical testing, control and mitigation measures. This includes, but not limited to: comprehensive discussion on nitrosamine formation mechanisms, extension of the scope from small nitrosamines toward Nitrosamine drug substance related impurities (NDSRIs), approaches toward theoretical risk assessments with purge factor calculations, guidance for detailed evaluation of risk from water, API testing, including methodology, with acceptable limit calculation, implementation of suitable control/mitigation strategies. At last, but not least, a separate chapter is dedicated to incorporation of nitrosamine risk management into the quality management system.	13 March 2025

3. Purpose and Scope

N-Nitrosamines (Nitrosamines) are organic compounds with a chemical structure R_2N-N=O, where R is usually an alkyl group (refer to section 4.1 for the detailed definition).

Nitrosamines are classified as probable human carcinogens on the basis of animal studies.[1] Nitrosamines need to be metabolized in the body to elicit their mutagenic or, in many cases, carcinogenic behavior. It is believed that most of these compounds are activated by alpha hydroxylation in presence of Cytochrome P450 and NADPH (nicotinamide adenine dinucleotide phosphate), which leads to the formation of an electrophilic carbocation and can alkylate the DNA.

Due to their extremely high carcinogenic potency, ICH M7 defines these substances as being part of the Cohort of Concern (CoC) [9], leading to much lower acceptable intakes than the typical threshold of toxicological concern (TTC) concept from ICH guideline M7 that limits exposure to mutagenic compounds to a maximum acceptable intake of 1.5 µg/day.

These substances are commonly found in food, tobacco and the environment, but since 2018 there have also been reports and recalls due to their presence in pharmaceuticals. Following notification of nitrosamines in some medicines in 2018, multiple health authorities request Marketing Authorization Holders (MAHs) to review all chemical and biological human medicines for the possible presence of nitrosamines and test products at risk.[2][3][4][5][5] API manufacturers are also required to assess a risk of nitrosamine presence to assure safety of APIs, address regulatory requests and to support MAHs.[6][7]

Regulatory guidance has been updated frequently as knowledge of nitrosamine risk has developed. There have been notable developments since the first version of this document was issued, particularly in the identification of risk factors relating to nitrosamine formation and nitrosamine acceptable limit determination. This guide is intended to support API/API intermediate manufacturers in evaluating

nitrosamine risk and provide information to supplement nitrosamine risk management practice based upon current experience and understanding of APIC member company representatives. However, readers should be aware that variation in interpretation and decision making is observed. Furthermore, knowledge and practice are still developing and acceptance by authorities might vary and change.

The guidance excludes expectations for regulatory submission content. Proposed content of risk assessment for health authorities submission is captured APIC template document.[18] in It also excludes assessment of N-nitroso compounds, such as nitrosamides, as well as nitrosoguanidines, N-nitrosocarbamates and N-nitrosoureas [2][8][56]. Current understanding at time of writing is that N-nitrosamides, N-nitrosoguanidines, N-nitrosocarbamates and N-nitrosoureas risk should be assessed as per the ICH M7 guideline.

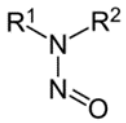
In scope of this APIC guidance document are chemically synthesized APIs and intermediates used for human medicinal products and will include evaluation of all materials used in the API synthesis (starting materials and intermediates, reagents, solvents and other raw materials).

- All chemically synthesized APIs and intermediates used for human medicinal products (including synthetic peptides, synthetic oligonucleotides)
- Fermentation APIs
- Herbal products, and crude products of animal or plant origin
- Semi-synthetic APIs

Although biological medicinal products and biological APIs are in the scope of Health Authorities requests to evaluate nitrosamines risk[2], this type of medicines are exempted for this APIC guidance document. For the nitrosamines risk assessment in biologics please refer to EFPIA guidance document [51].

4. Definitions and Abbreviations

4.1. Definitions

Term	Definition
Nitrosamine and N-nitrosamine	The terms "nitrosamine" and "N-nitrosamine" are used interchangeably within this document, and both are referring to the following structure as defined in EMA/409815/2020 document:  However, the structure of R^1 and R^2, not defined in guidelines, should be linked to the definition of vulnerable amines in the row below. Compounds for which amine precursor do not match bellow's definition (e.g. imidazole, indole, *N*-alkylamide (R^1NHCOR^2), N-alkylsulfonamide ($R^1NHSO_2R^2$), *N*-alkylguanidine ($R^1NH(C=NH)NHR^2$), diarylamine (ArNHAr), etc.) are considered as being N-nitroso compounds. These compounds are not part of the nitrosamine risk assessment, and should be considered under ICH M7 assessment.

Vulnerable / nitrosatable amines	The term “nitrosatable / vulnerable amines” corresponds to an amine function that have potential to react with nitrosating agents. According to EMA "nitrosatable" and "vulnerable" are used interchangeably. It corresponds to (but not limited to): Secondary amines (cyclic, acyclic), tertiary amines (cyclic, acyclic), secondary/tertiary amine precursors, such as (but not limited to) quaternary ammonium salts, N,N-dialkylamides (e.g. N-methyl-2-pyrrolidone, dimethylformamide, dimethylacetamide), N-chloroalkylamines, and N,N-dialkyl carbamates that can be converted to corresponding vulnerable amines. Secondary amines are of most concern (most vulnerable ones) as they can react with nitrosating agents significantly faster than most tertiary amines. Primary amines are no nitrosamine precursors (*i.e.* are not considered vulnerable amines). The nitrosation of an aliphatic primary amine yields an alkyl diazonium ion and water, not a nitrosamine. The alkyl diazonium ion is very reactive and will, *e.g.* form a hydroxyl compound and release N_2 in a reaction with water.
Vulnerable hydrazine, hydrazide or hydrazone	The term “vulnerable hydrazine, hydrazide or hydrazone” corresponds to a hydrazine, hydrazide or hydrazone function that can lead to N-nitrosamine derivative by means of an oxidation step.
Nitrosating agent	The term “nitrosating agent” corresponds to (but not limited to): nitric acid, nitrite salts, organic nitrites, nitrosonium salts, nitrogen oxides and nitro compounds.
Nitrosamine Drug Substance-Related Impurity	Nitrosamine Drug Substance-Related Impurities (NDSRI) are impurities which share structural similarity to the API (having the API or API fragment in the chemical structure) and are therefore unique to each API. NDSRIs generally form in the drug product through nitrosation of APIs (or API fragments) that have secondary or tertiary amines when exposed to nitrosating agents such as residual nitrites in excipients used to formulate the drug product, but could also be found in the API itself.
Enhanced Ames Test	Enhanced Ames Test (EAT) is a modified version of the classic Ames test, specifically modified to detect N-nitrosamines. For more details, refer to EMA/409815/2020.[2]
Structure Activity Relationships	Structure Activity Relationships (SAR) refers to the relationship between the molecular (sub) structure of a compound and its mutagenic activity using (Quantitative) Structure-Activity Relationships derived from experimental data.
Carcinogenic Potency Categorisation Approach	Carcinogenic Potency Categorisation Approach (CPCA) is an approach for assigning an N-nitrosamine impurity (including nitrosamine drug substance-related impurities [NDSRIs]) to a predicted carcinogenic potency category, with a corresponding acceptable intake (AI) limit, based on an assessment of activating or deactivating structural features present in the molecule. For more details, refer to EMA/409815/2020.[2]
Failure Mode and Effects Analysis	Failure Mode and Effects Analysis (FMEA) relies on product and process understanding. For more details refer to current ICH Q9 guideline (*ICH guideline Q9 on quality risk management*).

Acceptable intake	An intake level /limit associated with a theoretical excess lifetime cancer risk of 1:100,000 based on considerations in ICH M7(R2) for substances from the "cohort of concern".
Acceptable limit	According to EMA guideline[2] the term "acceptable limit" to avoid inclusion of nitrosamine impurity in API specification corresponds to 10-times lower limit than acceptable limit calculated from acceptable intake and maximum daily dose.
Confirmatory testing	Analytical testing of nitrosamines by a suitably validated method to confirm or refute experimentally the presence of nitrosamines. The purpose of the testing is to provide confirmatory testing data supporting the risk assessment in case a possible risk of presence of nitrosamines has been identified. The test methods need to be suitable for their intended use, using *e.g.* standard addition methodology or validation principles. The test should be capable to quantify the amounts of individual *N*-Nitrosamines or multiple *N*-Nitrosamines. The testing strategy for potential presence of the *N*-nitrosamine impurities consists of two consecutive steps for confirmatory testing: 1. Screening testing and/or 2. Confirmation testing (if required, based on outcome of screening testing)
Skip testing (periodic) vs. Routine testing	As per ICH Q6A definition periodic or skip testing is the performance of specified tests at release on pre-selected batches and/or at predetermined intervals, rather than on a batch-to-batch basis with the understanding that those batches not being tested still must meet all acceptance criteria established for that product.
Mutagenic impurity	An impurity that has been demonstrated to be mutagenic in an appropriate mutagenicity test model, *e.g.,* bacterial mutagenicity assay.
Purge factor	Purge reflects the ability of a process to reduce the level of an impurity, and the purge factor is defined as the level of an impurity at an upstream point in a process divided by the level of an impurity at a downstream point in a process. Purge factors may be measured or predicted.
Regulatory Starting Material	A raw material, intermediate, or an API that is used in the production of an API and that is incorporated as a significant structural fragment into the structure of the API. From the introduction of SM on, appropriate GMP should be applied to the intermediate and/or API manufacturing steps.

4.2. Abbreviations

Acronym	Abbreviation
API	Active Pharmaceutical Ingredient. Alternatively, referred to as Active Substance or Drug Substance
APIm	Active Pharmaceutical Ingredient Manufacturer
AI	Acceptable Intake

AL	Acceptable limit
CPCA	Carcinogenic Potency Categorisation Approach. For more details, refer to EMA/409815/2020.[2]
DP	Drug Product
FP	Finished Product (synonym of DP)
EAT	Enhanced Ames Test
HA	Health Authority
MAH	Marketing Authorisation Holder
MDD	Maximum Daily Dose
SAR	Structure Activity Relationships
NDSRI	Nitrosamine Drug Substance-Related Impurities
FMEA	Failure Mode and Effects Analysis
LOQ, LOD	Limit of quantitation, Limit of detection
MI	Mutagenic impurity
PF	Purge factor
RSM	Regulatory starting material
ESI	Electrospray ionization
APCI	Atmospheric Pressure Chemical Ionization
MRM	Multiple reaction monitoring

5. Nitrosamine Risk Assessment

5.1. General approach

Since March 2020 the request from health authorities is to perform the risk assessment to all market products, for new and ongoing marketing authorisation applications (MAA) and marketing authorisation variation (MAV) and made available to the Health Authorities.

However, in some circumstances the following criteria might be used for prioritization of risk assessment for different APIs/intermediates (“>” meaning “higher priority than”) depending on the information available:

- Higher daily dose taken
- Long duration of treatment
- Therapeutic indication
- Higher number of patients treated
- Commercial APIs > APIs used for clinical trials
- API manufactured in multipurpose equipment > dedicated equipment
- API manufactured in multipurpose equipment exposed to nitrosating agents
- API > Intermediate > RSM (for companies manufacturing the three categories)
- APIs still manufactured > APIs no longer manufactured but still on the market
- APIs sold to markets where risk assessment have already been requested by authorities > APIs sold to other markets

- Knowledge of the likelihood of a risk based on the chemistry of the process (presence of amine, nitro functionalities, nitrosating agents)

The risk assessment evaluates the items as potential sources of nitrosamines or their precursors in line with root causes described in the EMA/409815/2020[2] and other National Competent Authorities, as applicable. The assessment of N-nitrosamine potential presence is based on technical information from SM/intermediate/raw materials suppliers and scientific literature, if relevant. APIs and intermediates in scope are assessed by a targeted review of the following information: API structure and reactivity toward potential formation of nitrosamines, API/API intermediate and starting materials route of synthesis, quality aspects of starting materials, reagents and any other raw materials, incl. water and solvents, cross-contamination, including equipment and cleaning procedures. In addition, evaluation of presence of vulnerable amines in the final API/intermediate is an important highlight for the evaluations of subsequent DP/FP manufacturing by MAH/DP manufacturers, where risk of trace levels of nitrite in the excipients can potentially form N-nitrosamine during DP manufacturing process or within the shelf-life of FP.

In the process of nitrosamine risk assessment multiple (expert) functions, such as regulatory, chemists, toxicologist, quality and sourcing/procurement, might be involved.

An initial assessment outcome might change over time as new information becomes available (*e.g.,* supplier's information updates, analytical test results) and/or in case of changes in the manufacturing process, starting materials, suppliers, *etc.*. In this case impact of the change should be evaluated, re-assessment of relevant individual root might be needed, leading to a re-assessment of the overall API risk. The assessment circle is recommended to follow ICH Q9 principles.

5.2. Nitrosamine Formation Mechanisms

N-Nitrosamines are in scope depending on the API or impurity structural alert reactivity/route of synthesis, and/or information received from suppliers. Nevertheless, the main focus is on the following *N*-nitrosamines:

1) Small - low molecular weight *N*-nitrosamines: initial focus, named as standard 5 nitrosamines, such as:
   - *N*-Nitroso-dimethylamine (NDMA)
   - *N*-Nitroso-diethylamine (NDEA)
   - *N*-Nitroso-diisopropylamine (NDIPA)
   - *N*-Nitroso-isopropylethylamine (NEIPA)
   - *N*-Nitroso-dibutylamine (NDBA)
2) Other low molecular weight *N*-nitrosamines, such as, but not limited to:
   - N-nitroso-piperazine
   - N-nitroso-L-proline
3) Nitrosamine drug substance related impurities (NDSRIs)

In December 2021 EMA has started to receive reports of *N*-nitroso API impurities in finished products increasing number over time. These complex nitrosamines may be formed where the API itself is a vulnerable (secondary amine) or may contain such a vulnerable amine as an impurity or as a degradant. Since that time focus has been changed significantly from small nitrosamines to larger nitrosamines, such as, but not limited to:

- For example, over 7,100 bottles of Duloxetine have been recalled in US due to finding of Nitroso-Duloxetine above the acceptable limit [22].
- Several key authorities maintain and regularly update lists of NDSRIs with confirmed or proposed acceptable intakes (AIs) [47][48]

N-Nitrosamines can be formed when an amine and nitrosating agent are combined under favorable conditions although other generation pathways are also possible, such as oxidation and reduction processes from hydrazine-type compounds and *N*-nitro derivatives. [3][5] Root causes for *N*-nitrosamines in medicinal products identified to date can be grouped as risk factors linked exclusively with the manufacturing process and storage of active substance and/or as risk factors associated with manufacture and storage of the finished product. Moreover, there are also risk factors specifically linked to GMP aspects. Currently identified risk factors for *N*-nitrosamine impurities in medicinal products following health authorities guidelines[2][3][5] are described below. However, the list is not exhaustive and further root causes may also be applicable.

5.2.1. Direct use of Nitrosamines

Currently, nitrosamines are mostly considered as a consequence and a contaminant in synthesis and direct use of nitrosamines in synthetic chemistry is rare. However, nitrosamines were formerly used in non-pharmaceutical industries, such as in the production of rocket fuel, antioxidants, polymers or lubricant additives.

5.2.2. Nitrosation

N-Nitrosamines can be formed when a secondary/tertiary amine and nitrosating agent react under favourable conditions. Vulnerable amine can form nitrosamine in the presence of nitrosating agent in acidic conditions. The rate of nitrosation varies with pH and usually shows a maximum in the range of 2.0-3.5 (optimum pH range for the formation of nitrosamines) [29]. This range of pH where nitrosamine can form may be extended or narrowed depending on the vulnerable amine, nitrosating agent and reaction conditions (reaction time, temperature, ...), if supported by literature data.

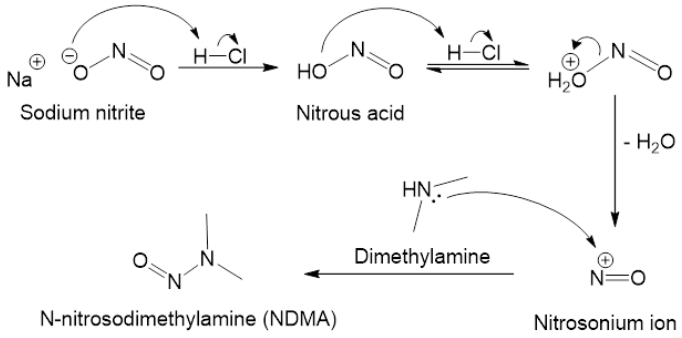


Figure 1: Schematic presentation of NDMA formation

According to EMA[2] nitrosating agents might be, but not limited to, as follows: nitrite salts and esters (e.g. $NaNO_2$, alkyl nitrites), nitroso halides, nitrosonium salts, nitrogen oxides, nitro alkanes, halogenated nitro alkanes, Fremy's salt, nitroso sulfonamides. Secondary or tertiary amines can be present within the same or different steps of the manufacturing process. Sources for secondary or tertiary amines can also be starting materials, intermediates, reagents, solvents (e.g. DMF, DMAc and NMP) and catalysts, which contain amine functionality, amine impurities (e.g. quaternary ammonium salts) or which are susceptible to degradation to reveal amines.

There are a wide variety of potential sources for both nitrosating agents and amines within the regulatory guidance. These need to be systematically assessed. Each potential source presents challenges that are commonly encountered in assessing the risk posed and in identifying and progressing mitigating actions.

These challenges and possible approaches to address them are expanded upon in the sub sections below.

5.2.2.1. Raw Materials, Intermediates or APIs as Nitrosamine Precursors

The evaluation of the nitrosamine formation in the API manufacturing process should consider the potential presence of a direct nitrosating agent and a secondary/tertiary amine source in the same manufacturing step (highest risk) or in different manufacturing step (lower risk, but would depend on carry-over from previous step(s)).

Some common process conditions known to potentially influence the formation of nitrosamines are the following:

- **acidic pH:** The rate of nitrosation varies with pH and usually shows a maximum in the range of 2.0-3.5 (optimum pH range for the formation of nitrosamines) [29]. Nitrosation reactions involving nitrite and secondary amines are more rapid at lower pH levels due to the increased presence of nitrous acid (HONO) and its protonated form (H_2ONO^+), which are potent nitrosating agents. [49][50]. At this pH the amine is mainly protonated (R_2NH^{2+}) and thus, the concentration of non-protonated amine is very low.
- **high temperatures**: The rate of nitrosation at high temperatures are expected to be higher [1][30][32][33][34].

It should also be considered that tertiary amines (and salts thereof) are significantly less reactive (typically about 1000-times more slowly than secondary amines to form nitrosamines), due to a rate-limiting dealkylation step that precedes the nitrosation of the resulting secondary amine [14][29].

Moreover, aromatic heterocyclic rings do not undergo *N*-nitrosation under usual nitrosating reaction conditions [35][36]. Aromatic heterocyclic rings are not chemically amines as the nitrogen atom is contained within the aromatic system and therefore, cannot form nitrosamines [52].

If the nitrosation occurred, the potential mutagenicity of the *N*-nitroso-heteroaromatic compound would involve a non-cohort of concern mechanism (not due to the alpha-hydroxylation and C-N cleavage) [8].

Electron-rich (hetero)aromatic substituents are the tertiary amines with an increased risk of nitrosamine formation compared to simple trialkyl amines [8][32][37][38][39][40].

It should also be considered that *N*-nitrosation rate is greatly affected by the tertiary amine structure because the size of the groups have a large effect on the C-N bond cleavage and bulky groups on the nitrogen atom appear to be cleaved less readily [1].

It is also known that nitrosamines could also be formed from reaction of nitrites with potential degradants from solvents (for instance, dimethylamine generation from degradation of solvent dimethylformamide). However, this risk could be discarded by profound knowledge of the process conditions (for example, hydrolysis may be discarded in absence of aqueous media and at mild temperatures).

5.2.2.2. Potential for Contamination of Raw Materials with Nitrosamines and/or Nitrosamine Precursors

The potential for nitrosamine presence in the raw material is directly related to the factors discussed in the previous section. If the API manufacturer is aware, or has reason to suspect, that both nitrosating agent(s) and vulnerable amine(s) are used in the raw material manufacturing process then process details should be sourced from the raw material manufacturer to allow an assessment similar to that described in 5.2.2.1.

The potential for nitrosamine precursor contamination is complex to assess. Since there are several different nitrosating agents and nitrosable substances, there are a wide variety of contaminants with the potential to be of significance.

Nitrosamines can be toxicologically significant at low concentrations and therefore precursor contamination could be significant at similarly low concentrations, *i.e.,* ppm or even ppb with consequent challenges in analytical method development and validation.

Full testing of all materials is typically impractical and risk assessment is necessary. Request of nitrosamine risk assessment to suppliers of key starting materials and key intermediates should be part of the qualification process (see section 10.3). Each material and supplier

combination should be assessed independently as risk factors may differ between suppliers.

Multiple risk assessment methodologies are of course available. A possible approach is FMEA assessment which offers the potential to allow quantification of the risk presented with numerical risk factors, such as severity, probability, and detectability.

5.2.2.3. Recovered materials

Using contaminated recycled or recovered materials, such as solvents, reagents, and catalysts, might pose a risk of nitrosamine impurities due to presence of residual amines (such as trimethylamine or diisopropoylethylamine). In some facilities, in which low risk of nitrosamine formation was identified in the manufacturing process of the API itself, cross-contamination has been observed due to use of recovered materials. [11] Use of recovered tributyltin chloride, triethylamine or solvent are confirmed sources of nitrosamine contamination.[11]

If the recovery process involves a quenching step (*i.e.,* nitrous acid used to decompose residual azide), nitrosamines could form during solvent recovery. These nitrosamines may be entrained if they have boiling points or solubility properties similar to the recovered materials, depending on how recovery and subsequent purification takes place (*e.g.,* aqueous washes or distillation). This further increases the risk of contamination in material recovery.[5]

This risk of introducing nitrosamines into the API manufacturing process increases when these materials are handled by third-party contractors who are unaware of the material content and/or use non-dedicated equipment. Contractors might employ general recovery procedures without considering source quality, pool materials from different sources, or use shared equipment. In addition, cross-contamination can also occur if the equipment is not properly cleaned and if there are no preventive measures to minimize the risk of nitrosamine formation. The same risk factors apply to materials recovered internally by manufacturers.[4][11]

To mitigate these risks, contractors should be qualified based on knowledge of their recovery processes, equipment cleaning procedures, validation programs, and proactive information sharing. Limiting the reuse of recovered materials to their original point of use in manufacturing processes can further reduce the risk of nitrosamine contamination.

According to APIC best practices, dedicated solvents, although recycled/recover bear no risk for nitrosamine cross-contamination. Furthermore, the use of recycled solvents, such as methanol, ethanol, 2-propanol bear no risk for contamination with amines or nitrosamines - the boiling point of NDMA (151 ^{0}C) and other nitrosamines is much higher compared to the ones of solvents recycled in the manufacturing process, which implies that even if traces of NDMA would be present, in distillation operations they would potentially remain condensed in the residue that remains in the distiller and is discarded as residue.

5.2.2.4. Cross contamination from multi-purpose equipment

If a manufacturing line is not dedicated to a specific API manufacturing process, and multipurposes equipment is used then there might be a risk for cross-contamination due to different processes being run successively on the same manufacturing line. Inadequate and unvalidated cleaning procedures can also lead to cross-contamination if precautions to avoid nitrosamine contamination are not in place. Nitrosamine precursors (i.e. nitrites, vulnerable amines, alkylamides) or nitrosamines themselfs can be transfered from one API process to another in case if not sufficient cleaning procedures are in place and if cleaning procedures are not validated according to ICH Q7: Good Manufacturing Practice Guide for Active Pharmaceutical Ingredients. API manufacturers should follow recommendations in ICH Q7 for ensuring that cross-contamination with nitrosamine or nitrosamine precursors can be prevented. On the other hand, even though the ability of cleaning procedures to remove certain nitrosamine impurities have not been demonstrated, nitrosamine impurities that could be present as traces are soluble in common organic solvents used for cleaning (water, ethanol, 2-propanol, acetone, ethyl acetate). API intermediates or API used in the manufacturing line are removed to trace amounts by mean of validated cleaning procedure (GMP requirement). The trace amounts of *N*-nitrosamine or *N*-nitrosamine precursor impurities in these API intermediates or APIs are similarly removed. [12][13]

5.2.2.5. Risks from Water

The risk of formation of nitrosamines during the manufacturing process by the reaction of amine sources with nitrite traces coming from the water used in the manufacturing process of the active substance is very unlikely because of the very low levels of nitrites in the water.

However, considering that we are looking at the formation/presence of nitrosamines at the ppb level, nitrite at these levels is almost ubiquitous. Thus, the use of potable water during later stages of drug synthesis is expected to be evaluated as a risk factor [1]. The conclusion is that the levels of nitrite that may be present in water used during API processing are very low, typically <0.01 mg/L [14]. Simulations based on the published kinetics of secondary amine nitrosation have shown that there is no risk that significant levels of dialkyl *N*-nitrosamines will be formed by traces of basic secondary amines (pKa > 9.5) encountering water containing this level of nitrite at temperatures below 55 °C. Synthetic concentrations of basic secondary amines are also not expected to form significant levels of dialkyl *N*-nitrosamines through reaction with trace nitrite at temperatures up to 55 °C provided the pH is 7 or above. Higher nitrite levels, up to the WHO limit of 3 mg/L, may, in some circumstances, for example, at low pH values or elevated temperatures, give rise to significant levels of *N*-nitrosamines.

The minimum acceptable quality of water used during the manufacture of active substances is potable water.[14] Nitrite is a controlled impurity in potable water, with a WHO guideline limit of 3 mg/L [16] and a European limit of 0.5 mg/L[17]. Moreover, according to European legislation[17], nitrate is controlled in potable water at 50 mg/L, which corresponds to 37 mg/L of nitrite assuming 100 % conversion as worst-case scenario (even though it is unlikely to assume 100 % conversion since enough reductive media should take place for conversion of nitrate into nitrite, which would require the presence of a reductive agent in very specific scenarios).

Based on the above, the nitrosamine formation from the nitrites present in the water and vulnerable amines should be assessed on case-by-case basis, considering different type of water used (*e.g.* potable, process, purified) and consequently levels of nitrites (concentration), vulnerable amines and reaction conditions (pH). Under acidic conditions and presence of vulnerable amine in the same process step as water, the risk for formation of nitrosamine should be properly addressed. It is recommended that the risk is excluded/confirmed by means of theoretical assessment via purge factor calculation considering levels of nitrites in water.[28] Indeed, risk for formation of nitrosamines due to nitrites in water can often be considered negligible:

> Since the concentration of nitrites in water is considered to be very low, and the rate of nitrosation is proportional to the concentration of non-protonated amines and nitrites (k_1 $[R_2NH][HNO_2]^2$), the potential conversion of amines to nitrosamines by nitrites is insignificant [30][31].

On the other hand, it is known that use of disinfected water (chlorination, chloro-amination, ozonisation) in the presence secondary or tertiary amines within the same or different steps of the manufacturing process could be a source of nitrosamine formation. However, this risk could be discarded in case that the disinfected water is suitably treated to remove any potential traces of chlorine, chloramine and ozone before its use in the manufacturing process (if risk is discarded, no testing should be required).

According to APIC experiences in most cases when water is disinfected with sodium hypochlorite, excess of sodium hypochlorite is quenched with sodium metabisulfite and further purified by RO before used in the manufacturing process, this should lower the risk. If water is treated by ozonization then it is important if compounds such as ammonia, hydroxylamine, hydrazine, hydrazone or hydrazide used in the manufacturing process that could oxidize. If not present, it could be concluded that there is negligible risk for nitrosamine formation due to water.

5.2.2.6. Water Testing

The risk assessment should consider all potential sources of nitrosamines from the water supplier to the manufacturing process.

Water is a potential source of nitrosamines or nitrosatable substances (chloramines and nitrites) therefore a risk assessment should be performed by the company to evaluate the

potential risk for nitrosamine contamination/formation in the manufacturing processes. As part of the risk assessment, the water should be evaluated as possible nitrosamine source in the risk assessment, but may not systematically imply nitrosamine testing in the water used.

Additional controls and preventive measures could also be implemented to ensure the quality of the water used in the manufacturing process.

5.2.2.7. Risk of Leaching from Ion Exchange Resins

Reaction of amines leaching from quaternary ammonium anion exchange resins (*e.g.* used for purification steps) with nitrosating agents present in the liquid phase might pose a risk for nitrosamine contamination.[2] A recent example of this was in the production of water for injections where residual chloramine used to disinfect incoming water reacted with dimethylamine leaching from the anion exchange resin used in the demineralisation step to form NDMA. In addition, disinfection procedures such as chlorination, chloro-amination and ozonisation can lead to significant *N*-nitrosamine generation as by-products in case vulnerable amines are present. Given the source of contamination, risk is related to the concentration of the reactive agent(s) and thus to the volume of water or used to dilute a particular product and is expected to be appropriately considered.

5.2.2.8. NOx in air

Nitrogen oxides (NOx) are present in the environment at parts-per-billion (ppb) levels. The family of NOx represents a complex mixture of species, with the equilibrium states of these species depending on the medium (gas or liquid), pH, and temperature. The two main components of NOx are nitric oxide (NO) and nitrogen dioxide (NO_2). NOx in the air can react with secondary amines present in drug products to form nitrosamines. This underscores the importance of stringent control measures in the pharmaceutical industry to prevent the formation of these harmful compounds.

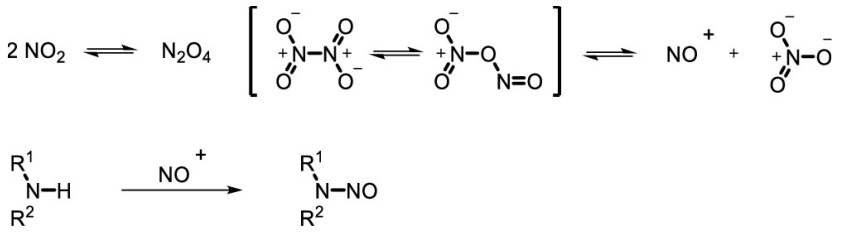


Figure 2: Mechanism of formation of N-nitrosamine with NO_2 [43].

NOx risk involves any operation conducted under air (without inert atmosphere). Processing operations under inert atmosphere do not present this potential risk. Certain

operations performed under air may have to be assessed (*e.g.,* certain drying and milling operations) as an additional source of nitrosating agent.

5.2.3. Oxidation

Here we consider the unintentional oxidation reactions within the manufacturing process.

N-Nitrosamines can be formed through oxidation's reactions of hydrazones, hydrazides and hydrazines compounds. This nitrosamine should as well be able to lead to an alkylating diazonium salt as described in below scheme. Sources of oxidant can come from hypochlorite, air, oxygen, ozone and peroxides in the manufacturing process or during storage. [10]

Agents as hydrazines, hydrazides and hydrazones need to be considered. Their use in potential presence of oxidation sources (hypochlorite, air, oxygen, ozone and peroxides) should be assessed as indicated by EMA.[2] In addition, nitrite formation by oxidation of hydroxylamine or nitrite release from nitro-aromatic precursors (*e.g.* by fluoro de-nitration), in the presence of secondary or tertiary amines within the same or different steps of the manufacturing process, should be considered as well.[2]

Antioxidants have been shown to be effective inhibitors in reducing formation of nitrosamine impurities during the manufacture and storage (*e.g.* ascorbic acid, caffeic acid, ferulic acid) [23][24].

5.2.4. Degradation

Nitrosamines generation from the degradation of the drug substance by inherent reactivity (presence of nitro-alkyl, oxime, or other functionality) or by an exogenous nitrosating agent, need to be considered in the risk assessment [25]. A good example is generation of NDMA from Ranitidine [26].

5.2.5. Packaging

Whereas the initial concern for formation of nitrosamines rested on the presence of small aliphatic secondary amines and their precursors (predominantly from solvents and reagents used in the synthesis of drug substances) it became apparent that other mechanisms could exist. Nitrocellulose blister materials, in which the nitrocellulose primer in a lidding foil acts as a nitrosating agent, can be sources of *N*-nitrosamines in drug product by the *N*-nitrosylation of secondary amines, present in printing ink, especially in liquid formulations where risk of leaching is higher. *N*-Nitrosamines can then transfer from foil to the drug product during the blistering operation. To avoid *N*-nitrosamines in drug product, nitrocellulose-free blister material should be considered [27]. The related risk can be further assessed by using statements of the packaging component suppliers.

Nevertheless, this packaging is mostly related to finished drug product and not drug substance, and for this reason it is considered that this risk factor is not so relevant for API itself and could be considered as negligible.

5.3. Purge Assessment

In each root cause assessed it is recommended to be clearly justified and concluded whether risk is identified or not (please see APIC template)[18]. It might be that a certain root cause is not applicable (*e.g.* when recovered solvents are not used in the API manufacturing process, the risk factor from this root cause is considered as not applicable). Purge assessment can be used in initial assessment to make conclusion regarding nitrosamine risk potential (*i.e.* negligible risk/no risk or risk of potentially present/risk) and/or to justify control strategy following conclusion of potentially present.

Purge assessment (ICH M7 Option 4)[9] without analytical data may be accepted or not by Health Authorities, evaluation on a case-by-case basis is usually expected. According to Health Canada guidance[3] an Option 4 control strategy proposal may not be appropriate when the concentration of any nitrosamine impurity in an API is greater than 30% of the AI limit. However, such a strategy may be acceptable when process understanding has been demonstrated by fate-purge studies, identification of process parameters that impact nitrosamine impurity levels and when supported by appropriate analytical data. According to current experiences by APIC with Health Canada, sole purge factor (PF) calculations are not accepted but should be supported by experimental data. On the other hand, according to the guideline ANVISA [19] might accept Option 4 as a control strategy for nitrosamines solely based on process control, with no need for analytical tests when it is possible to determine that the risk of the presence of nitrosamine(s) above the maximum permitted limit(s) is negligible. European Health Authorities normally does not accept predicted PF without testing results, but according to limited experiences within APIC predicted PF calculation (without suporting analytical testing) to exclude nitrosamine risk could be sufficient also for EMA.

For nitrosamine impurities, ICH M7 option 4 control strategy (understand process parameters and impact on residual impurity levels with sufficient confidence that the level of the impurity in the drug substance will be below the acceptable limit and as such no analytical testing / control of the nitrosamine impurity in any specifications is needed) may be acceptable. Option 4 can be justified by the calculation of predicted purge factors, a concept first proposed by Teasdale et al. [20] based in profound process understanding and identification of the key physicochemical parameters of the given nitrosamine impurity that may influence its removal throughout the manufacturing process, in combination with the specific process conditions. The key physicochemical parameters of the given nitrosamine identified to be considered in the predicted purge factor calculation are reactivity, solubility, volatility, ionizability (supported by literature or experimental data) and any additional physical process designed to eliminate impurities (such as chromatography). For instance, the basicity of the amines (pKa) should be considered, as less basic amines (pKa ≤ 9.5) are being nitrosated much more rapidly [21]. Another example is the fact that NDMA is described as very soluble in water (13.5 mol/L) [1], so in aqueous washes in work-up operations could be effectively purged.

In order to calculate the purge factor, scores for each parameter [41] are assigned on the basis of the physicochemical properties of the nitrosamine impurity relative to the process conditions. The scale is based on the premise that a high purge factor equates to high nitrosamine impurity clearance. Thus, a high purge factor value indicates a low probability that a nitrosamine impurity will be observed on the basis of knowledge of physicochemical properties of the nitrosamine and understanding of the synthetic process. The overall predicted purge factor is the multiplication of the individual ones from each manufacturing step.

The calculation of the predicted purge factor should determine the maximum realistic theoretical level which could be formed as the worst-case scenario and what estimated levels could remain in the active substance considering the purging capability of the manufacturing process. The concentrations of both the secondary/tertiary amine and nitrosating agent in the manufacturing process should be considered in the calculation.

After obtaining the predicted purge factor, it should be compared to the required purge factor (defined by the initial estimated potential concentration of the nitrosamine as worst-case scenario divided by its acceptable concentration limit calculated with the Acceptance Intake for the given nitrosamine and the Maximum Daily Dose of the medicinal product).

According to the literature [42] [54] [55], when calculated theoretical overall purge factor is more than 1000× higher than the required purge factor, then it can be concluded that Option 4 is considered acceptable and there is no risk for the given nitrosamine without need of pursuing further confirmatory testing. Meanwhile, when the theoretical overall purge factor is from 1× to 100× than the required purge factor, there is a potential risk and further analytical data would be needed by confirmatory testing to confirm/exclude the risk.

Below it is included the recommended decision tree [42]:

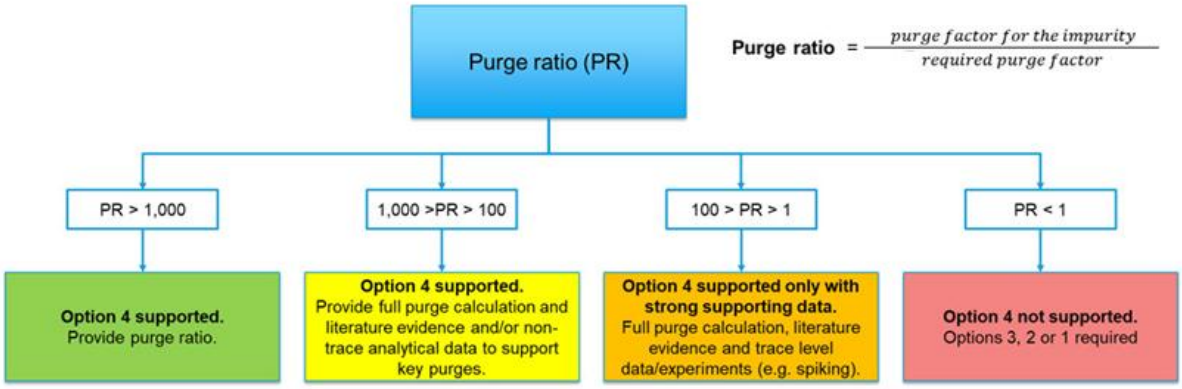


Figure 3: Decision tree of evidential requirements for option 4 control based on purge ratios. [54]

Option 4 control strategy could also be justified if the predicted level of the nitrosamine at the final API is < 1% of the AI-based limit.

Notwithstanding, the acceptability of theoretical purge calculations approach depends ultimately by the Health Authorities.

Furthermore, in some circumstances theoretical purge factor cannot be calculated due to proximity of nitrosamine formation and final drug substance isolation or lack of information. In this case, testing is recommended to exclude/confirm the risk.

6. API Testing

If the risk assessment indicates a potential risk for the formation of, or contamination with, nitrosamines, confirmatory testing of API should be performed or theoretical predicted purge calculations on case-by-case basis under acceptability of HAs could be pursued (refer to section 5.3).

In case of analytical confirmatory testing (most conservative approach), the test should be capable of quantifying the amounts of individual or multiple *N*-nitrosamines. Testing results should be provided to HA and/or customers, if required. Depending on the test results, investigation, implementation of control and/or mitigation strategies might be required.

To support customers to evaluate the risk at DP level, sometimes analytical results of precursors, like nitrites, vulnerable amine impurities are needed to be tested.

6.1. Limit Calculation and Setting

a) <u>Dose assumptions</u>

Acceptable Intakes (AIs) for nitrosamine compounds are set on a total daily intake basis for the impurity. The concentration limit in the API should be established considering the AI set for the impurity and the Maximum Daily Dose (MDD) for the API, according to the following:

$$\boldsymbol{Limit\ (ppm) = \frac{AI\ (ng/day)}{MDD\ (mg/day)}}$$

According to EMA guideline [2] for a control point in the API, the limit should be expressed in general per drug substance (i.e. relating to form of salt, hydrate, solvate etc. where relevant).

MDD determination for the API should be based on the maximum recommended daily dose in the approved drug product labelling. It is strongly recommended that API manufacturer should take Martindale as a reference value for MDD, but other sources could be used alternatively for MDD selection on case-by-case basis under acceptability by HAs. In case of different MDD recommendations for different medicinal products formulated with the same API, the higher Maximum Daily Dose should be considered for calculations in order to provide an acceptable limit based on a worst-case scenario as higher MDD will lead to lower Acceptable Limit.

Published Acceptable Intakes for the specific nitrosamine impurity might not be harmonized between different Health Authorities; in such case, internal limit calculation should be based in the worst-case AI (*i.e.* lowest AI) where medicinal product with the API is marketed.

Dialogue with MAHs is recommended in the acceptable limit calculations in order to guarantee a control strategy based on the different approved drug product.

The same risk approach is applicable to all routes of administration. Corrections to limits are generally not acceptable unless route-specific differences are justified by data. Current nitrosamine guidelines do not differentiate between chronic/non-chronic in terms of limit calculation, meaning that current nitrosamine guidelines only consider limit calculation for lifetime exposure regardless of the duration of the treatment.

Less-than-lifetime (LTL) approach can be used temporarily only after discussion by Health Authorities on case-by-case basis in case of non-chronic applications.

b) <u>Limit setting for individual nitrosamines:</u>
For details please see EMA guideline.[2]

- If the nitrosamine impurity has substance specific animal carcinogenicity data, the TD_{50} should be used to calculate the AI.

- If no specific animal carcinogenicity data is available for a given nitrosamine:

    - the Carcinogenic Potency Categorization Approach (CPCA) should be used to establish the AI: CPCA is a convenient and conservative methodology to set AI limits for nitrosamines based on general structure-activity relationship (SAR) concepts for *N*-nitrosamine compounds. As the science is evolving in the prediction of mutagenic and carcinogenic potency of nitrosamine compounds, the CPCA is expected to be further refined and expanded as new data become available. Lists of AI limits of known nitrosamines are regularly updated by the authorities. These AI limits should be used for calculation. As a default and conservative general approach, the CPCA is expected to be used when more specific data for a nitrosamine impurity is not available for AI calculation.

    - If a surrogate nitrosamine is available with sufficiently robust carcinogenicity data, the TD_{50} from the surrogate substance can serve as a point of departure for derivation of AI by SAR and read across.

    - A negative result in a GLP-compliant Enhanced Ames Test (EAT) allows control of the nitrosamine impurity at 1.5 µg/day (ICH M7).

    - A negative result in an *in vivo* mutagenicity study allows control of the nitrosamine impurity as a non-mutagenic impurity (*i.e.* according to ICH Q3A limits)

    - For products intended for advanced cancer only, nitrosamine impurities should be controlled according to ICH Q3A guideline.

Current regulatory expectation is that, if potential presence is concluded via risk assessment, confirmatory testing is required regardless of which CPCA category is assigned [2][3][5], even though purge assessment approach may be explored (see section 5.3).

For EMA, Health Canada, Swiss Medic, TGA, HAS (Singapore), ANVISA as per guidelines a negative result in a GLP-compliant enhanced Ames test (EAT) allows control of the *N*-nitrosamine at 1.5 µg/day. However, in addition to the EAT, US FDA is currently requesting a second in vitro mammalian cell mutation assay and in vitro metabolism data to support an AI limit of 1500 ng/day.

The AI of 1.5 µg/day for a nitrosamine impurity testing negative in the EAT is still a conservative approach, as limits for non-mutagenic impurities are generally established based on ICH Q3A guidance. However, this conservative approach is the acceptable regulatory strategy to date due to the uncertainties for nitrosamine compounds and the high carcinogenic potential identified for some nitrosamines.

From a scientific and toxicological perspective, it seems reasonable that as more scientific evidence becomes available for nitrosamine impurities with structural features minimizing the risk of mutagenic and carcinogenic properties, as well as more in vitro and in vivo experimental mutagenicity data confirms the low mutagenic risk for several nitrosamine-class compounds, a negative EAT with additional SAR supporting evidence of the low mutagenic and carcinogenic risk for the impurity, may be able to justify higher acceptable limits for nitrosamine impurities according to general ICH Q3A requirements.

For EMA, Health Canada, Swiss Medic, TGA, HAS (Singapore), ANVISA as per guidelines a negative result in suitable *in vivo* tests should be sufficient for ICH Q3A limits, meanwhile for US FDA a negative result in an *in vivo* mutagenicity study may not be supportive of AI equal to ICH Q3A/B limits. FDA acknowledges that these recommendations may differ from those of other drug regulatory agencies.

c) <u>Limit setting for total nitrosamines (if applicable):</u>

It is considered that *N*-Nitrosamines present below 10% of their respective AI (*i.e.* not specified in the API specifications) constitute a negligible toxicological risk and, thus, they do not need to be specified and/or to be factored into the calculation of limits for individual or total *N*-nitrosamine impurities.
This approach is specified in the EMA guidance [2] and is also in accordance with the ICH M7 principles for mutagenic impurities.

- **Option 1:** the AI limit for total *N*-nitrosamines should be set in ppm/ppb according to the most potent *N*-nitrosamine present at ≥ 10% of its AI. The most potent nitrosamine is the one with the lowest AI. Limits for individual *N*-nitrosamines can be defined but are not necessarily needed. However, it should be clearly stated which *N*-nitrosamines are included in the calculation of total *N*-nitrosamines.
- **Option 2:** the limits for *N*-nitrosamines should ensure an overall risk of not more than 1 in 100,000. Different approaches can be employed to achieve this risk requirement:

- ***Fixed approach:*** fixed AI limits (in ppm/ppb) are set for individual nitrosamines and no limit for total *N*-nitrosamines is needed. The limit for each *N*-nitrosamine should be set at a percentage of its AI limit such that the sum of the % AI limits for each specified nitrosamine does not exceed 100%.
- ***Flexible approach:*** each *N*-nitrosamine should be specified at its AI limit in ppm/ppb and an additional limit for total *N*-nitrosamines is required. The calculation for total *N*-nitrosamines could be written as:
-

$$\sum_{i=2}^{n} \frac{Xi}{AIi} \; x \; 100\% \leq 100\%$$

Where:
Xi is the amount of each single N-nitrosamine i in ppm
AIi is the AI limit of each N-nitrosamine i in ppm.

For each batch, to determine whether the limit for total *N*-nitrosamines is met, the amount of each *N*-nitrosamine present (in ppm/ppb) should be converted to a percentage of its respective AI limit. The sum of % AI limits of specified *N*-nitrosamines should not exceed 100%.

d) <u>Customer expectations for limit below calculated max</u>

The AI limits published in the guidelines are considered qualified for nitrosamine impurities in the drug product at the end of shelf life. In some cases, tighter limits in the API specifications and in the drug product release specifications may be warranted to ensure that the drug product shelf life specification will be met. Therefore, it is reasonably expected that some customers for certain drug products require APIs with nitrosamines of lower than accetable limits calculated based on accpetable intake indicated in guidelines.
Likewise, vulnerable/secondary amines in APIs could be potentially converted to the corresponding NDSRIs during final product manufacturing processes and/or storage. Therefore, the acceptable levels for vulnerable amine impurities in APIs, which are set in line with ICH Q3A/compendial monograph, are sometimes not acceptable from the perspective of corresponding NDSRI formation at drug product level and required to be lower.
APIC expresses willingness to collaborate with DP manufacturers/MAHs in respect to specific nitrosamines and vulnerable amines limit settings in API that are suitable for specific drug product, as reflected in APIC letters **Error! Reference source not found.Error! Reference source not found.**.

The following might be requested by customers:

1) <u>Limits of *N*-nitrosamine in API below 10% of AI</u>

Limits of nitrosamine in API are desired to be below 10% of AI in order to justify omission of the test for the nitrosamine from the API specification after analysis of at least three production scale batches or six pilot scale batches.

2) Very low limits for amines

Precursors of nitrosamines in the API (secondary amines) should be controlled in the API at a reasonable level. It is not feasible for API industry to work at level that will enable the limit equals/below the limit calculated from AI for the corresponding nitrosamine in the FP (assuming 100% conversion).

Reality is that some API companies are facing challenges from customers to verify that amine content is very low relative to historical impurity setting limits (*e.g.* 0.05% disregard limit). However, no current convention as to what is reasonable.

In general, APIC is of opinion **Error! Reference source not found.** that tightening per default (including where not justified/needed) of the limits for NDSRIs / vulnerable amine stricter than current guidances (*i.e.* nitrosamine guidances, ICH Q3A, Ph.Eur. monograph) would be too much of a burden for the API manufacturers.

3) Requirement from customers to do confirmatory testing for nitrosamines despite conclusion of negligible risk

Confirmatory testing of the API for nitrosamines might be conducted when the risk assessment indicates that there is a potential source of nitrosamine impurities in the formulated product. Notwithstanding, if the nitrosamine risk is just at the formulated product and not in the API, it is more reasonable to do nitrosamine testing at DP level than at API level.

4) Limits for nitrites

Justifying absence of nitrosating agent might be also required. 1 ppb of nitrites already could lead to nitrosamine formation under suitable conditions during DP manufacturing/storage. Nevertheless, there is no industry expectation to prove nitrite content in API unless a clear direct source of nitrite is identified (e.g. use of water is in general not considered a sufficient cause to trigger nitrite testing). It could be considered as an unrealistic expectation bearing in mind that nitrites may come from the excipients used in the DP manufacturing [53].

6.2. Test methodologies

Nowadays GC-MS/MS and LC-MS/MS are commonly used for the analysis of *N*-nitrosamine in the active pharmaceutical ingredients. Considering the possibility of generating nitrosamine during the analytical process, LC-MS/MS is considered the most acceptable technique in terms of the reliability of the results, avoiding the in-situ generation of *N*-nitrosamine giving false positive results.

The analytical procedures for *N*-nitrosamines should be carefully chosen taking into account:

- potential presence of precursors (secondary amines; nitrite) in the sample
- workup procedures must be validated for any potential interferences
- hydrophilicity / lipophilicity as well as volatility / non-volatility of the target analyte
- LOQ provides the minimum level at which an analyte can be quantified with acceptable accuracy and precision and is thus preferred over LOD for impurity testing and decision-making

- LOQ should be minimum at or sufficiently below the toxicologically required limit, taking into account the purpose of testing (*e.g.* routine testing, justifying skip testing, justifying omission of specification)

For confirmatory testing, a limit test may be sufficient (not all validation parameters are tested). For routine testing after confirmatory testing (if ≥ 10 % of the limit), full validation of analytical method is needed.

The use of a limit test could be acceptable to confirm the negligible risk for presence of nitrosamines such as NDEA, NDMA (nitrosamine of small structures) and it should be in line with the validation requirements outlines in ICH Q2. If limit test is performed to justify omission of specification, the LOD of the analytical method should be ≤ 10% of the acceptable limit based on the AI.

If quantitative testing is performed as a routine control, the limit of quantification (LOQ) should be at or below the acceptable limit for the respective nitrosamine impurity.

The selected method should have good specificity to ensure the unequivocal determination of *N*-nitrosamines in accordance with scientifically recognized guidelines, such as ICH Q2 (R2). The common challenge faced involves the separation of the API peak from the *N*-nitrosamine peak.

If the same analytical method is used to test for multiple nitrosamines, then the selectivity of the method should be demonstrated at the LOQ for each nitrosamine. Preferably, employing a working range extending from below 10% of the most rigorous specification to up to 120% of the least stringent is recommended to forestall future method revalidations. Furthermore, it is suggested to establish a nominal concentration positioned centrally within the calibration curve to ascertain method applicability across diverse specifications. A sensitivity solution is recommended to be included in the system suitability testing to ensure the method's sensitivity on a daily basis.

The mass detection is usually used in positive mode. The ionization principle used for MS plays a relevant role regarding sensitivity and detectability. ESI source is much more suitable to ionize large polar molecules like NDSRIs, and APCI helps ionize neutral molecules as all classic nitrosamines.

To quantify an amount of substance, tandem MS generates a spectrum in MRM for a selected precursor ion and product ion. Only those mass fragments are detected by the mass spectrometer lowering the limit of detection by several orders of magnitude and allowing an unequivocal identification of the analyte since the specific collision induced by dissociation reactions is monitored.

The optimization of the mass spectrometry leads to the monitoring of the most intense two fragments of the compound generated from the molecular weight peak. To ensure the correct identification of nitrosamines, two MRM transitions are selected in the analytical method. One is described to be the quantifier transition, which will be used for the quantitation of the

nitrosamines in every method. The second one will be the qualifier transition, which is also selected from the mass spectrum of the target compound. The presence of this transition in the correct amount relative to the quantifier gives evidence of correct target compound identification. The relative intensity of the quantifier/qualifier transition (qualifier ratio) from the reference solutions must be compared to the qualifier ratio of the samples. The maximum accepted relative qualifier ratio tolerance is set as follows (taken from the Commission Decision (2002/657/EC) amending Directive 96/23/EC): Deviation ± 20%.

Regarding the sample preparation, the new trend is working with solid-phase extraction (SPE) or solid-phase micro extraction (SPME). However, the concentration levels for most substances can be achieved through direct dissolution, avoiding longer or more laborious processes while yielding acceptable impurity recovery results. In the realm of non-specific nitrosamines such as NDMA or NDEA, various analytical methodologies utilizing sample preparation via dispersion have been documented. It is recognized within these methods that while the nitrosamine is effectively extracted into the solvent, the API may persist in suspension. Conversely, in the context of NSDRI analytical methods, careful consideration must be given to the solubility of the matrix in the diluent during the development of the workup procedure. This is imperative as the optimal approach to ensure complete dissolution of the analyte in the solvent lies in utilizing a solvent capable of dissolving the entirety matrix. Although it has been observed that the solvent which shows the highest sensitivity for nitrosamines is water (or an aqueous solvent), some matrixes do not fully dissolve in this solvent in the concentration needed for the analysis. Thus, an equilibrium must be fulfilled in order to obtain the best recovery and sensitivity combined with the complete solubility of the matrix, and consequently of the nitrosamine. Nevertheless, it is important to treat the sample in the same manner as the references.

With the aim to minimize the increase in nitrosamine/NDSRI during analysis, it is recommended that the samples used should have minimal contact with the atmosphere, avoiding exposure to air as much as possible. Preferably, the sample for analysis should be packaged in a plastic bag directly from the storage drum.

Based on the nitrosamine levels obtained different control strategies should be performed, refer to section 6.4 for more detail.

6.3. Confirmatory testing

If the risk assessment indicates a potential risk for the formation of, or contamination with, nitrosamines, further mitigation/control strategy, including confirmatory testing of API should be performed and suitable mitigation strategy (refer to section 7) should be in place.

The test should be capable of quantifying the amounts of individual *N*-nitrosamines or multiple *N*-nitrosamines to assure levels of the nitrosamine(s) well below the AI-based limit in the API (refer to section 6.1 for limit calculation). It is essential to establish and validate analytical methods for the accurate detection and quantification of nitrosamines for confirmatory testing (refer to section 6.2).

Testing results should be provided to HA and/or customers, if required.[18] Depending on the test results, investigation, implementation of control and/or mitigation strategy might be required.

To support customers to evaluate the risk at DP level, sometimes analytical results of precursors, like nitrites, vulnerable amine impurities are needed to be tested. [18]

For confirmatory testing ("one time testing"), a minimum of 3 consecutive production scale batches or 6 consecutive pilot scale batches should be tested. For skip testing, a minimum of 3 batches or 10% of the yearly production should be tested (whichever number is the highest) [2]. Depending on the results found in confirmatory testing, different control strategies (see section 6.4) and/or mitigation measures (see section 7) may be established. If less than 3 batches have been manufactured, then a routine limit for the given nitrosamine is expected until a third batch is manufactured and tested to prove the nitrosamine level is below 10% of the AI-based limit in at least 3 batches to support absence of routine testing.

6.4. Control Strategy

When the presence of nitrosamines is confirmed in pharmaceutical products, it is crucial to understand the risk factors and root causes of these impurities. This understanding provides the basis for implementing effective risk mitigation and control strategies, to ensure that nitrosamine levels remain within the acceptable intake (AI) limit.

Based on available analytical results from confirmatory testing, the following scenarios are possible:

- if the analytical testing results are < 10 % of the AI-based acceptable limit for the given nitrosamine then Option 4 control strategy (*i.e.* nitrosamine not controlled in any specification or in other words, omission of routine testing) would be justified.
- If the analytical testing results are ≥ 10 % and < 30 % of the AI-based acceptable limit for the given nitrosamine then Option 1 control strategy (skip testing of the nitrosamine at the API specifications) would be justified. Skip testing is not applicable for US scope.[5]
- If the analytical testing results are ≥ 30 % and ≤ 100 % of the AI-based acceptable limit for the given nitrosamine then Option 1 control strategy (routine testing on batch-to-batch basis of the nitrosamine at the AI-based limit in the API specifications) would be justified.
- If the analytical testing results are > 100 % of the AI-based acceptable limit for the given nitrosamine, health authorities and customers (MAH) should be informed and may imply product recall. Risk mitigation actions should be undertaken to reduce the risk (refer to section 7), such as changes in manufacturing process.

7. Risk mitigation and CAPAs

These strategies may include (but not limited) changes to the manufacturing process and/or the introduction of appropriate specifications. Measures related to facilities and equipment, such as cleaning procedures and environmental monitoring, should also be considered.

To mitigate the risks associated with nitrosamine impurities, the manufacturing process may need to be modified to eliminate or reduce the conditions that lead to the formation of nitrosamines.

Stringent cleaning procedures and regular environmental monitoring should be implemented to prevent (cross-)contamination, and the cleaning and change-of-line procedures should be evaluated and updated to mitigate potential risks to the GMP line. A detailed action plan should be defined to document the measures taken to mitigate the risk of formation of nitrosamines. All necessary changes should be recorded in the change control system. It is important to notify competent authorities and customers, as appropriate, about the presence of nitrosamines and the steps being taken to address the issue.

8. Risk Assessment Conclusion

Based on the risk evaluation conducted and mitigation measures (see section 7), the risk for presence of nitrosamines is evaluated as: negligible (no risk identified) / potentially present (risk identified).

For the final risk assessment outcome (considering API manufacturer's control strategy), the risk is defined as follows in line with the APIC template (Report on the risk of potential presence of nitrosamine impurities) for API manufacturers [18]:

Negligible (No risk identified):

- No risk according to theoretical risk assessment if the predicted nitrosamine level calculated by theoretical purge assessment is < 1% of the AI-based limit in final API (see section 5.3): Option 4 control strategy (no routine testing).
- Risk assessment is set as a potential based on risk evaluation, however appropriate control strategy for nitrosamine impurity is applied by API manufacturer:
    - Based on confirmatory testing results levels of the respective nitrosamine are ≤ acceptable limit based on the AI: control strategy following Option 1 (batch-to-batch testing) according to ICH M7 is necessary if confirmatory testing shows levels > 30% of the AI-based acceptable limit. When the levels are < 30% of the acceptable limit based on the AI, skip testing is justified (Option 1). Please note that skip testing is not applicable for US FDA.
    - Control Option 2 or Option 3 are applied for control of nitrosamine impurity in previous API stages (e.g. setting a limit for the nitrosamine in the raw material, key starting material or intermediate, or as an in-process control).
- Mitigation based on analytical testing:

- When the analysed levels for nitrosamine according to confirmatory testing (see section 6.3) in a minimum of 3 consecutive production scale batches or 6 consecutive pilot scale batches are consistently < 10% of the acceptable limit based on the AI, and the root cause is identified and well-understood, no further testing is justified.

Potentially present (Risk identified):

- Risk assessment is set as a potential, however no appropriate control strategy is applied by API manufacturer yet. Suitable control strategy should be further considered.

In case that the conclusion is "potentially present (risk identified)", after establishing a proper control strategy which allows to mitigate the risk, an updated version of the nitrosamine risk assessment may be generated to update the conclusion to "negligible (no risk identified)".

9. Presentation to Customers

As indicated in regulatory guidances [2][6][7] MAHs are responsible for quality, safety and efficacy of medicinal product. MAHs/Applicants and APIM should work together and take precautionary measures to mitigate the risk of presence of nitrosamines during the manufacture and storage of all medicinal products containing chemically synthesized APIs. In this context, APIC is well aware of APIM / CEP holders responsibilities, namely to provide MAH the most recent version of CEP, and support with any necessary information that is related to safety, quality and efficacy, including information on nitrosamines, MIs and other pivotal information not necessary included in CEP, but needed for comprehensive risk assessment for medicinal product. In general, APIC is willing to support and collaborate with DP manufacturers / MAH as expressed with APIC letters **Error! Reference source not found.Error! Reference source not found.**.

It is expected that the level of shared knowledge should be such that permits MAHs to take responsibility for the quality of active substance as incorporated into the finished product. Therefore, as recommended by regulatory guidances, timely cooperation, communication and information/data sharing between APIM and MAH is expected. For this purpose, APIC established a common basis among API industry regarding sharing nitrosamine risk assessment information with Applicants and HAs with the main purpose to have harmonized approach. Within the APIC template (Report on the risk of potential presence of nitrosamine impurities) API manufacturer[18] is recommended to provide to Applicant all the information needed to support risk assessment for medicinal drug product, such as:

- All risk factors according to EMA/369136/2020 and EMA/409815/2020 evaluated
- List of potential nitrosamines
- Risk outcome
- Mitigation plan if required (testing, incl. testing results, theoretical derisking)
- Presence of nitrosating agents
- Presence of nitrosatable/vulnerable substances that are needed for MAH to evaluate the risk for Nitrosamine formation at DP level

According to APIC experiences, some of the information can be shared in advance with customers and API manufacturers can ask for support or consultation on risk assessments (*e.g*. mostly connected to correct limit calculations) before the final risk assessment is available and further shared.

Some APIC members have also reported experiences on customers requesting testing of nitrosamines in APIs at the end of the re-test period due to possibility of nitrosamines formation by degradation throughout storage. It is in general considered that observation of nitrosamine formation under stress testing conditions at the FP or API should not trigger systematically confirmatory nitrosamine testing in the API, as the conditions in forced degradation studies are not likely to take place during long-term storage, even though it may be useful information for some customers.

10. Incorporation of Nitrosamine Risk management into the QMS

10.1. Organizational Awareness / Training

Implementing organizational awareness and risk management to prevent or reduce the presence of nitrosamine impurities in active pharmaceutical ingredients requires a comprehensive approach. It is recommended that the entire organization be made aware of the risk of nitrosamine impurities in APIs and understand the risks associated with these potentially harmful impurities.

Manufacturers should conduct awareness campaigns in nitrosamine-related topics across the whole organization. Training programs on nitrosamine awareness and risk management should be implemented in key areas of the organization, including Quality Assurance, Quality Control, Research & Development, Manufacturing, Supply Chain and Regulatory Affairs. The training should be tailored to the needs and role of each key area, without neglecting the general overview of what is necessary and required for a complete assessment of nitrosamines risk and how to proceed if these impurities are detected.

The Quality Management System (QMS) should define nitrosamine-specific policy and procedures for risk evaluation, detection, and control of nitrosamines impurities in APIs. Regulatory Agencies and Competent Authorities expect from API manufacturers to comply with the established guidelines. A continuous review and update of the QMS to address emerging risks and regulatory changes should be considered. Any changes related to nitrosamine risk management or required to comply with updated regulations should follow a robust change control process.

Manufacturers should assess the risk of nitrosamine impurities in their APIs, identify potential sources and evaluate the likelihood of nitrosamine formation during their manufacturing processes, and to further implement mitigation strategies to minimize or eliminate risks. Analytical methods for the detection of nitrosamines should be developed, validated, and integrated into the QMS. Raw materials, API intermediates, and final APIs should be monitored for nitrosamines impurities.

The API supply chain should be assessed for nitrosamine risks, and manufacturers shall collaborate with suppliers to ensure that the presence of nitrosamines is controlled and kept as low as possible in all materials and perform regularly audits to suppliers' processes and quality control measures.

Accurate records of nitrosamine risk assessments, testing, and corrective actions should be maintained and deviations, corrective and preventive actions (CAPA) should be adequately documented.
Manufacturers are responsible for maintaining open communication within the organization regarding nitrosamine risk management and to notify the Competent Authorities of the outcome of nitrosamine risk evaluations.

10.2. Regulatory Intelligence

A survey of the various Regulatory Agencies and Competent Authorities guidelines in force around the world should be carried out and analyzed for implementation within the organization. Internal measures should be taken to successfully implement the guidelines into the specifics of the industry.

It will be the responsibility of the Regulatory Intelligence team or other relevant area (such as Quality Assurance) to compile, interpret and monitor the guidelines, taking into consideration their suitability for the organization and to establish the appropriate communication channels to disseminate the information to all key areas involved. Collaboration with regulatory affairs, quality assurance, and other relevant teams is crucial.

Organizations should foster collaboration between different key areas. An open communication will ensure that all stakeholders understand the importance of nitrosamine risk management for patient safety and product quality.

Implementing regulatory intelligence for nitrosamine risk management in APIs involves staying informed about the latest regulatory updates, guidelines, and requirements related to nitrosamine impurities; It requires a full understanding of the regulatory landscape, familiarization with the regulatory guidelines, and stay updated on any changes or new requirements.
Addressing nitrosamine challenges requires vigilance, robust testing, and proactive risk management to ensure patient safety and product quality.

10.3. Supplier Quality Management

Nitrosamine risk assessment should be an integral part of the supplier qualification process. By incorporating this assessment, pharmaceutical companies can proactively manage potential risks, ensuring the consistent quality and safety of their products.

In the pre-qualification phase, pharmaceutical companies should request detailed documentation on the supplier's nitrosamines risk assessments for the key starting material(s) and key intermediates, including any historical data on nitrosamine levels in their materials.

In case suppliers are not able or willing to perform a nitrosamine risk assessment or missing documentation companies should have internal processes to evaluate potential risk of the material to the manufacturing process.

Thorough on-site audits to suppliers focusing on potential sources of nitrosamine formation within the supplier's processes (including solvents, reagents and raw materials used) and facilities (prevention of cross contamination) should be performed.

A critical component of the supplier qualification process is the supplier risk assessment which should involve assessing the risk of nitrosamine contamination in the supplier's raw materials, starting materials or intermediates, and processes, by examining chemical reactions, storage conditions and handling procedures. Additionally, the supplier risk management strategies should be evaluated, including control measures and corrective action plans for nitrosamine contamination.

As part of the supplier qualification process, it should be ensured that the supplier uses validated analytical methods for detecting and quantifying nitrosamines, if a risk is identified from their nitrosamine risk assessment which triggers the need of confirmatory testing. These methods should be sensitive, specific, and reliable.

When the nitrosamine is part of the purchasing specifications, it is recommended that the Quality Agreement with the supplier clearly defines responsibilities for nitrosamine testing and immediate reporting of any detected nitrosamine levels.

Continuous monitoring and re-qualification of suppliers are necessary to maintain control over nitrosamine risks. This involves implementing processes for continuous monitoring of nitrosamine levels in supplied materials, periodic testing, regular reviews of supplier performance, and re-qualification audits.

Suppliers should be required to notify the pharmaceutical company of any changes in their processes or materials that could potentially lead to nitrosamine formation or cross-contamination.

10.4. Lifecycle management

Lifecycle management of the risk of formation of nitrosamines involves continuous monitoring, evaluation and mitigation of risk from initial product development through to GMP production. Any potential change that may impact on the risk assessment (*e.g.* change of supplier, manufacturing process, packaging) should be evaluated to prevent nitrosamine formation as well as cross contamination and should be an integrated part of the evaluation of change control.

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