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White Paper

Managing Nitrite Impurities: A Supplier-Manufacturer Approach to Mitigating Nitrosamine Risk

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Nitrosamines emerged as a public health concern in relation to pharmaceuticals in 2018 when N-nitrosodimethylamine (NDMA) was found in products containing valsartan, an angiotensin II receptor blocker.[1] Other pharmaceuticals containing unacceptable levels of nitrosamine impurities have since been identified and include ranitidine, nizatidine, and metformin HCl.[1] NDMA is a genotoxic and carcinogenic agent in animals and is classified as probably carcinogenic to humans by the International Agency for Research of Cancer.[2]

A comprehensive assessment of active pharmaceutical ingredients (APIs), formulated drug products, and packaging subsequently revealed the presence of nitrosamine drug-substance-related impurities (NDSRIs). Formation of NDSRIs may result from the presence of potentially nitrosatable secondary or tertiary amine moieties in APIs or API impurities and nitrosating agents formed from low levels of nitrite present as impurities.[1]

In response to these concerning findings, regulatory authorities published guidance requiring pharmaceutical companies to assess both chemical and biological products for the possible presence of nitrosamines, and if needed, define appropriate mitigation actions. While the exposure to nitrosamines from pharmaceuticals is likely to be orders of magnitude lower than from food, in order to protect patients, drug manufacturers should adopt effective nitrosamine control strategies.[1]

This whitepaper describes how nitrosamines are formed, the prevalence of nitrosamines and their chemical precursors in pharmaceuticals, methods for quantitation, and strategies for managing nitrite impurities. In addition, a case study highlighting strategies to avoid NDMA formation in metformin drug products is also provided.

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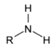
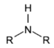
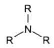
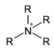
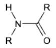
Molecule class	Structure	Nitrosamine precursor?
Primary amine		**No**
Secondary amine		**Yes**
Tertiary amine		**(Yes)**
Quaternary ammonium		**No, but could contain secondary or tertiary amine impurities**
Secondary amide		**No**

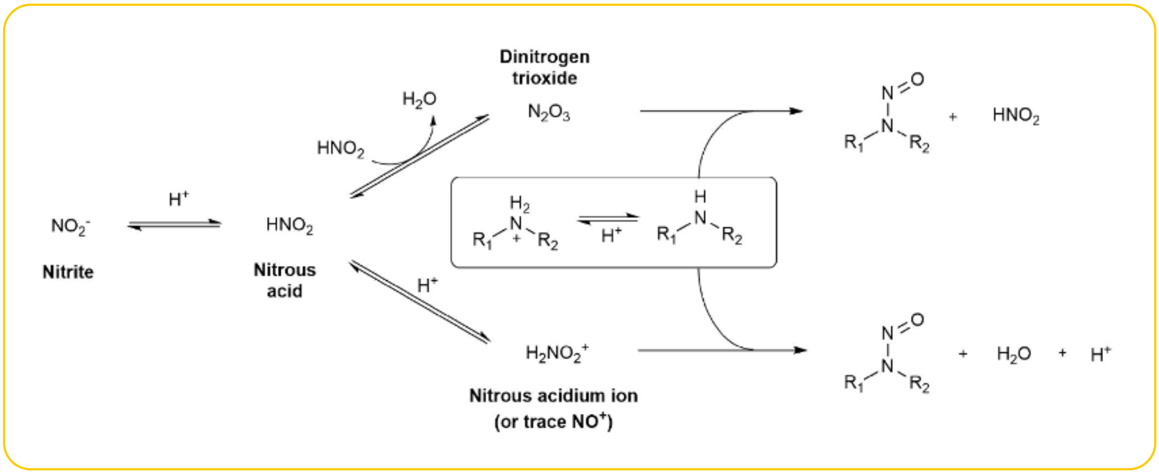
Table 1: Secondary amines are the most frequent nitrosamine precursor.

How Nitrosamines are Formed

Nitrosamine impurities are most frequently derived from secondary amines, which can be starting materials for the synthesis of active pharmaceutical ingredients (APIs), APIs themselves or API related impurities. Primary amines cannot form nitrosamines, tertiary amines require nitrosative de-alkylation, which are much slower processes, and quaternary ammonium ions may degrade to secondary and tertiary amines or contain those as impurities. (Table 1).

In addition to secondary amines, nitrosating agents, most frequently derived from inorganic nitrite, must be present to generate nitrosamines (Figure 1). In acidic pH conditions, nitrite forms nitrous acid and can then further converge to either dinitrogen trioxide or the nitrous acidium ion; these nitrosating agents can then nitrosate the secondary amine.

Figure 1: Nitrosating agents can be derived from inorganic nitrite under acidic conditions.[3]



When nitrosamines are metabolized via the cytochrome P450 enzymes (CYPs), they form a diazonium ion which leads to DNA alkylation. If this damage is not properly repaired, or if the DNA replicates and the corresponding base is not properly incorporated, the DNA will be mutated, potentially leading to an uncontrolled proliferation of the affected cells.

Prevalence of Nitrosamine Precursors in Pharmaceuticals

The first nitrosamine contamination issues focused on small molecule amine impurities generating nitrosamines in the presence of nitrosating agents. Nitrite, however, appeared to be more prevalent in excipients than originally anticipated and by 2021 it was clear that many substances feature 2°/3° amine functionality and could form nitrosamine drug substance-related impurities (NDSRIs).

Beta blockers are one example of a drug family that is affected by nitrosamines. The scaffold of beta blockers has a secondary amine moiety and hence they are susceptible to forming their respective nitrosamines. The same is true for ACE inhibitors such as ramipril and lisinopril which have a secondary amine functionality in the molecule.

The potential prevalence of nitrosamine precursors in pharmaceuticals was recently reported by Schlingemann, et al.[4] In silico tools were used to identify secondary and tertiary amines in the drug impurity structures in databases of drugs and drug impurities. Table 2 provides a summary of possible nitrosamines identified through searches of the US Food and Drug Administration's Global Substance Registration System hosted by the United States Pharmacopeia (USP) and filtered for APIs and API impurities, the World Health Organization (WHO) list of essential medicines, the top 200 drugs by sales volume, and the FDA orange book.

EMA[5], FDA and other Health Authorities have established the Carcinogenic Potency Categorization Approach (CPCA) to classify nitrosamines in five categories. Interestingly nitrosamines derived from secondary amines mainly fall into the less potent categories four and five, with an acceptable intake limit of 1,500 ng/day. A small fraction of nitrosamines are classified as potency category one with limits of 18 ng/day (EMA) or 26.5 ng/day (FDA)[6]. Nitrosamines derived from tertiary amines are distributed relatively evenly among the potency categories.

Figure 2 shows the distribution of potential nitrosamines of small molecule drugs from the FDA's global substance registration system (GSRS) across the EMA potency categories.[7] Potent NDSRIs are more likely to be derived from the less reactive tertiary amines than from secondary amines.

Source	No. of Entries	Structures featuring [Absolute (%)]			Possible nitrosamines		
		2° amine	3° amine	2° and/or 3° amine	Total	from 2° amine	from 3° amine
USP – APIs	8611	1268 (14.7)	2517 (29.2)	3536 (41.4)	7895	2375	5525
USP – Imp	3564	459 (12.8)	681 (19.1)	1077 (30.2)	2213	720	1493
WHO EML	563	58 (10.3)	95 (16.9)	140 (24.9)	287	79	208
Top 200	210	41 (19.5)	43 (20.5)	71 (33.8)	170	81	89
Orange Book	2211	296 (13.4)	526 (23.8)	757 (34.3)	1739	512	1227

Table 2: A large proportion of drugs can theoretically form nitrosamines.[4]

Structures featuring secondary or tertiary amines ranged from nearly 25% to over 40%. The percentage of secondary amines ranged from just over 10% to more than 19%.

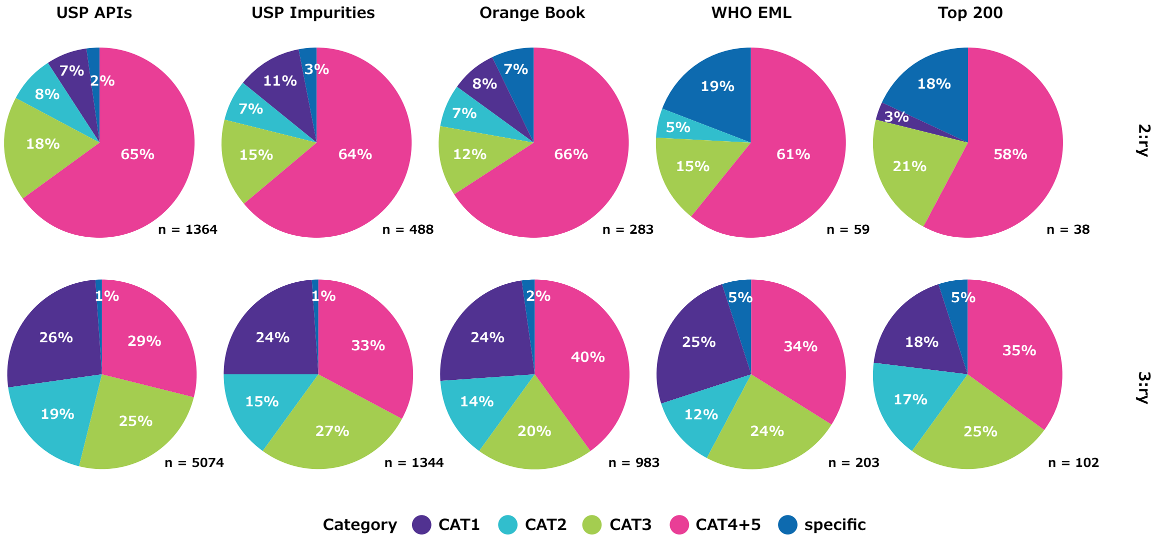


Figure 2: Distribution of potential nitrosamines of small molecule drugs from the FDA's GSRS into the EMA potency categories.[5]

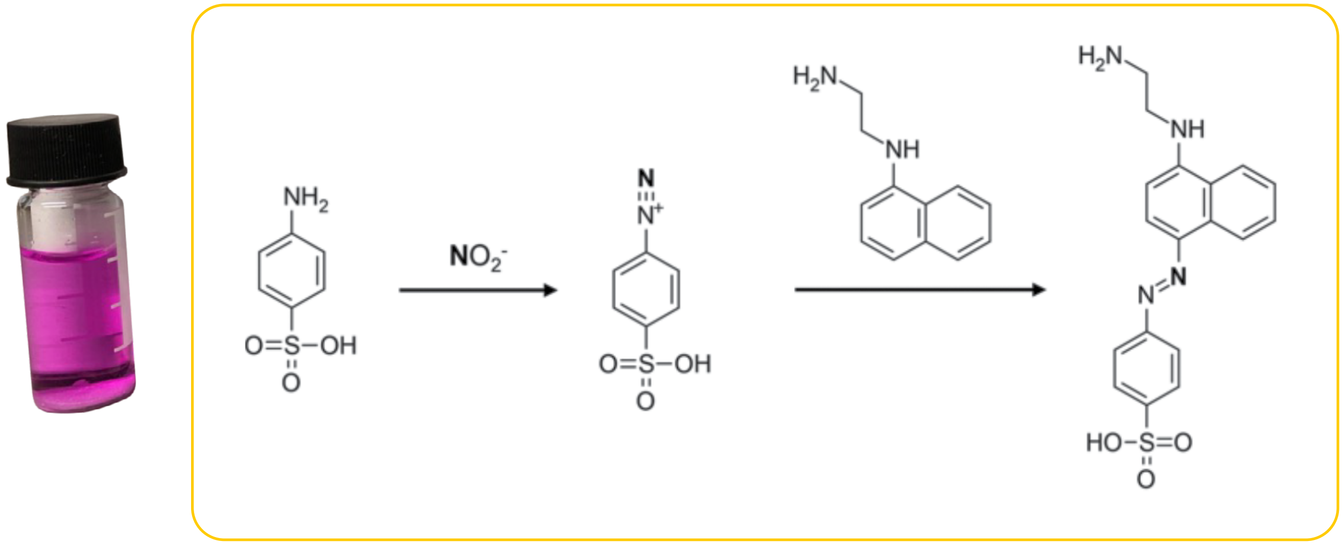


Figure 3: Griess derivatization to detect nitrites.

Methods for Nitrite Quantification

Nitrite is a challenging analyte to detect due to its widespread presence in solvents, excipients, and packaging materials and its presence at trace levels. It is highly polar, has low UV absorbance, and is reactive. There are many approaches for detection, but not all methods are applicable for identifying the presence of trace amounts at the ng/g level.[8]

Griess derivatization is an indirect method for nitrite detection and was first described in 1879. It is a simple reaction that transfers nitrites to a pink azo dye (Figure 3). The derivatization increases retention in RP-HPLC. The chemical reaction, however, can be inhibited by the sample matrix, and if the full conversion does not occur, the nitrite content in a sample may be underestimated.

HPLC-MS/MS following Griess derivatization compensates for varying yields in the Griess reaction by stable isotope dilution analysis. This method offers very high sensitivity and specificity due to the use of MS/MS. Mathematical correction for spectral isotope overlap is required when using this method.

Anion exchange chromatography with conductivity detection is a direct method for nitrite detection but offers lower sensitivity and specificity compared to other approaches as there are frequent interferences. A combination of ion chromatography (IC) with a post-column Griess derivatization allows UV detection and removal of interfering compounds that might suppress the Griess reaction.

The presence of challenging matrices drives the need for orthogonal methods of nitrite detection. UV-active matrices can lead to co-elution in HPLC-UV which prevents accurate integration. In this situation, two options can be used including higher specificity techniques such as LC-MS/MS or a different separation mechanism such as ion chromatography with post-column derivatization (PCD) to separate and then derivatize the nitrite (Figure 4).

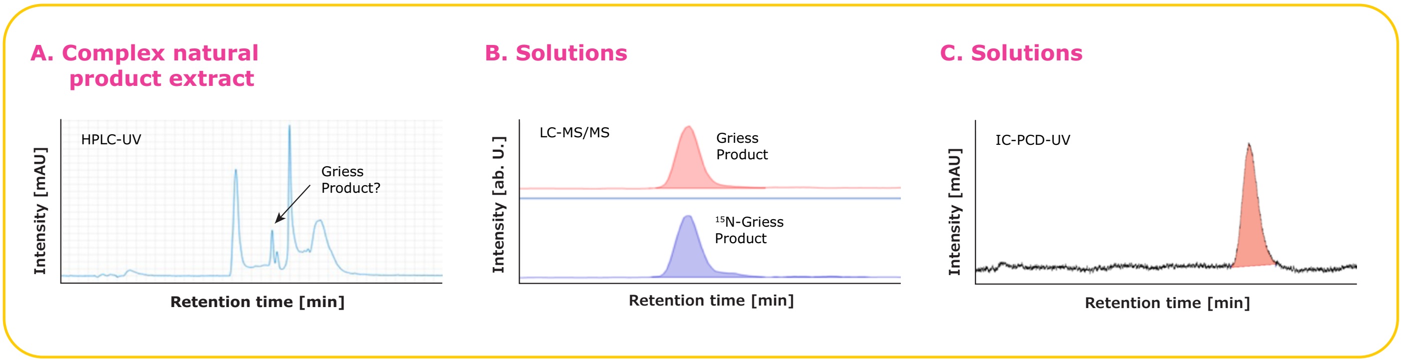


Figure 4: Co-elution in HPLC-UV (A) requires higher specificity (B) or a different separation mechanism (C).

Salts or other materials that bring high ion loads into the chromatography process can saturate the column when using IC, preventing integration of nitrite (Figure 5). While selectivity in IC-PCD-UV is higher, the nitrite peak is distorted and elutes much earlier. Removal of halides by silver filtration can improve separation and result in a signal that can be integrated.

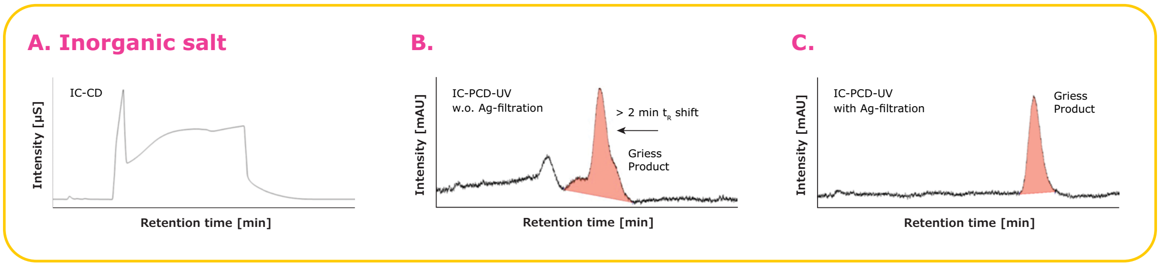


Figure 5: Co-elution in ion chromatography-conductivity detection (IC-CD) or IC-PCD-UV (A and B) with silver filtration (C).

Another challenge is that the Griess reaction itself can be inhibited by matrix components which is often observed for bases and polymers. Lowering sample weights can be beneficial but this negatively affects the limit of quantitation (LOQ). Alternatively, direct detection via IC-CD or matrix removal with IC-PCD-UV allows for quantitation (Figure 6).

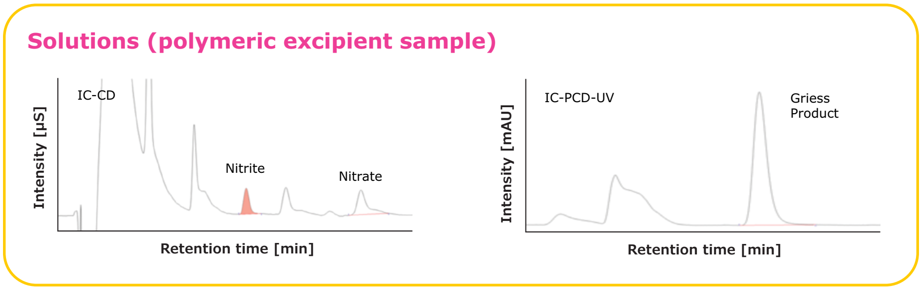


Figure 6: Matrices that inhibit the Griess reaction calls for direct detection (IC-CD) or matrix removal (IC-PCD-UV).

Table 3 summarizes the methods described above for nitrite detection and the LOQ of each. In our labs, HPLC-UV and ion chromatography with post-column derivatization are primarily used.

Analytical technique	~ LOQ [ng/g]	Prevalence of instrumentation	Cost (instrument + reagents)	Selectivity	Limitations	Advantages
HPLC-UV	20-30*	high	low	high	co-elution Griess inhibition	Easy, generic workflow
HPLC-MS/MS	20-30*	scarce	high	highest	Griess inhibition	Highest sensitivity & selectivity, generic workflow, SIDA
IC-CD	100-150	medium	medium	lowest	co-elution, method development (sample prep. & chromatography) required	Direct method, nitrate data available
IC-PCD	20-30*	scarce	medium	high	(co-elution)	Generic workflow, most versatile

Table 3: Comparison of methods used for nitrite detection.

*For Griess derivatization-based methods, the LOQ is not limited by instrument sensitivity but by ubiquitous nitrite background contamination.

Table 4 provides a quantitative comparison of the analytical techniques for nitrite detection in the presence of an insoluble inorganic salt and a halide salt. There is a high level of agreement in the results from the four different methods.

A. Inorganic Salt

Analytical technique	Results [ng/g]	Result [ng/g], corrected by recovery
HPLC-UV	1071	1071
HPLC-MS/MS	1113	1199
IC-CD	1235	1189
IC-PCD	1034	1167
SD [ng/g]	76	51
RSD [%]	6.8	4.4

B. Halide Salt

Analytical technique	Results [ng/g]	Result [ng/g], corrected by recovery
HPLC-UV	43417	43417
HPLC-MS/MS	38740	n. a.*
IC-CD	n. a.	n. a.
IC-PCD	46920	45512
SD [ng/g]	3351	1048
RSD [%]	7.8	n. a.

*recovery not determined as sample has to be diluted prior to measurements

Table 4: Quantitative comparison of the results of nitrite detection in the presence of an inorganic salt (A) and halide salt (B).

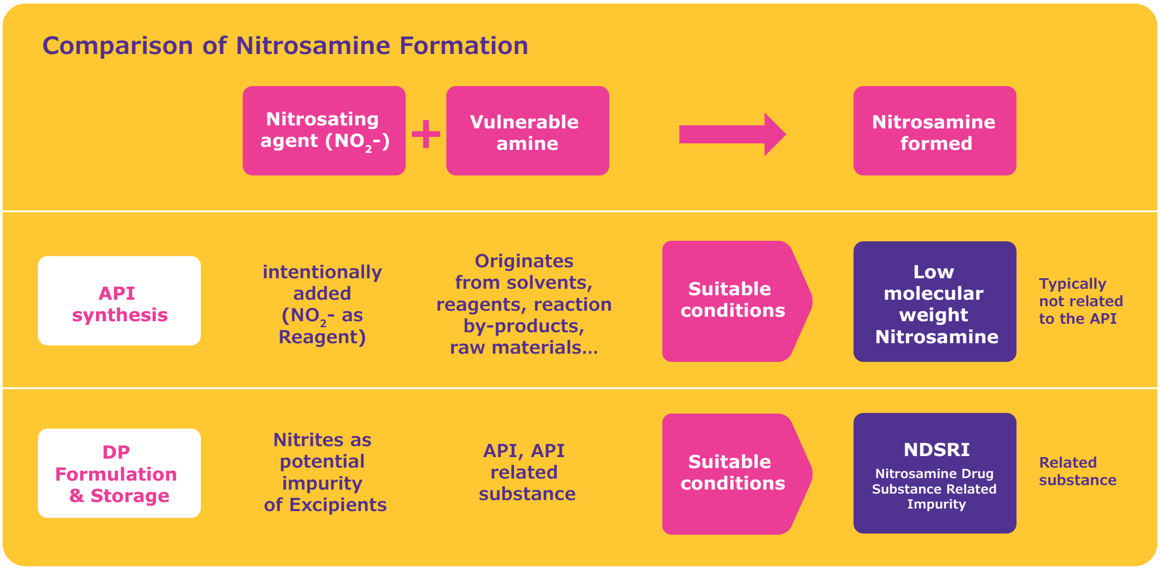


Figure 7: Typical routes to generate nitrosamines of low molecular weight and NDSRIs.

Regulatory Expectations for Nitrites in Excipients

The presence of nitrites in excipients poses the risk of nitrosamine formation in drug products. Formation can result from the API synthesis process in which a nitrosating agent is added and where a vulnerable amine may originate from solvents, reagent, reaction byproducts, or raw and starting materials or degradation products thereof. Under suitable conditions, nitrosamines of low molecular weight such as NDMA could be formed (Figure 7). Nitrosamines can also form during drug product formulation or during storage due to their presence in excipients.

The FDA highlights nitrosamines in their 2021 guidance for industry. As more data are published on this topic, it is expected that these guidelines will continue to evolve.[9] In Questions and Answers (Version 15, March 30, 2023), the EMA noted that nitrites have been identified as impurities and many common excipient marketing authorization holders or applicants should be aware that nitrosamines or API impurities can form at levels exceeding acceptable intake levels.[10]

Sources of Nitrites in Excipients

The presence of nitrites in excipients are a probable root cause for formation of NDSRIs, and may arise from several sources including:

- Process water has been considered as source of nitrite contamination in excipients, but this is typically a route of low concern.
- Raw materials used in the manufacture of excipients can contain nitrites and this can be an important root cause.
- Nitrite impurities are introduced to raw materials used during production of the excipients, such as during the spray drying process; this is a well-identified root cause.

Recalls Due to the Presence of N-Nitroso APIs in Drug Products

API nitrosamines are likely formed through an interaction between secondary amines in the API chemical structure, with nitrites presence in the formulation. The nitrites are possibly introduced from the excipients used in this formulation and their presence can create a risk of nitrosamines formation depending on other factors present. Varenicline, Quinapril, and Rasagline are all secondary amines which were nitrosated, leading to recent recalls. Orphenadrin is an example of a tertiary amine of which an NDSRI can be formed; this also resulted in a recall. A recall for the beta receptor antagonist metoprolol was initiated in 2023 in Germany due to the presence of N-Nitroso-metoprolol.

Conclusion

If APIs, API impurities, or degradation products thereof show a nitrosatable structure of a vulnerable amine, nitrosamines may be generated in the presence of nitrosating agents. The risk of generating nitrosamines must be assessed during the manufacture of the drug product, including formulation and during storage of the finished product. The presence of these impurities in human medicinal products shall be mitigated as much as possible and shall be at or below the acceptable intake published by the competent authorities. Several drugs have already been recalled since the levels of nitrosamines exceeded the acceptable intake levels.

However, nitrite data for excipients are often not available, and there is no regulatory requirement for nitrite testing of excipients. To address this important safety issue, nitrites levels contained within three batches of each product of our Emprove® Essential and the Emprove® Expert portfolio have been assessed.

As shown in Table 5, three product categories have been defined based on the amount of nitrites detected. For some products, the nitrite concentrations are not more than 200 ng/g and in many cases are below the LOQ. These products present a low level of risk for introducing a relevant level of nitrites into a process. The second category of products have nitrite levels between 200 and 500 ng/g. For most products in this category, the presence of nitrites has been detected in at least one batch; these products are periodically tested to monitor the level of nitrites. The final category contains products for which one batch was found to have a nitrite concentration exceeding 500 ng/g. While this is still in the sub-PPM range, further control steps are being applied and more batch data are being collected to derive a feasible limit.

Information related to the presence of nitrite in these products is provided in the Emprove® Material Qualification and Operational Excellence dossiers. This information supports the risk assessment of nitrosamine formation in drug products and contributes to improvements in patient safety and risk mitigation, and relies on collaboration of excipients and drug product manufacturers to reduce the risk of nitrosamine formation in the drug product.

Not more than 200 ng/g	**>200 ng/g up to 500 ng/g**	**> 500 ng/g**
Typically below LOQ	Where presence of nitrites is identified:	Where presence of nitrites is identified:
• Due to low level, a process risk for introduction of nitrites is not expected	• Periodic retesting	• Gain more batch data to derive feasible limit
• Low concern	• Moderate concern	• Develop method for regular control

Table 5: Emprove® products are categorized into three groups based on the quantity of nitrites detected.

Case Study: Avoiding NDMA Formation in Metformin Pharmaceuticals

The following case study is summarized from Schlingemann, et al.[11] Metformin is the most frequently used drug to treat type 2 diabetes and is synthesized from 2-cyanoguanidine and dimethylamine, which is the precursor of NDMA. Testing all stages of metformin API synthesis starting from DMA revealed that there was approximately 30-50 parts per billion (ppb) NDMA present in the DMA starting material and this was formed in several process intermediates. Two crystallization steps and a drying process, however, significantly reduce the NDMA content to below 1 PPB or to an undetectable level (Figure 8).

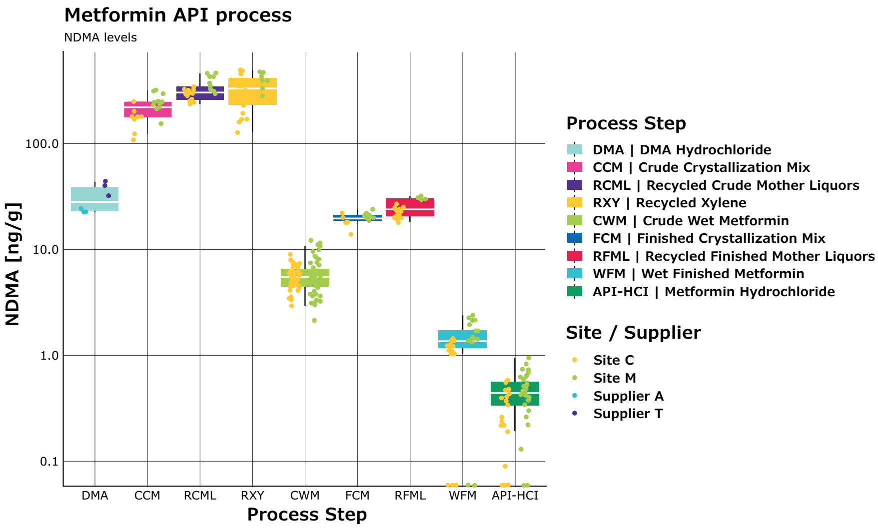


Figure 8: Metformin API is essentially free from NDMA.

Analysis of process intermediates revealed that for the immediate release formulation of metformin, NDMA was formed during wet granulation; for the extended-release formulation, NDMA was only present in the final blend (Figure 9). In the case of metformin, the presence of NDMA can be attributed to the use of excipients with a high nitrite content.

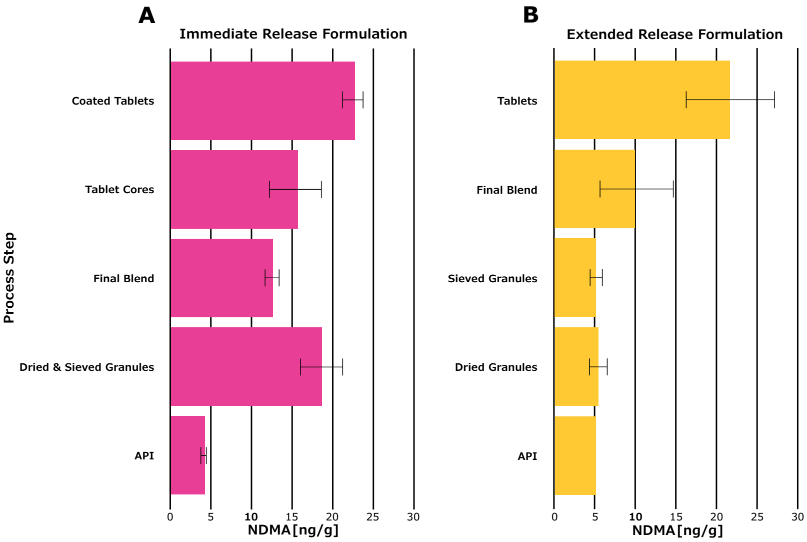


Figure 9: NDMA was formed when the immediate release (A) or extended release (B) API was combined with nitrite-containing excipients under promoting conditions.

The relevant excipients were hydroxypropyl methylcellulose (HPMC) for the extended-release formulation and polyvinylpyrrolidone (PVP) for the immediate release formulation.

The nitrite content in these excipients varied considerably depending on the supplier (Figure 10).

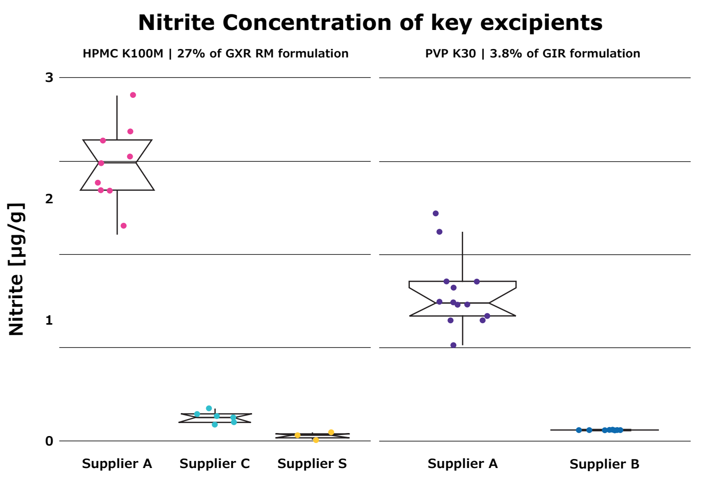


Figure 10: Considerable differences observed in the nitrite content of key excipients from different suppliers.

Drug product batches made with high-nitrite excipients had significantly more NDMA. As shown in Figure 11, the red dots represent the NDMA content in drug product batches made with high nitrite excipient and the green dots represent the NDMA in drug product batches made with low nitrite excipient.

Another factor influencing the amount of NDMA in the drug product is dimethylamine in the API. If the amount of dimethylamine is relatively lower, less NDMA will be formed. The amount of nitrite is also important to consider as both must react to form NDMA. It is also important to note that the current compendial limit for DMA in metformin API is 500 PPM; 150 PPM was far below the compendial limit, but obviously still too much for in terms of NDMA formation, when a high level of nitrites is introduced through the excipients.

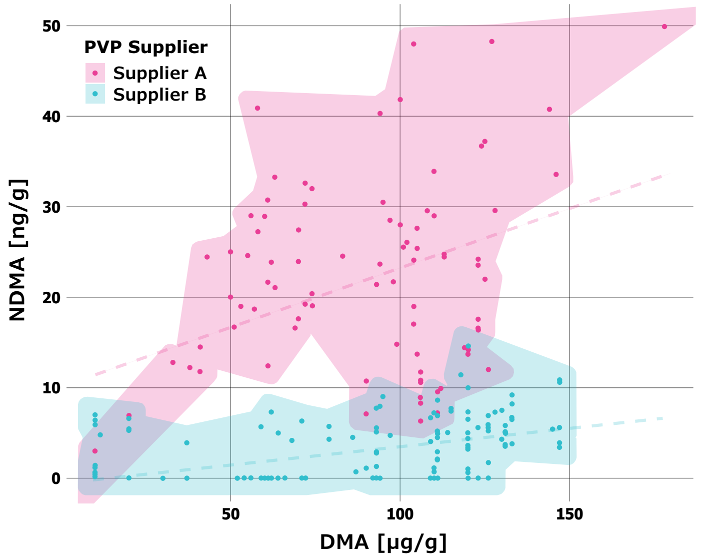


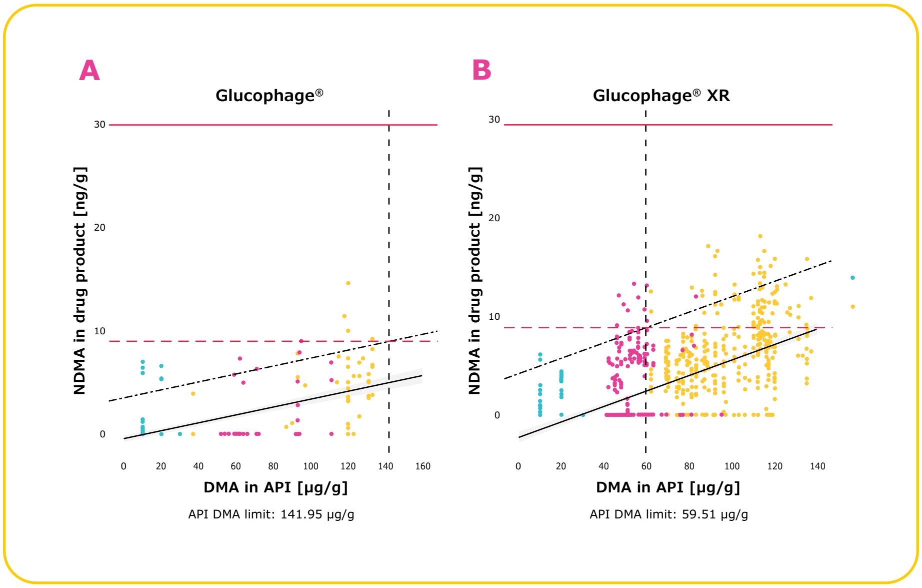
Figure 11: Drug product batches made with high-nitrite excipient had significantly more NDMA.

NDMA Control Strategy

The proposed NDMA control strategy for excipients included two approaches:

- Discontinue the use of high-nitrite excipients by moving to a different supplier offering consistently low nitrite content
- Reduce the amount of residual DMA content in the API synthesis process to an internally-established limit

The internal limit for DMA was established by measuring thousands of drug product batches and correlating the DMA and NDMA content and then taking 30% of the acceptable intake of NDMA which is 96 ng/day. Figure 12 shows the linear regression of DMA versus NDMA, and the 95% prediction interval. The intersection of the 95% prediction interval with 30% of the content limit defined a DMA content that will be below the 30% NDMA limit in 95% of the cases. For the immediate release and extended-release formulations, 140 PPM and 60 PPM DMA are permissible.



API DMA limit: 141.95 µg/g

API DMA limit: 59.51 µg/g

Figure 12: Deriving a DMA limit was essential for the NDMA control strategy for immediate (A) and extended-release formulations of metformin.

It is important to note that other excipients may also contain nitrites and potentially impact the drug product.[9] As shown in Figure 13A, crospovidone can have a variable and relatively high nitrite concentration ranging from practically nothing to 15 PPM. While the concentration of nitrites in magnesium stearate can also be relatively high, it is likely to have a relatively low impact in practice as only 0.1 to 0.5% is typically added to the drug product.

Figure 13B shows the importance of controlling the nitrite content both in excipients and the drug product. The amount of nitrosamine that can form in an API is related to the content of the vulnerable amine and the nitrite. With excipient loadings ranging anywhere from 0.12% to almost 50%, total nitrite in the drug product may range from 0.1 to several PPM. Considering that perhaps 1-10% of this nitrite is converted and reacts with the amine to nitrosamine, amounts well above acceptable intake limits may result. For example, in the case of 10% conversion of the amine to nitrosamine, up to 540 ppb of the respective nitrosamine can result (Figure 14).[1]

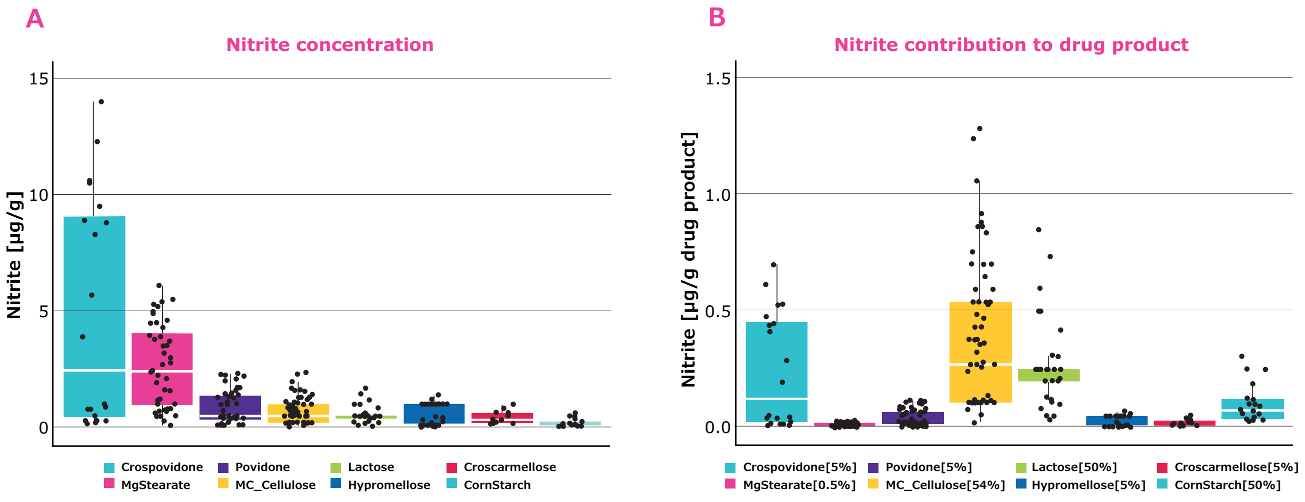
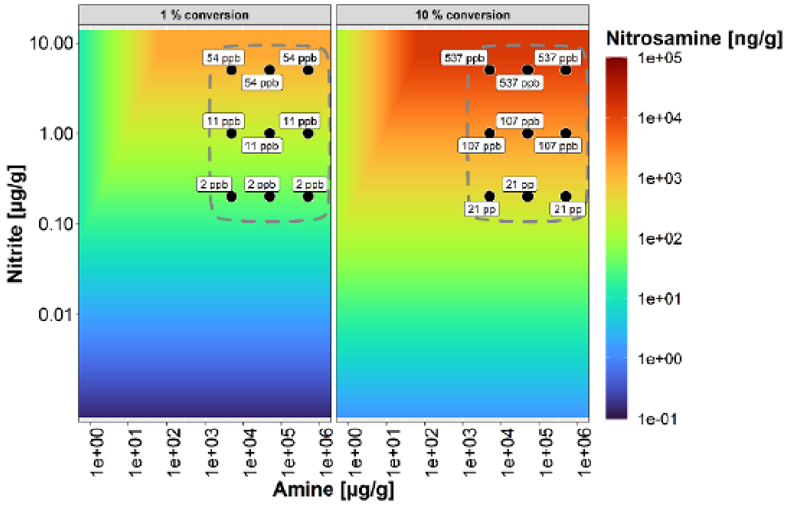


Figure 13: Nitrite concentration in excipients (A) and nitrite contribution to the drug product (B).[3]



Source: https://doi.org/10.1021/acs.oprd.3c00100

Figure 14: Typical amine and nitrite content in drug products can lead to considerable nitrosamine formation. In many cases, the API itself is the vulnerable amine.[1]

A recent publication provides additional information on nitrosamine assessments in excipients and drug products.[3] The article describes a database constructed by a consortium of pharmaceutical companies that lists the nitrite content of approximately 100 excipients. The database can be used for risk assessment purposes, to determine if an excipient may contain problematic amounts of nitrite.

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