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Nitrosamine Impurities in Pharmaceuticals: Chemistry, Toxicology, and Advanced Analytical Approaches

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Nitrosamine impurities, composed of nitroso and amine functional groups, pose significant carcinogenic and mutagenic risks. An extensive literature survey was undertaken, encompassing peer-reviewed research articles, regulatory authority guidelines, and market recall data, to thoroughly explore the occurrence, sources, and regulatory perspectives related to nitrosamine impurities. The survey revealed that the formation of nitrosamine impurities in active drugs is attributed to factors like reactants and process parameters used in their synthesis and their generation in the drug products can be associated with the active drugs, excipients used, packaging material employed during manufacture and the storage conditions maintained. Stringent regulatory requirement for control of nitrosamine impurities to trace levels in pharmaceuticals necessitates the requirement of highly sensitive advanced analytical techniques for the accurate and precise detection and quantitation of nitrosamine impurities. This review is an elaborative account of the Nitrosamine impurities which gives information related to types of nitrosamine impurities, their toxicological concerns, chemistry behind their formation, and major contributors in their formation along with analytical methods used for their detection with appropriate examples wherever possible.

Keywords: Analytical techniques; Mutagenic impurities; Nitrite; Nitrosamine impurities; Secondary amine.

Impurities present in drug substances and drug products are detailed in the International Council for Harmonisation (ICH) quality (Q-series) guidelines. ICH Q3 defines impurity in DS as any compound from the new DS that is not the chemical entity as called the new DS. It establishes an impurity in DP as any constituent of the new DP that is neither the DP nor an excipient of the said DP.[1,2] Nitrosamine impurities are a class of organic impurities that have significantly impacted in the pharmaceutical industry and have stringent regulations for their control. Impurities belonging to the nitrosamine category in drug formulations (pharmaceuticals) were observed first in 2018, where the United States Food and Drug Administration (US FDA) reported their presence in Valsartan batches manufactured by Zhejiang Huahai Pharmaceutical (ZHP).[3] Since then, these

impurities have become a topic of growing concern throughout the pharmaceutical industry owing to their carcinogenic potential.

Nitrosamine impurities can be observed in both DS and the DP. The formation of Nitrosamine impurities in DS is attributed to factors such as the reactants used and synthesis process parameters, including pH and temperature of the reaction. The generation of NI in the DP can be associated with the active drugs, excipients used, packaging materials employed during manufacture, and the storage conditions maintained.

Detection of NI in both DS and DP involves sensitive analytical techniques. This article aims to provide a comprehensive insight into the world of NI. It has covered information related to the types of NI, their toxicological concerns, the chemistry behind their formation, and the major contributors to their formation, along with an elaborate and detailed account of the analytical methods used for their detection, accompanied by appropriate examples wherever possible.

MATERIALS AND METHODS

This review involved a comprehensive study and search through various science-based databases which included PubMed, Scopus along with Google Scholar, using keywords such as "nitrosamine impurities", "carcinogenicity", "conditions leading to NI formation in DS and DP", "sources", "analytical techniques", "regulatory control". No restrictions were applied regarding the publication year or language of the studies. A total of 50 studies were reviewed including case studies, original research work, industry reports, review articles. The research uses a comprehensive approach based on the unity of theory and practice.

RESULTS

NI and their types

NI contain a nitroso and an amine functional group as shown in Figure 01.

US FDA has reported following seven types of NI that are most commonly encountered in DS and DP.[4] These are given in Table 01:

Toxicological Concerns Associated with Nitrosamines

The rising concern over the NI presence in DP is due to its mutagenic and potentially carcinogenic nature in humans.[3-5] ICH multidisciplinary (M-series) guideline M7(R2) defines mutagenic impurity as "An impurity which has been demonstrated to be mutagenic in an appropriate mutagenicity test model, e.g., bacterial mutagenicity assay".[6] The National Toxicology Program, US Department of Health and Human Services, classifies NI as "reasonably anticipated to be human carcinogens".[7] NDMA comes in the class, which are termed as "probably carcinogenic to humans" by the International Agency for Research on Cancer (IARC).[8]

As per a study performed by Yamazaki *et al.*[9] in 1992, NIs in the body are oxidised by cytochrome P450 (CYP450), leading to the formation of a reactive intermediate alkyldiazohydroxides (ADH), which irreversibly alkylate nucleic acids, thereby supporting the probable carcinogenic and mutagenic nature of NIs. This study shows that the liver microsomal enzymes CYP2E21 and CYP2A6 catalyse the activation of nitrosamines in the body; other microsomal enzymes, such as CYP3A4 and CYP1A2, may also contribute. The carcinogenic potential of NDMA is attributed to the formation of two reactive intermediates, namely formaldehyde

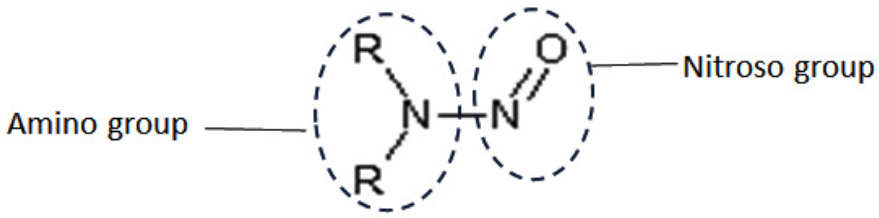
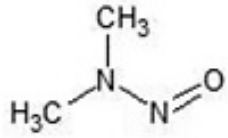
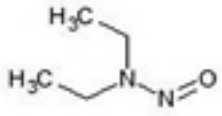
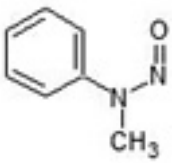
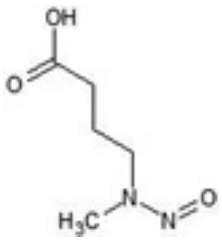
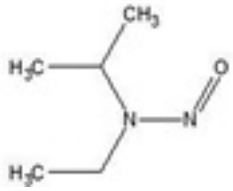
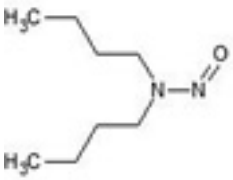
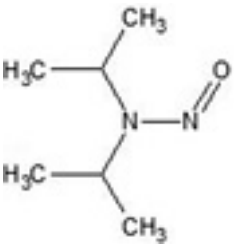


Fig. 1. General Structure of Nitrosamine Impurity

Table 1. Nitrosamine Impurities listed USFDA

Nitrosamine Impurity	Abbreviation	Chemical Structure
N-nitrosodimethylamine	NDMA	
N-nitrosodiethylamine	NDEA	
N-nitrosomethylphenylamine	NMPA	
N-nitroso-N-methy-4-aminobutanoic acid	NMBA	
N-nitrosoisopropylethylamine	NIPEA	
N-nitrosodibutylamine	NDBA	
N-nitrosodiisopropylamine	NDIPA	

and methyldiazonium ion, produced during the catalysis of nitrosamines by CYP450 enzymes, that alkylate the DNA base guanine irreversibly. The methyldiazonium ion is mainly responsible for methylating DNA, leading to carcinogenesis. This carcinogenic nature is due to the possible adduct formation which involves methylation at O6 and N7 of guanine.[10]

Table 2. Acceptable Intake of NI as per USFDA

Nitrosamine Impurity	Acceptable Intake (nanograms per day)
NDMA	96
NDEA	26.5
NMBA	96
NMPA	26.5
NIPEA	26.5
NDIPA	26.5

Acceptable Intake and Acceptable Daily Intake

ICH M7(R2) defines acceptable intake (AI) as "an intake level that poses negligible cancer risk, or for serious/life-threatening indications where risk and benefit are appropriately balanced".[6] Acceptable Daily Intake (ADI) is the daily intake of a substance which, over the entire life time of a human, will not have any adverse effects or will not cause any harm to the health of that human.[11] Based on the threshold of toxicological concern (TTC), the US FDA defines the ADI for NI.[4] According to ICH M7(R2) guideline on mutagenic impurities,

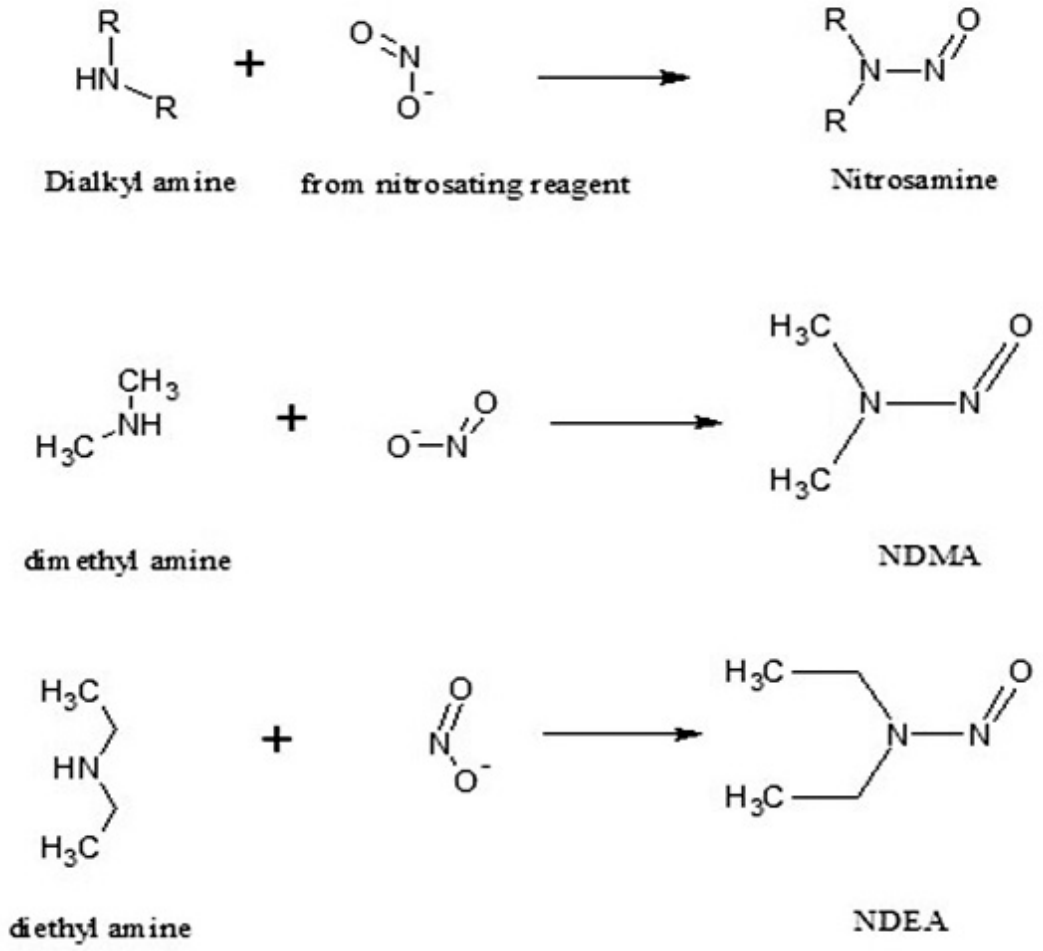


Fig. 2. Chemical reactions involved in formation of Nitrosamine Impurities

"known mutagens with unknown carcinogenic potential need to be maintained at or below acceptable limits, i.e., appropriate TTC". Known mutagens with unknown carcinogenic potential are those that exhibit bacterial mutagenicity but do not induce carcinogenicity in rodents. According to the TTC-based AI for mutagenic impurity, 1.5 µg of mutagenic impurity is allowed per person per day, which poses a negligible risk, meaning the cancer risk is less than 1 in 1,000,000 over a lifetime of exposure. This approach is utilized for mutagenic impurities present in pharmaceutical products that are to be used for long course treatment of more than 10 years and wherein no carcinogenic data is available for such pharmaceuticals.[6] European Medicine Agency (EMA) also follows the ICH M7(R1) principles for NI. The AI limits for NI are based on TTC. These guidelines require all Marketing Authorization Holders (MAHs) to perform risk analysis for the occurrence of NI in pharmaceuticals.

ADI limits for NI as provided by USFDA are summarized in Table 02.[4]

Sources of Nitrosamine Impurities in Pharmaceuticals

The formation of nitrosamine impurity is possible in most circumstances where both nitrite and amine sources are available.[5] Different sources reported to contribute to the formation of NI in pharmaceuticals are discussed below.

Nitrosamine Impurity in Drug Substance (Active Pharmaceutical Ingredient API)

According to ICH Q1A (R2), DS is "the unformulated DS that may subsequently be formulated along with excipients to produce the dosage form".[12] Reagents and reaction conditions used during the preparation of synthetic DS contribute to the generation of NI. Two reagents

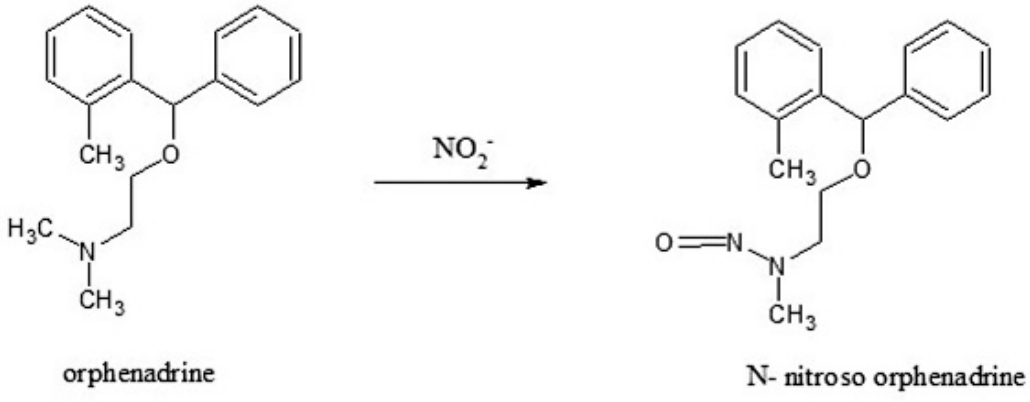


Fig. 3. Formation of N-nitroso orphenadrine (NDSRI) from orphenadrine

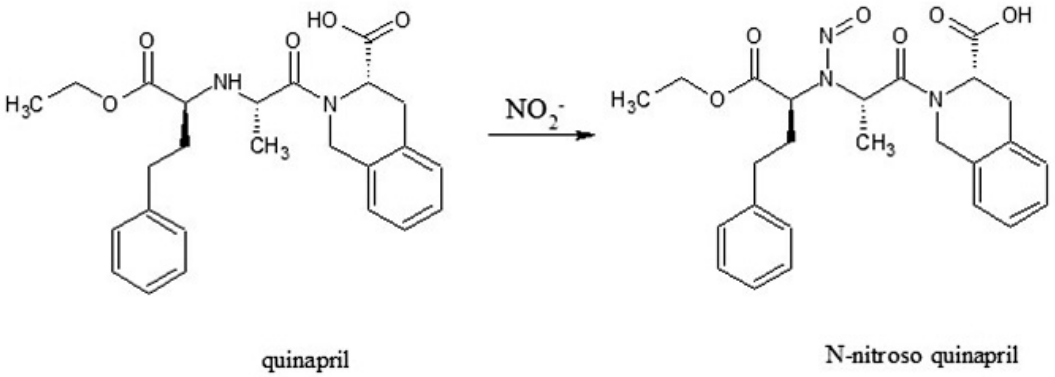


Fig. 4. Formation of N-nitroso quinapril (NDSRI) form quinapril

may react with each other to generate NI. NDMA was reported in 2018 to originate from a reaction between sodium azide, used to form the tetrazole ring in Valsartan synthesis, and sodium nitrite, employed to quench the excess sodium azide.[5,13] NI formation is possible if the manufacturing conditions are not monitored. Similarly inappropriate reaction conditions like pH, temperature have also reported to be responsible for formation of nitrosamine impurities.[14]

Synthesis of API sometimes involves use of recycled or recovered materials. If these recycled materials are contaminated with untreated nitrite or NI, API and other raw materials synthesized using them are definitely expected to contain these impurities.

Availability of both an amino and a nitro functional group in the molecular structure itself is a favourable condition for the NI formation as the API itself becomes the source for both amine and nitrite. The typical example of this is Ranitidine.

Nitrosamine Impurity in Drug Product

Excipients used in the formulation of the DP are sometimes contaminated with nitrite and thus contribute to the formation of NI in the final product. Few excipients commonly used in dosage form preparation, such as diluents like microcrystalline cellulose, from the class of super disintegrants, like croscarmellose sodium, sodium starch glycolate, and crospovidone, and glidants like magnesium stearate and corn starch, are reported to be contaminated with nitrite.[15,16] Certain excipients, such as L-proline, meglumine, triethylamine, ethylene diamine tetraacetic acid (EDTA), having amine functionality present, can also lead to NI formation. Certain polymeric excipients, such as povidone and crospovidone, are reported to contain peroxides, which are responsible for generating nitrosating agents. These agents can react with labile amines and/or acidic degradants, catalysing nitrosamine formation.[5,16] A classic example of such involvement of

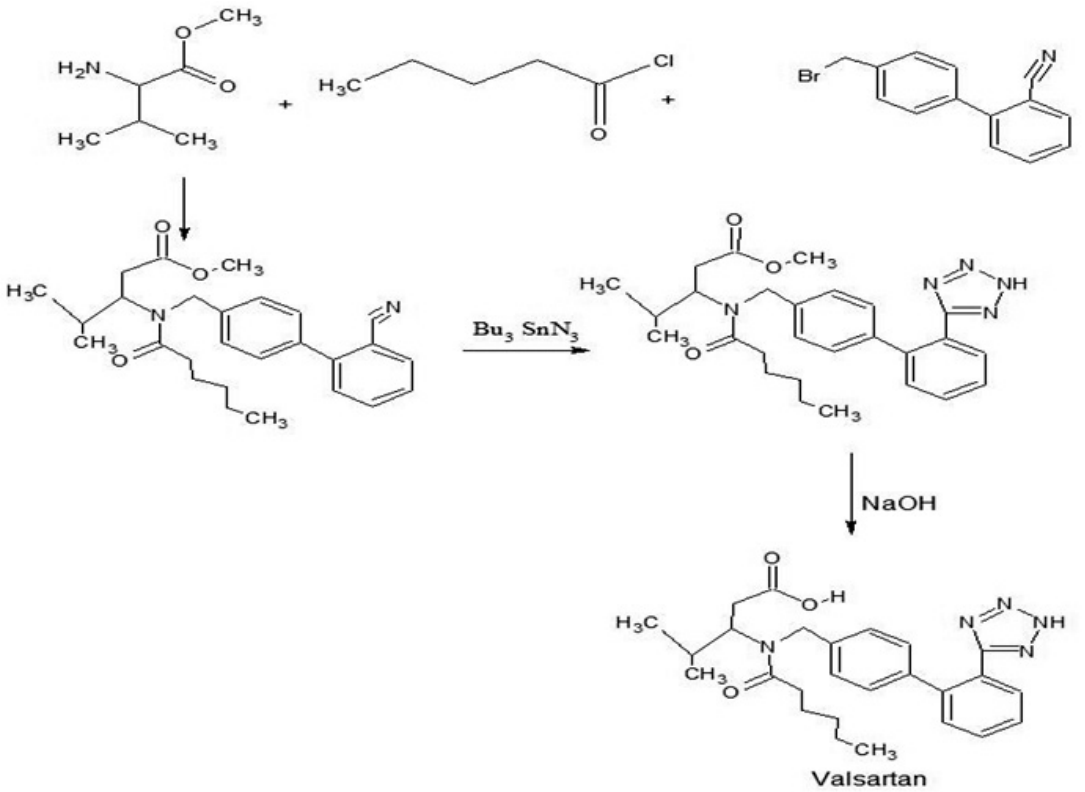


Fig. 5. Route of Valsartan Synthesis as per European Pharmacopoeia

crospovidone is studied by N. Golob *et al*. [17] in Afitinib tablets (2023). They had evaluated two tablet formulations, which were prepared by using the excipient crospovidone with different nitrite content. It was observed upon accelerated storage that formulation with crospovidone having higher nitrite content showed a greater proportion of NDMA formation when analysed with liquid chromatography-high resolution mass spectrometry (LC-HRMS).[17]

NI are even reported to be formed during the storage of DP, for example, metformin hydroperoxide. This metformin hydroperoxide is reported to bring about oxidation of other metformin molecules or intramolecular oxidation ultimately leading to formation of NI.[3]

NI is also reported to occur in DP due to their interaction with the packaging material used to store it. Nitrocellulose lidding foils are commonly employed for packaging purpose

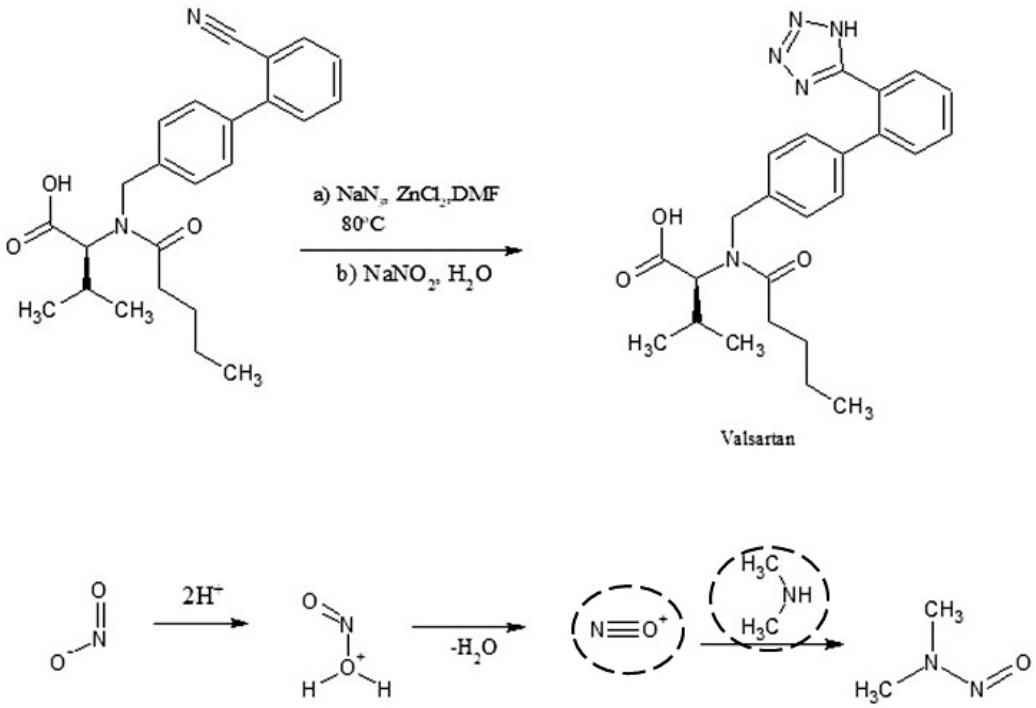


Fig. 6. Chemical reaction involved in the modified reaction involved in Valsartan Synthesis

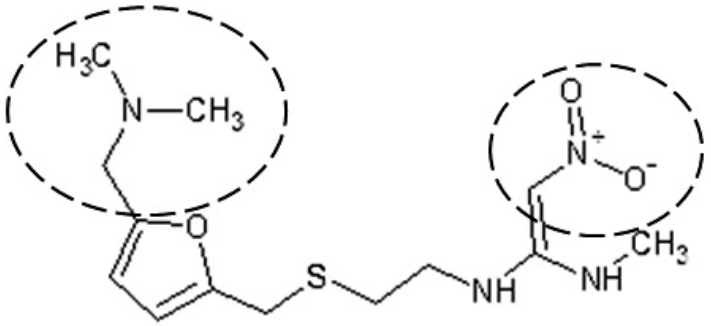


Fig. 7. Structure of ranitidine

which are labelled using printing ink. Literature has stated the evidence of reaction between nitrite from nitrocellulose and secondary amine functional group in drug resulting in the formation of nitrosamine impurity. During storage these may be transferred to the DP causing contamination of the DP with NI.[18,19]

Chemistry of Nitrosamine Impurity Formation

The reaction involved in the formation of NI includes the reaction of nitrites with amines, carbamates, amides, and urea derivatives.[20] Amongst amines, the secondary amino group is found to be the most susceptible to nitrosamination as it directly forms nitrosamine upon reaction with nitrosating reagent. Aromatic secondary amines undergo faster reactions compared to aliphatic secondary amines. The primary amine group generally does not form nitrosamine upon reaction with nitrite, as it forms an unstable nitroso species and ultimately forms diazonium ion when protonated. However, in the presence of a second primary amine molecule, it can form a secondary amine, which in turn can form nitrosamine. Tertiary amino groups are significantly less susceptible to the formation of NI and form nitrosamine only after their subsequent rearrangement to secondary amine.[5] Protonated amines are more susceptible than their free bases, mainly due to the reduced solubility of free bases and the need for acidic conditions to generate the active nitrosating agent.[5,20] The general reaction leading to the formation of nitrosamine impurities can be depicted as follows:

Nitrosamine Drug Substance-related Impurities (NDSRIs)

Along with NI listed in Table 01, nitrosamine drug substance-related impurities (NDSRIs) have also been cited to be present in DP.

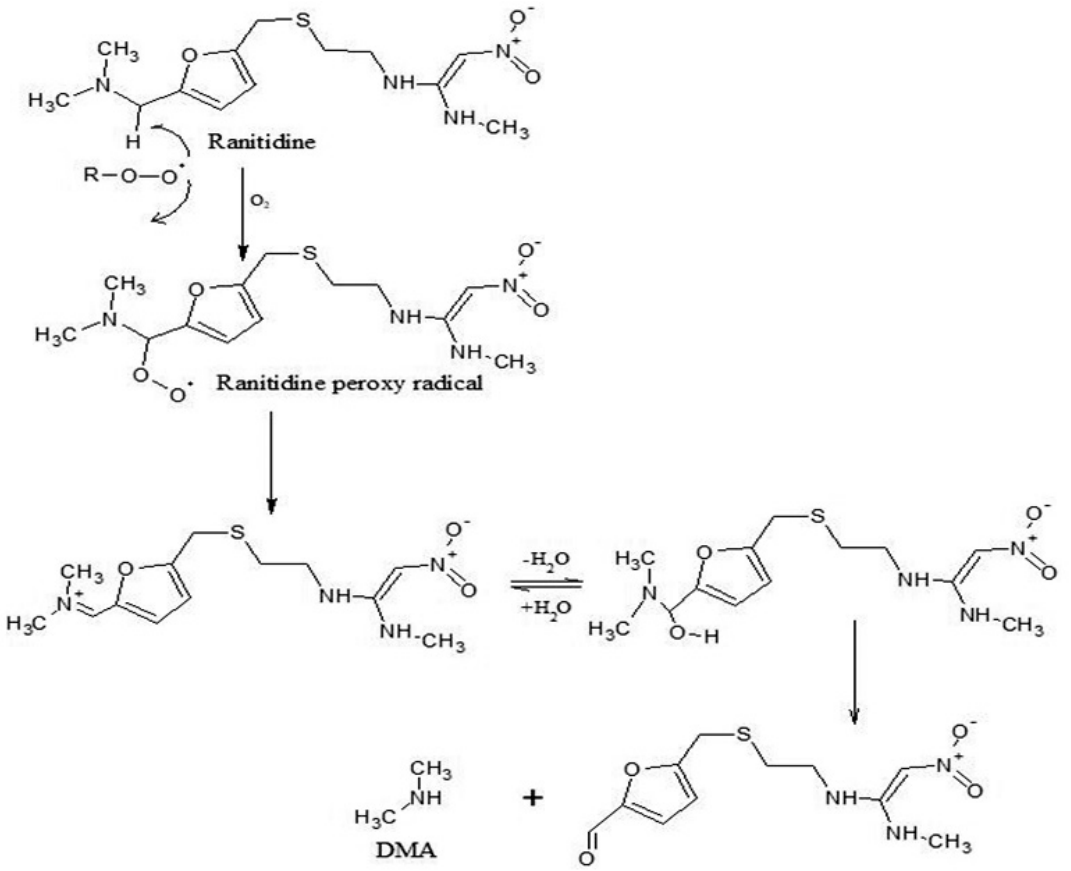


Fig. 8. Formation of DMA from Ranitidine

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As mentioned in Federal Register, "NDSRIs are a class of nitrosamines having structural similarity to the API, and thus, differ in certain respects from small molecule NI. NI that donot share structural similarity to the API, and are therefore, not considered to be NDSRIs".[21] Formation of NDSRIs in DP may occur either during manufacturing of DP or during storage. This is attributed to the presence of nitrite impurities at parts per million (ppm) level in common excipients such as poly vinyl pyrrolidone (PVP), sodium starch glycolate, croscarmellose sodium and pre-gelatinized

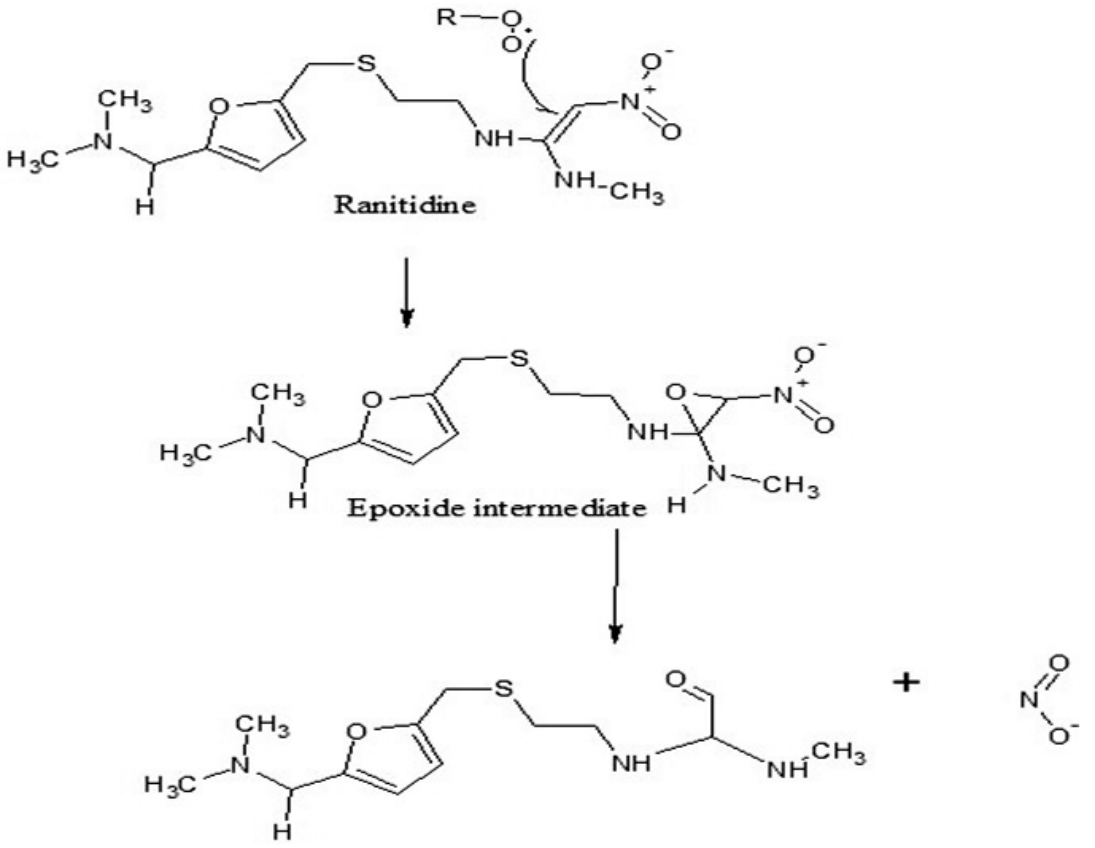

Fig. 9. Formation of nitrite from Ranitidine

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Table 3. Summary of Nitrosamine Impurities Formation in Valsartan, Ranitidine and Afatinib

API	Impurity Formed	Source	Recall Year
Valsartan	NDMA	Change in synthesis process to use of tributylin azide and DMF as solvent	2018
Ranitidine	NDMA	Autooxidation	2019
Afatinib	NDMA	Excipient crospovidone containing nitrite	Not applicable (Research study)

Table 4. LC-MS methods used for separation and detection of NI in DS and DP

Drug (s)/ Drug Class	Nitrosamine Impurities Detected	Stationary Phase	Mobile Phase	Elution Mode	Ionization Mode	LOD	LOQ	Reference Number
Rivoroxban(DS)	*N*-(2-hydroxyethyl)-*N*-phenylnitrous amide	ODS	Aqueous solution of methanoic acid (0.1%): methanol 1:1	Isocratic	-	0.045 ng/mL	0.15 ng/mL	30
Sitagliptin phosphate(DS)	7-nitroso-3-(trifluoromethyl) -5,6,7,8-tetrahydro-[1,2,4] triazolo[4,3-*a*]pyrazine (NTTP)	ODS	A: 0.1% methanoic acid in 0.01M ammonium formate in waterB: ACN	Gradient	ESI	0.037 ppm	0.098 ppm	31
Candesartan cilexetil Irbesartan LosatanValsartan Olmesartan medoxomil (DS and DP)	NDMA NDEA NDPA NMBA NMEA NDiPA NDiPLA NDELA NEIPA NMOR NPYR NPIP	HSS T3 (ODS)	A: Aqueous solution of methanoic acid (0.1%)B: 0.1% methanoic acid in acetonitrile/ methanol (2:8)	Gradient	APCI	20 ng/g	50 ng/g	32
Mab A expressed by Chinese hamster ovary cells (DP)	MeNP NMOR NPYR NDEA NPIP NEIPA NDPA NDIPA NMPA NDBA NDIBA NDBzA	-	-	-	-	0.005-0.025 µg/L	0.010-0.250 µg/L	33
Acebutol hydrochloride Bisoprolol fumarate Metoprolol succinate Metoprolol tartarate Sotalol hydrochloride (DS)	*N*-nitrosoacebutolol, *N*-nitrosobisoprolol, *N*-nitrosometoprolol, and *N*-nitrososotalol	HSS T3	Aqueous solution of methanoic acid (0.1%) combined with methanol/ acetonitrile	-	ESI	0.02–1.2 ppb	2–20 ppb	34
Ranitidine (DS and DP)	NDMA	ODS	A: Aqueous solution of methanoic acid (0.1%)B: Methanol	Gradient	APCI	0.01 ppm	0.03 ppm	35

Varenicline tartrate (DS and DP)	NDMA NDEA N-nitroso varenicline	ODS	A: Aqueous solution of methanoic acid (0.1%)B: Methanol	Gradient	APCI	0.22 ppm	0.66 ppm	36
Duloxetine hydrochloride (DS)	NDMA NDIPA N-nitroso duloxetine	Phenyl C18	Methanoic acid and methanol mixture	Gradient	APCI	0.0001 ppm	0.003 ppm	37
Empagliflozin (DS and DP)	NDMA NDEA NEIA NDIPA	-	0.1% aq. ammonia buffer: methanol5:95	Isocratic	-	NDMA, NEIA, NDIPA 0.03 ppm NDEA0.02 ppm	NDMA, NEIA, NDIPA 0.03 ppm NDEA0.02 ppm	38
Sartan group (DS and DP)	NDMA NDEA NDIPA NMPA NDBA NMBA NEIPA 5-[4'-(azidomethyl)-[1,1'-biphenyl]-2-yl] -2H-tetrazole (AZBT)	ODS	A: Aqueous solution of methanoic acid (0.1%)B: Methanol	Gradient	APCI	Range:NMBA, NDEA, NDIPA, NMPA, NDBA0.2-50 ng/mL NDMA, NEIPA0.5-50 ng/mL AZBT2.0-100 ng/mL		39
Rifampicin(DP)	1-methyl-4-nitrosospiperazine (MNP)	Phenyl column	A: 10mM ammonium formate (pH 9) B: Methanol	Gradient	ESI	0.51 ng/mL	1.52 ng/mL	40
Rifampin Rifapentine (DS and DP)	MNP CPNP	Phenylhexyl column	A: 10mM ammonium formate (pH 9) B: Methanol	Gradient	ESI	-	0.05 ppm	41
Metformin tablet formulation (DP)	NDMA NDEA NDPA NDBA NPYR NPIP NMOR	ODS	A: Aqueous solution of methanoic acid (0.1%) B: Acetonitrile	Gradient	-	-	0.1-5.1 pg/mg API	42
Nizatidine (DS and DP)	NDMA	-	-	-	-	0.25 ng/mL	0.50 ng/mL	43
Levocetrizine (DS and DP)	NPIP	F5 column	A: 2mM ammonium formate in water B: Acetonitrile	Gradient	ESI	-	1 ng/mL	44

NDPA: N-nitrosodipropylamine, NDIBA: N-nitrosodiisobutylbutylamine, NDBzA: N-nitrosodibenzylamine, NDiPLA: N-nitrosodiisopropanolamine, NDELA: N-nitrosodiethanolamine, NEIPA: N-nitrosoethylethylpropylamine, MeNP: 4-methy1-nitrosopiperazine, NEIA: N-nitrosoethylisopropylamine,s MNP: 1-methyl-4-nitrosopiperazine, CPNP: 1-cyclopentyl-4-nitrosopiperazine

Table 5. Brief Summary of Different Analytical Techniques for Nitrosamine Impurities Detection

Name of the analytical technique	Sensitivity	Suitability
LC-MS/MS	Highly sensitivity	Ideal for non-volatile and thermolabile nitrosamines, most widely used for estimation of NI in DS and DP
GC-MS	High	Suitable for volatile nitrosamine such as NDMA. Good resolution, but limited to thermostable and volatile NI
SFC-MS	High	Useful for complex matrices and chiral separation
LC-UV	Low	Low sensitivity, not preferred for trace level analysis of NI in pharmaceuticals
LC-Fluorescence	Low	Applicable after derivatization with suitable reagent

starch. The exact source of nitrites and nitrates in excipients remains unknown, but they may be attributed to their presence in trace amounts in the water used during manufacturing, processing steps involving acid titration, and air oxidation during the drying process. These excipients, when combined with APIs containing secondary, tertiary or quaternary amines, can potentially lead to the formation of NDSRI in the drug product.[15] This reaction is reported to proceed under acidic pH conditions via the formation of reactive NO^+ species. Therefore, depending upon the structure of the API, the excipients used and the manufacturing steps, there exists a risk of NDSRIs or simple NI formation in different DP.

One such example is the formation of N-nitroso orphenadrine, an NDSRI found in Orphenadrine citrate ER tablets. Orphenadrine is an anticholinergic agent used as a skeletal muscle relaxant. Orphenadrine citrate ER tablets were recalled from the market because the NDSRI in them was found to exceed the acceptable limits.[22,23] Chemical structure of Orphenadrine and its NDSRI, N-nitroso orphenadrine, is given in Figure 03.

Another example of product recall from market due to NDSRI exceeding the acceptable intake, is in combination product of quinapril and hydrochlorthiazide (Accuretic™).[22,24] Chemical structure of quinapril and its NDSRI, i.e. N-nitroso quinapril, are given in Figure 04.

The formation of NI in selected DS and DP are discussed in detail in the following section.

Valsartan

NI in pharmaceutical drug products were for the first time observed in 2018 by the US FDA in Valsartan batches manufactured by Zhejiang Huahai Pharmaceutical (ZHP). Valsartan is an antihypertensive agent from angiotensin (AT_1) receptor blocker class approved by the US FDA in 2005. According to the articles, the formation of NDMA is attributed to the change in the process employed in synthesis of Valsartan API. This change in the synthetic process was undertaken in order to improve the yield but in turn resulted in the NI contamination in the finished product. Figure 05 depicts the original route of synthesis for valsartan as per European Pharmacopoeia.[25]

As indicated, this process involved the use of tributyltin azide (Bu_3SnN_3) to form the tetrazole ring in valsartan, as indicated in Figure 06.[25] The modified process involved the replacement of tributyltin azide with sodium azide, and dimethyl formamide (DMF) was employed as the solvent. Excess of sodium azide was quenched using sodium nitrite ($NaNO_2$). Dimethyl amine (DMA) was reported to be present either as an impurity in DMF or was formed as a result of disproportionation reaction catalysed by $ZnCl_2$.[5,13] Figure 06 depicts the modified route of synthesis for valsartan to improve yield, but which in turn led to NDMA formation.

Ranitidine

Ranitidine is an antihistaminic agent utilized for treatment of peptic ulcer that was approved by the US FDA in 1983. Its popular formulation Zantac® was found to be contaminated with nitrosamine impurities above acceptable limits in September 2019. The NI found was NDMA. Molecular structure of Ranitidine (Figure 07)

contains both dimethyl amino (secondary amine) and nitrite functional groups.

Ranitidine undergoes autooxidation and generates DMA and nitrite. This availability of amine and nitrite functional groups provides favourable conditions to form NDMA by autooxidation of ranitidine which takes place in two steps with the involvement of two peroxy radials.[26]

Formation of DMA from Ranitidine

The first step involves generation of ranitidine peroxyl radical formed by the reaction of ranitidine with peroxyl radical. This ranitidine peroxyl radical undergoes disproportionation to generate hydroxyl group which in turn undergoes hemiaminal rearrangement to form DMA. The sequence of the chemical reactions is given in Figure 08.

Formation of Nitrite from Ranitidine

Involves the reaction of the C=C bond adjacent to the nitro group with the peroxyl radical that results in the formation of an epoxide ring. This is followed by the rearrangement and liberation of the nitrite group [26] as indicated in Figure 09.

Afatinib

As mentioned previously in the article, Afatinib tablets are reported to generate NI due to nitrite contamination in the excipients. A comparative study was conducted by N. Golob *et al.*[17], 2023 to evaluate the NI formation in film-coated tablets of Afatinib formulated using crospovidone obtained from two different vendors. The two groups of film-coated afatinib tablet preparations (containing crospovidone from two other companies having different nitrite contents) were subjected to accelerated stability studies, and the LC-HRMS technique was used to analyse the content of the NDMA generated. The crospovidone from one company had lower nitrite content as compared to the crospovidone manufactured by the other company. The results of the LC-HRMS analysis showed that the film-coated tablets prepared with crospovidone having a greater nitrite content exhibited greater NDMA formation compared to the group formulated with low-nitrite-containing crospovidone. This reflected that the excipient crospovidone was the nitrite source. Further study showed that secondary amine was formed due to the hydrolysis of afatinib, thereby generating DMA, which in turn reacted with nitrite from the excipient to form NDMA.[17] The formation of NDMA was due to the degradation of the active ingredient and the nitrite from the excipient crospovidone. API afatinib has been shown to undergo hydrolysis, forming DMA. The presence of nitrite in crospovidone was evaluated using ion exchange chromatography, which revealed the presence of nitrite in the sample. The DMA and nitrite so formed lead to the formation of NDMA.

Table 03 summarizes the formation of NI in Valsartan, Ranitidine and Afatinib.

Analytical Methods for Detection of NI

Due to the stringent regulatory requirements for controlling NI to a low level in drug products, sensitive analytical techniques are employed in the detection of NI in pharmaceuticals. Chromatographic methods are reported for the separation of NI from drug substances and drug products.

NI Separation using High Performance Liquid Chromatography (HPLC)

HPLC technique is used to separate NI on octadecyl silyl (ODS) columns, biphenyl columns, phenyl columns, phenylhexyl columns, and pentafluorophenyl propyl (PFP) columns. Detection is carried out using various detectors.

HPLC using UV Detector

Patil *et al*.[27] have reported the use of a UV detector for detecting nitrosamine impurities in Losartan. The use of a diode array detector (DAD) at 230-233 nm and a UV detector at 228 nm for detecting NDMA in valsartan is reported by Li *et al.* and Al Kasem *et al.*[28].

HPLC using Fluorescence Detector

Dariusz Boscar *et al.*[29] (2021) used a fluorimeter as a detector for HPLC separation of NDMA and NDEA in Enalapril maleate. The method involved precolumn derivatization wherein nitrosamines were derivatized using agents like dansyl chloride, fluorenylmethoxycarbonyl chloride. The latter was shown to give better sensitivity and selectivity. The separation was done by HPLC with PDA detector followed by an external fluorescence detector. The method reported quantitation limit of 0.038 and 0.050 µg/g for NDMA and NDEA respectively.

HPLC using Mass Spectroscopic Detector (LC-MS)

Most of the analytical methods used for detecting NI employ liquid chromatographic

separation (HPLC or Ultra-Pressure Liquid Chromatography, UPLC) coupled with mass spectrometric detection. Hyphenated techniques, such as LC-MS or LC-MS/MS, are found to be the most commonly used methods for detecting NI in DPs due to their high sensitivity. Details of liquid chromatography analytical methods, including the stationary and mobile phases employed and mode of elution with MS detection, are given in Table 04.

Gas Chromatography using MS Detector (GC-MS)

Low molecular weight NI, which can be readily volatilized, are reported to be analysed by Gas chromatography using an MS detector (GC-MS). Kalauz *et al.*[45] utilized the GC-MS method to determine 12 low-molecular-weight nitrosamine impurities, including NDMA, NDEA, NDPhA, N-nitrosodipropylamine (NDPA), NMEA, NMOR, NPIP, N-nitroso-ethylisopropylamine (EIPNA), N-nitroso-diisopropylamine (DIPNA), N-nitroso-N-methylaniline (NMPA), 1-methyl-4-nitrosopiperazine (MeNP), and NPYR. The method reported a linearity range from 12-120 ng/mL. Shu-Han Chang[46] (2022) employed a GC-MS/MS method, utilising the headspace-solid phase microextraction technique (HS-SPME), for the determination of fourteen nitrosamine impurities in forty-four pharmaceuticals, with a limit of quantitation of 0.05 µg/g. Liu *et al.*[47] (2021) developed a sensitive GC-MS/MS for the simultaneous determination of four nitrosamine impurities in pharmaceuticals with detection limits from 0.002-0.150 ppm. Anna B. Witkowska *et al.*[48] (2022) developed a sensitive method using GC-MS for the simultaneous determination of nine NIs in Cilostazol, Sunitinib malate, and Olmesartan medoxomil, with a quantitation limit of up to 21.6 ng/mL. Tummala and Amgoth[49] (2021) reported the estimation of four NIs in valsartan using the GC-MS technique, with the method having a detection limit of 0.02-0.03 ppm and a quantitation limit of 0.06-0.09 ppm.

Supercritical Fluid Chromatography with MS Detection (SFC-MS)

Schmidtsdorff *et al.*[50] (2019) have reported Quality by Design (QbD) approach and supercritical fluid chromatography (SFC) for the detection of NI in valsartan and losartan. The method utilized ultra-performance convergence chromatography (UPC) with PDA detector. Detection was performed using MS detection. The method was capable of detecting NDMA, NDEA, NMEA, NDPA, NDBA, N-nitrosodiphenylamine (NDPhA), NPYR, NPIP and NMOR with detection limits of 4.55, 1.58, 1.81, 0.24, 0.34, 0.22, 3.71, 2.26 and 4.20 ng/mL, respectively.

A concise comparative view of different analytical techniques based on sensitivity of detection and selectivity for NI in pharmaceuticals is illustrated in Table 05.

DISCUSSION

NI in pharmaceuticals is a potential health threat because of the mutagenic and carcinogenic properties it possesses. This is attributed to the irreversible alkylation of DNA bases upon activation by CYP450 enzymes. NI in pharmaceuticals is attributed to APIs containing amine functionality (mainly secondary amines), excipients contaminated with nitrite, and APIs containing both amine and nitrite groups (e.g., ranitidine). Due to the mutagenic nature of NI, they need to be controlled to trace levels in pharmaceuticals. Regulatory agencies exercise strict control over levels of NI in pharmaceuticals; hence, accurate and precise quantitation of NI in DS and DP is crucial. To accurately detect and quantify NI in pharmaceuticals at such low levels, highly sensitive and advanced analytical techniques are required. There is a possibility of false positive detection of NI during analysis of pharmaceuticals due to interference from solvents such as DMF, interference from the complex matrix encountered in pharmaceuticals which can mimic NI, interference from the excipients. Mostly the separation is reported by various chromatographic techniques including LC, GC and SFC. Owing to the presence of weakly absorbing chromophoric group, mainly showing absorption maxima below 220 nm (where maximum solvents show interference), LC separation coupled with UV detection have low sensitivity. However, fluorometric methods involving post separation derivatization with agents such as dansyl chloride have been reported to provide low sensitivity detection. The most sensitive detection and quantification of NI is provided by MS detection

capable of quantifying upto ng/mL level (parts per billion). Gas chromatographic methods coupled with MS detection are mainly reported for the volatile, low molecular weight NI. LC-MS methods provide the most reliable means to detect and quantify the presence of NI at such low levels in pharmaceuticals, hence LC methods coupled with MS detection have been mainly reported for the detection and quantitation of NI and NDSRIs in pharmaceuticals. It is equally important that the samples are handled properly maintaining their stability by proper storage, protection from light and heat to ensure accuracy in their detection.

CONCLUSION

Carcinogenic potential associated with NI has seriously impacted the pharmaceutical industry, with significant concern increasing day by day. This review provides a comprehensive overview of nitrosamine impurities, elucidating their types, toxicological concerns, and acceptable limits, alongside a detailed analysis of the chemistry underlying their formation and the critical contributors to their generation. Through an in-depth discussion of advanced analytical techniques, including HPLC-UV, HPLC-fluorescence detection, LC-MS, GC-MS, and SFC-MS, this article highlights state-of-the-art methodologies for the precise and reliable detection of nitrosamine impurities, supported by relevant case studies of Valsartan, Ranitidine, and Afatinib.

Addressing nitrosamine impurities effectively requires a combination of stringent regulatory guidelines, proactive risk assessments, and advancements in analytical capabilities. Since, formation of these impurities can take place during storage as well by migration of nitrite from packaging material, continuous monitoring of NI formation in DP and post-marketing surveillance is essential for DP susceptible to NI formation. By exploring the intricate interplay between their formation pathways and detection strategies, this review aims to equip researchers and industry professionals with the necessary insights to mitigate nitrosamine impurities contamination in pharmaceuticals. Ultimately, the collective efforts of scientists, regulatory authorities, and pharmaceutical manufacturers are pivotal in ensuring drug safety and maintaining public health.

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This research does not involve any clinical trials.

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Authors contribution

Vineeta Khanvilkar: Selection of the title and pathway for the data search; Deepali Jagdale: Checking the chemistry part of the data collected; Abhay Shirode: Drafting of the article; Ammara Sahibole: Systematic literature search, study of the data and its compilation, typing of the manuscript, drawing the chemical structures; Gayatri Vinchurkar: Drafting of the article.

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