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Review Article

NAVIGATING NITROSAMINES: ORIGIN, DETECTING, ANALYSING AND REGULATING IMPURITIES IN PHARMACEUTICALS

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ABSTRACT

N-nitrosamines are carcinogenic impurities mostly found in groundwater, treated water, foods, beverages, and consumer products like processed meats, alcoholic beverages, cosmetics, and cigarette smoke. The recent discovery of N-nitrosamines in pharmaceutical products and subsequent recalls pose a significant health risk to patients. Nitrosamine impurities in drug products have appeared as a critical concern in pharmaceuticals prompting extensive scrutiny from regulatory agencies and stakeholders. To avoid carcinogenic and mutagenic effects in patients relying on these medications, authorities have established specific guidelines in risk assessment scenarios and proposed control acceptable limits for nitrosamine impurities in pharmaceuticals. This review provides an information on historical background of Nitrosamine impurities; its carcinogenic effect; the sources and formation of impurities; associated risks of nitrosamines in drug formulations; different analytical techniques for nitrosamine detection. It also gives an understanding of the general Quality Risk management (QRM) process, techniques for measuring nitrosamine impurities with control strategies as directed by the regulatory authorities and how to avoid them in pharmaceutical drug products. A brief review on recalls of drug classes including angiotensin II receptor antagonists, histamine-2 receptor antagonists, antimicrobial agents, and antidiabetic drugs by regulatory bodies due to its potential harm produced by nitrosamine have been discussed. Moreover, the regulatory landscape governing nitrosamine impurities are explored, encompassing recent guidelines from major regulatory bodies such as the United States Food and Drug Administration (USFDA), European Medicines Agency (EMA) and Health Canada (HC) in controlling/eliminating the nitrosamine impurities in pharmaceuticals.

Keywords: N-nitrosamines, Impurities, Analytical techniques, Recall, Detection, Quantification, Regulatory

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INTRODUCTION

Organic substances with the general structure $R_2N–N=O$ are known as N-nitrosamines. Nitrosamines is generated when urea derivatives, amides, carbamates, and secondary and tertiary amines react with nitrites or nitrogenous groups. Because of their ability to cause cancer and the potential implications for regulations, nitrosamine impurities have become a major concern in the pharmaceutical sector and require thorough investigation. In recent years, allegations of nitrosamine contamination in various medicinal products have drawn increased attention from regulatory bodies across the globe. Pharmaceutical product's safety and efficacy is compromised in the presence of these contaminants, which makes it imperative to completely understand their sources, detection techniques, and regulatory consequences [1, 2].

The objective of this review is to give an in-depth overview of the present scenario of regulatory affairs with nitrosamine-related contaminants in pharmacological compounds. These molecules were found in many pharmaceutical products, which has led to regulatory responses that are stringent and creates concern about patient safety. It is essential to understand the mechanisms that underlies in the synthesis of nitrosamines to implement efficacious control techniques during the medicine development and manufacturing processes [3].

Under this context, we examine nitrosamine exposure to different endogenous and external causes. To comprehend the complexities of nitrosamine occurrence in medicines, the purpose of precursor materials, manufacturing processes, and storage conditions in nitrosamine generation was investigated. A thorough examination of analytical techniques for the identification and characterization of nitrosamines is provided, ranging from standard methods to innovative technologies that provide enhanced sensitivity and specificity [4].

Moreover, this analysis illuminates the dynamic regulatory environment encompassing nitrosamine contaminants. International regulatory bodies have promptly responded to this emerging obstacle by issuing directives, frameworks for risk assessment and recommendations to ensure the initiative-taking identification and mitigation of risks associated with nitrosamines in medicinal products [5, 6]. Moreover, instances of specific pharmaceutical compounds facing challenges with nitrosamines are also investigated, offering practical insights into the implementation of strategies for detection and reduction.

The review aims to combine existing information about nitrosamine impurities in pharmaceutical compounds, thereby serving as a valuable resource for scientists, analysts, and regulatory experts which will ensure the integrity and safety of pharmaceutical products by evolving a global regulatory guideline [7, 8].

Search strategy

The search engines used for this review article were Scopus, PubMed, Web of Science, Google Scholar, UCLA library journal source and Article Plus within a search timeline from 2005 to 2024. Keywords like Nitrosamines, Nitrosamine impurities, History of Nitrosamine, Total Quality Risk Management, Regulatory guidelines, Formation of Nitrosamines, Carcinogenicity of Nitrosamines, Risk Assessment, USFDA guidelines, EMA, Marketing Authorization Holder (MAH), Valsartan Recall, Ranitidine Recall, Regulation and control, Nitrosamine in Pharmaceutical products were used.

Historical background

The history of nitrosamines in drug products can be traced back to the 1950s and 1960s when they were first identified as contaminants in food and beverages. Studies at that time linked nitrosamines to cancer in laboratory animals, leading to concerns about their potential health risks to humans [9].

1960s-initial discovery

When researchers discovered that nitrosamines caused cancer in lab animals, they were originally identified to be potential carcinogens

in the 1960s. Concerns have been raised by discovering the potential existence of nitrosamines in food, water and among other products used by consumers.

1970s-identification in pharmaceuticals

Studies showing the detection of nitrosamines in pharmaceutical products stimulated greater investigation. It was discovered that these contaminants, which may be present in some pharmaceutical formulations, are the consequences of the interaction with secondary amines and nitrosating agents.

The 1980s-regulatory response

To solve the problem, regulatory bodies introduced rules for reducing the number of nitrosamines in medications in 1980s. The primary goal of these guidelines was to prevent the formation of nitrosamines throughout the drug manufacturing process.

2000s-advances in analytical techniques

With the development of analytical techniques, particularly mass spectrometry and chromatography, the pharmaceutical industry was able to detect and quantify nitrosamine contamination in smaller amounts with greater complexity.

2018-ranitidine contamination

Ranitidine, a common medicine used to treat heartburn showed presence of N-nitroso dimethylamine which was a key turning point. As a result, ranitidine products were recalled around the world, and people were made more aware of the potential existence of nitrosamine contaminants in pharmaceuticals.

2019-widespread recall of valsartan

Nitroso dimethylamine was found to be present in Valsartan, an Angiotensin II Receptor Blocker (ARB). It quickly became known through investigations that greater amounts of nitrosamines were present in other ARBs including Losartan and Irbesartan. This prompted regulatory agencies around the world to increase their investigation.

2020s-global regulatory response

Authorities from all over the world stepped up their scrutiny of nitrosamine contaminants in the years that followed. Health professionals updated regulations and published suggestions for evaluating and decreasing risks related to the substances in drugs.

2020s-expansion of nitrosamine concerns

Regulatory bodies extended their investigations to include other medicine classes, which prompted manufacturers to perform comprehensive evaluations of their products with respect to nitrosamine contamination. More stringent inspection on manufacturing of the drug products by the regulators focused more consideration on reviewing and overseeing the production of nitrosamines. This includes assessing the raw materials, nature of the reactions and sources for contaminants in the production.

To solve this issue, international regulatory bodies have responded to the problem by increasing their inspection on drug manufacturing facilities and setting up acceptable daily intake limits of nitrosamines [10]. Also, they have raised a safety concern on nitrosamine contamination in several drug classes including angiotensin II receptor antagonists, histamine-2 receptor antagonists, antimicrobial agents and antidiabetic drugs. USFDA and other regulatory agencies have collaborated closely together to monitor and manage the problem and United States Pharmacopeia (USP) has established guidelines and methods for detecting and controlling nitrosamine impurities [11].

Carcinogenicity of nitrosamine impurities

Nitrosamine contaminants including N-Nitrosodiethylamine (NDEA) and others, have the potential to cause cancer by inducing mutagenesis effects. It is shown that N-N-nitroso dimethylamine causes mutations because of its genotoxicity in *in vivo* studies [12-14]. n-Nitrosamines are metabolized by cytochrome P450 enzymes, including Cytochrome P450 2E1(CYP2E1) and Cytochrome P450 2A6 (CYP2A6) to produce N-nitroso dialkylamines. For example, CYP2E1participates in the breakdown of several substrates in rodent models such as organic solvents, nitrosamines, and various pharmacological compounds [15]. Furthermore, Cytochrome P450, family III, subfamily A (CYP3A4) contributes to the activation of bigger N-Nitrosamines by catalysis. *In vitro* as well as *in vivo* models showed nitrosamine metabolism modulated by variable expression of CYP2E1 [16].

Nicotine-Derived Nitrosamine Ketone (NNK) mediates damage to the DNA which is cytotoxic and may result in the creation of methyl DNA base adducts like N7-Methyldeguanosine (N7-Me-dGuo) and O6-Methyl-2′-deoxyguanosine (O6-Me-dGuo). It can also create other genotoxic activity in healthy cells including Pyridyloxobutyl-DNA (POB-DNA) base adducts like O2-[4-(3-pyridyl)-4-oxobut-1-l] thymidine (O2-POB-dThd) and O6-Pyridyloxobutyl-2′-Deoxyguanosine (O6-POB-dGuo). Major genotoxic compounds produced because of nitrosamine impurities include aldehyde DNA adducts [17]. However, in-depth research is required to fig. out the precise composition of the nitrosamine impurities in polyhydroxybutyrate (PHB) Deoxyribonucleic Acid (DNA) adducts.

Additionally, these nitrosamine activity in drugs or medications causes DNA damages resulting in the creation of O6-methylguanine (O6-MeG) or 3-Methyladenine (3MA). The primary cause of DNA methylation is N-Nitroso Dimethylamine (NDMA) and unrepaired 3MA which increases mutagenicity and in turn can cause cancer. Alkyl adenine glycosylase (AAG) usually repairs the DNA damage resulting from 3MA by removing the methylated bases and initiating repair with base excision. Thus, AAG activity plays an essential part in reducing the mutagenicity caused by nitrosamine toxins like Nitroso dimethylamine [18, 19].

Sources of nitrosamine impurity in pharmaceuticals

Nitrosamines in pharmaceutical products arise from Active Pharmaceutical Ingredient (API), product manufacturing processes, direct and indirect cross-contamination from solvents and equipment, and chemical degradation during storage. If the impurities appear or is present throughout these steps, it might be integrated and delivered to the drug product, as shown in fig. 1.

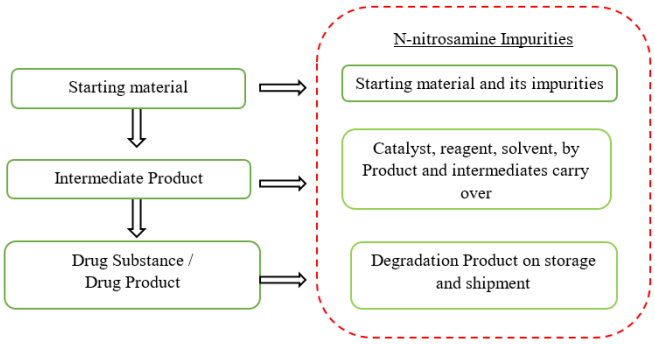

Fig. 1: Sources of nitrosamine impurities [19]

Most potential root causes of nitrosamine contamination can be classified into three groups based on the pharmaceutical manufacturing process flow: factors associated with API, manufacturing of the product and packaging procedures [19].

Alachlor

Geochemical processes of decomposition including biotransformation, photochemical deterioration, precipitation, and volatilization are known to occur with alachlor. It is proved that acetanilide and aromatic chemicals are formed by such geochemical degradation. Also, it is claimed that soil fungus and aquatic insect larvae break down alachlor metabolically to produce 2,6-diethylaniline. Although a lack of direct evidence of 2,6-diethylaniline forming nitrosamines, the mammalian metabolite 2,6-diethyl nitroso benzene had characteristics that were proper for the formation of DNA conjugates [20].

Atrazine

Two nitrosatable secondary amine moieties found in atrazine can combine to generate either mono-N-nitroso atrazine or di-N-nitroso atrazine. These substances are not likely to originate in the groundwater itself. Desethyl-Atrazine (DEA) and Deisopropyl-Atrazine (DIA), which are nitrosatable and can generate the associated nitrosamines, are the other consequences of atrazine decomposition [20].

Carbamate derivatives

Aldicarb is a nitrosatable carbamate pesticides. In the presence of NO_2, aldicarb's secondary amine group is known for producing.

Cyanazine

Soil extract have shown to have nitrosamine derivatives of cyanazine and its related chemicals. The most popular chlorotriazine herbicide for managing broad-leafed weeds in corn and maise is cyanazine. Ten percent of the nitroso-species were produced when cyanazine was nitrosated in lab. However, the addition of Sodium Thiocyanate (NaSCN) increased the reaction yield by up to 40–50% [19].

Linuron

Linuron is an herbicide containing phenyl urea and dichlorobenzene. When NO_2 is present in groundwater, it can produce N-nitroso linuron. Using amide hydrolysis, linuron is bio-transformed into 3,4-dichloroaniline, yielding N, O-Dimethyl Hydroxylamine (N, O-DMHA) as a byproduct [20].

Formation of nitrosamines

In general, the formation of nitrosamines requires the presence of a nitrosatable compound (amine) and inorganic nitrite. Nitrite ions (NO_2^-) are not effective nitrosating agents directly. To enable nitrosation, nitrite first reacts with protons (H+or H_3O+) to convert to nitrous acid (HNO_2, pKa 3.37). However, HNO_2 is also an intermediate in forming the nitrosating agents and is converted into its active nitrosating species dinitrogen trioxide (N_2O_3) [21, 22].

The next step is the reaction between the nitrosatable amine (e. g., secondary amine, R_2NH) and the nitrosating agent (e. g., N_2O_3), forming the nitrosamine (R_2NNO). The nitrosatable amine can be a primary, secondary, tertiary amine or even a quaternary ammonium compound. However, secondary amines are the most reactive compounds towards nitrosating agents, generating stable nitrosamines.

Nitrosation of primary amines, tertiary amines, and quaternary ammonium compounds leads to the formation of deaminated products or the corresponding secondary amines, which can then be nitrosated to the corresponding nitrosamines. The rate of nitrosation is proportional to the concentrations of the reacting amine and dinitrogen trioxide (N_2O_3) and is pH dependent. Nitrosation reactions are influenced by a range of factors, and the inhibition of nitrosamine formation is achieved by compounds that react with nitrite such as primary amines, sulfhydryl compounds, certain aromatic compounds, and ascorbate.

Formation of nitrosamines by using reagents and solvents

This method is not commonly used in product formulation but widely used in API manufacturing when reagents and solvents are employed significantly. Numerous chemicals and solvents used in the production of API are recognized as genotoxins and carcinogens. Consequently, even though expected solvents and reagents are used during synthesis, it cannot be predicted that they will be also included in the finished product. Using different processes and methods it has shown that the residual amount of these substances and solvents contributes to the production of nitrosamine impurities. Nitrosamine precursors are included in the reagents and solvents throughout their manufacturing process. Furthermore, when nitrosating sources or amines are present during synthesis, they have the potential to cause the creation of nitrosamines directly or indirectly through their breakdown into products. Solvent recycling and recovery are popular and beneficial to the environment practice from waste streams.

Role of Dimethyl formamide (DMF) in the production of nitrosamines in valsartan is examined. One of the reactant substances used in the industrial manufacture of DMF is dimethylamine. On the other hand, heating DMF is known to convert it back to dimethylamine and carbon monoxide. Moreover, it showed that DMF hydrolysis produces dimethylamine and formic acid. It is necessary to keep an eye on the amount of dimethylamine in DMF since it is a prerequisite for the synthesis of nitroso dimethylamine when Sodium nitrite is present [21, 22].

Formation of nitrosamines during synthesis

The pathways leading to nitrosamine synthesis is divided into classical and non-classical types based on how often or preferentially they develop in the setting of synthetic chemistry. Most of the methods accounts in the literature related to the traditional formation of nitrosamines, which also happens to be the most common pathway of nitrosamine creation. Nitrosamines are typically prepared by reacting with a nitrosating agent and an amine source that matches amine-containing molecules, forming an N–N link in the process [21, 22].

The literature has also shown the existence of other less well-known pathways that result in the synthesis of N-nitrosamines, aside from the N-Nitrosation of NH-containing molecules. They are referred to as non-classical routes since they are less commonly described and have limited experimental explanations. If nitrosating chemicals are unintentionally present in the acidic medium simultaneously with an amine-containing reagent, the traditional approach generates a large amount of N-nitrosamine during active pharmaceutical ingredient production, as shown in fig. 2.

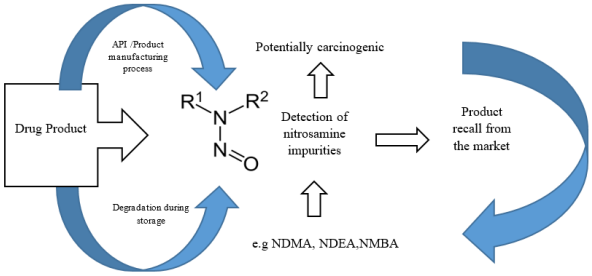

Fig. 2: Nitrosamine formation during synthesis

Formation of nitrosamine from excipients

Excipients are substances added to a formulation that are non-toxic, chemically inert, and inert in terms of sources and structure. They are more intricate than well-defined API. Excipients were historically often simple in structure and derived from natural sources, but recent developments in chemistry and materials research have led to an increase in their complexity. They also come from a variety of sources, including mining for materials, biotechnology, animals, chemical synthesis, and harvesting plants.

Excipient complex makeup and variety of origins often add to their impurity. Since most excipients are inactive, they do not react with API. Excipients and API may, however, occasionally react during processing or storage, leaving evidence of degrading contaminants in the finished product. In Pharmaceutical products, nitrosamine contamination has not linked to excipients yet. However, nitrosamines can be introduced into the finished pharmaceutical product from a variety of possible excipient-related sources [21, 22].

Formation of nitrosamines from deterioration during processing or conserving

They are considering the amount of ingredients and procedures in the manufacturing process. Both the API and the excipient/s could be degraded with close interaction that they have with other substances under different processing circumstances, such as high energy mixing, elevated temperature, and moisture. Additionally, during storage of finished product, excipients might break down. As a result, it is crucial to take into consideration the potential for nitrosamine production because of degradation during processing or storage.

After storing at 25 °C for 12 d, a study showed that the amount of N-N-nitrosodimethylamine in 150 mg ranitidine tablets increased significantly from 18 ng to 25 ng and N-nitroso dimethylamine concentration increased to 142 ng after 14 d of storage at 70 °C. The findings imply that the ranitidine formulation may degrade during its shelf life with N-nitrosodimethylamine serving as the breakdown product. There have been reports of stability problems with Ranitidine [21, 22].

Formation of nitrosamines from printing and packaging processes

The printing processes used in the production of primary packaging materials is recognized as a potential source of contamination, even though primary and secondary pharmaceutical packaging materials are unlikely to significantly contribute to nitrosamine contamination. Azo-based pigments and dyes, which are frequently used in printing inks are known to have high amine contents which can result in the synthesis of nitrosamine. Furthermore, nitrates may occur from the use of nitrocellulose as a binding agent in solvent-based inks, especially at high-temperature and challenging conditions.

Amines found in API or excipients and nitrite ions produced from nitrocellulose breakdown during printing can move through primary packaging and react with amines in ink or final products causing nitrosamine formation. Nitrosamines has shown to migrate from blister lidding foil during packaging potentially contaminating the product. The lidding foil may have embedded nitrosamines due to amines and nitrocellulose and heat from sealing can release these nitrosamines into the unsealed blisters contaminating the product.

Regulatory authority requires pharmaceutical manufacturers to assess the risk of nitrosamine formation during synthesis, production, or stability and Manufacturers must develop a plan to mitigate this risk. Regulatory guidelines are available to help manufacturers reduce nitrosamine contamination and the specific mitigation strategies depending on the source of the contamination [21, 22].

Total quality risk management process

The methodical process of identifying, evaluating, communicating, and controlling threats to the quality of pharmaceutical goods across the course of their lifecycle is known as Quality Risk Management (QRM). It entails structured procedures to support risk-based scientific decision-making.

Risk identification

The process of starting a QRM program entails defining the issue or risk questions including assumptions about hazards and obtaining pertinent background data for risk evaluation.

Risk control

Risk control is the process of making decisions with the intention of either accepting or lowering the risk. To achieve this goal, risk control activities may focus on allowing queries to reduce risk to an acceptable level such as-Is the risk above an acceptable level? What can be done to reduce or drop risks? What is the balance among benefits, risks, and resources? Is the new risk introduced because of the identified risks being controlled?

Risk communication

Consists of decision makers and exchanging information about risk and risk management. The output or outcome of the QRM procedure needs to be properly conveyed and recorded. Information about hazards to quality may be given that relates to their existence, nature, form, probability, severity, acceptability, control, treatment, and detectability [23].

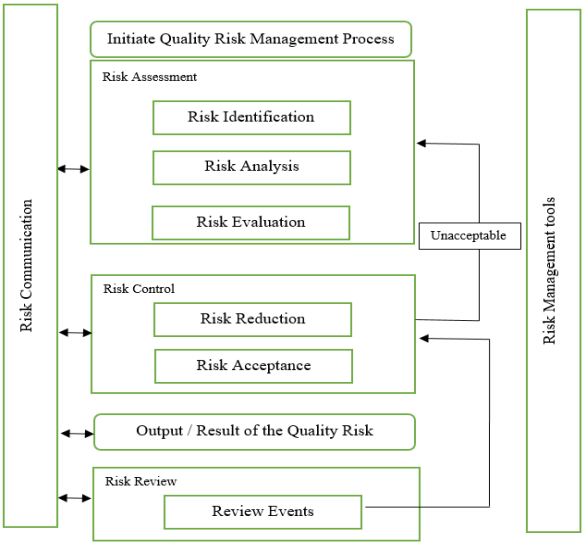

Fig. 3: Quality risk management process

Review

Systems should be in place to guarantee that the QRM process output is seen routinely and examined as needed to evaluate fresh data that might have an impact on the initial QRM. Any review frequency ought to be found by tools for risk management using established risk management techniques, risk can be evaluated and controlled. The following advanced tools are also potentially used in quality risk management: flowcharts, check sheets, and cause-and-effect diagrams are examples of basic approaches that are particularly helpful in structuring risk management processes through data collection and organization.

European Medicines Agency (EMA) recommended techniques like (FMEA) or Failure Mode Effects and Criticality Analysis (FMECA) to analyse the identified pharmaceutical product that were at risk. FMEA offers higher dependability and can break down the analysis of complicated processes into manageable phases and is extremely useful in all stages of the risk assessment process (risk identification, risk analysis, and risk evaluation) [23].

A fishbone diagram (fig. 4) was utilized in conjunction with this risk management tool [23]. The fishbone diagram also known as the cause-and-effect diagram, or the Ishikawa diagram offers a visual representation of effects and the factors that result in or influence them. Being the problem/effect diagram, the fishbone diagram is mostly used in failure investigations and root cause analysis.

Techniques for measuring nitrosamines in pharmaceutical drug products

The USFDA, EMA and other regulatory bodies have released multiple analytical techniques for nitrosamine detection since July 2018. These techniques are mostly chromatographic combining Mass Spectrometry (MS) and either Liquid Chromatography (LC) or Gas Chromatography (GC). For these analytical techniques, single and tandem mass spectrometers are often suggested. In addition, some applications recommend using hybrid quadrupole ion-trap mass analysers, which offer better specificity and mass resolution than traditional quadrupole mass analysers [24-30].

Nuclear Magnetic Resonance (NMR), Ion mobility spectrometry, Microfluidic devices, electrochemical detection, and others are used for the detection of nitrosamines. High-Resolution Mass Spectrometry (HRMS) has been added to the USFDA changed procedures for nitrosamine testing in medicines. More specifically, the q exactive tm hybrid quadrupole-orbitrap mass analyser offers increased performance in terms of Limit of Quantification (LOQ) and isotopic resolution by being able to discriminate isotopic masses of residual solvents as 15N Drug Master File (DMF).

There are some worries and potential problems with the analytical techniques that are now in use. Furthermore, recommendations are provided for the advancement of upcoming analytical techniques in this field [24-30].

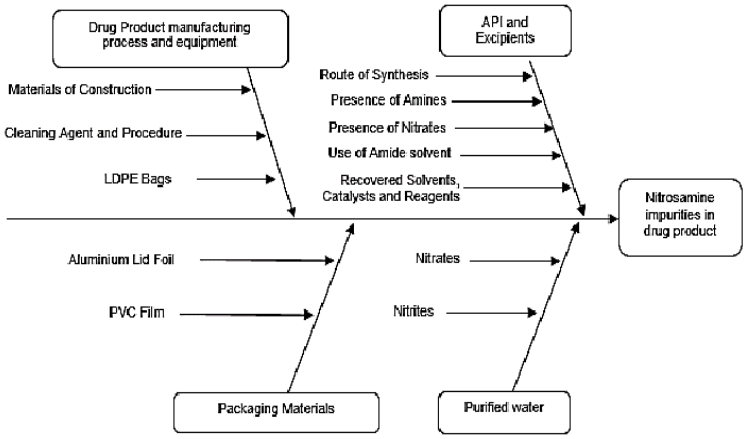

Fig. 4: Fish bone diagram for presence of nitrosamine impurity in medicines

Spectrophotometric detection accompanied by LC

A popular laboratory instruments for impurity analysis includes High Performance liquid Chromatography (HPLC) and UV–visible spectrophotometers are advised in standard quality-control procedures. Photodiode Array (PDA), spectro fluorescence and chemical luminescence detectors are among the most often reported spectrometric detectors for nitrosamine measurement. Additionally reported on is Supercritical Fluid Chromatography (SFC). Nitrosamine analysis in food and dairy products has been done using HPLC coupled with a UV or Photodiode Array (PDA) detectors [24-30].

GC-based techniques using diverse types of detectors

Both liquid and gas chromatographic methods have certain advantages as well as disadvantages. In contrast to LC, GC is less expensive to use and comes with an integrated chemical library and environment friendly instruments. Pharmaceutical contaminants that are volatile or semi-volatile, such as the leftover solvents in pharmacopeia monographs, can be effectively qualified and quantified using GC. In the analytical procedures utilized by the USFDA and other scientific publications, a PEG-coated column is employed. Additional coating materials such as cyano-propyl phenyl, dimethylpolysiloxane concentrations of impurities, are based on deactivated fused silica [24-30].

LC–MS-based methods

The recommended technique for quantitatively evaluating trace contaminants in food, environmental, and pharmaceutical samples is reverse-phase LC combined with MS. In intricate matrices, MS can recognize and detect charged analytes. Nitrosamines, especially n-nitrosodimethylamine are often detected in water using triple quadrupole mass analysers. For the analysis of polar and nonpolar non-volatile chemical compounds, LC-MS is more effective than Gas Chromatography–Mass Spectrometry (GC-MS). It is also used for the analysis of thermally unstable drugs such as metformin and ranitidine.

The USFDA stated that Liquid chromatography-high resolution and Liquidchromatography-Mass spectrometry/Mass spectrometry (LC-MS/MS) are better techniques because GC which tends to overstate n-nitrosodimethylamine levels in ranitidine. Various ionization techniques are available LC-MS. For polar and thermolabile chemicals, Electrospray Ionization (ESI) works well because of its capacity to ionize in the liquid phase. For compounds that are less polar and thermostable, Atmospheric Pressure Chemical Ionization (APCI) and Atmospheric Pressure Photoionization Ionization (APPI) are used to ionize in the gas phase. The very non-polar compounds that ESI has trouble with can be ionized by them. Whereas APPI ionizes gaseous analytes using photons from a discharge lamp, APCI

employs a corona discharge needle. Matrix-assisted Laser Desorption/ionization (MALDI) is a mild ionization technique for polymers and biomolecules like DNA, proteins, and sugars used to aid desorption under pulsed laser irradiation [24-30].

Technologies based on fluorescence sensors

Apart from chromatographic analysis, some sensors are employed for the measurement of nitrosamine. Cucurbit[n]uril (CB[n]) and its derivatives have been used to detect and measure nitrosamines linked to cancer. Although CB[n] molecular probes have been used for fluorescence sensors, their non-chromophoric nature needed that their employment was limited to dye displacing tests. Scientists have created supramolecular sensors for the evaluation of cancer-associated nitrosamines namely N-nitrosopiperidine (NPIP), N-Nitrosonornicotine (NNN) and NNK using both cyclic and acyclic CB[n]-type fluorescent probes.

Using statistical multivariate analysis and linear discriminant analysis, the sensor's response was found and assessed. Even in the presence of a high concentration of nicotine matrix interference, quantitative analysis was able to successfully find the concentration range of 0–18 ppm and 0–21 ppm in the mixes of tobacco-specific N-nitrosamines and NNK respectively with LODs 0.05 ppm and 0.27 ppm, respectively [24-30].

Methodology for N-nitroso compound (NOC) detection and screening

NOCs are defined as nitrosamines having a nitroso functional group attached directly to a nitrogen atom, such as n-Nitrosodimethylamine (NDMA), n-Nitrosodiethylamine (NDEA) and N-Nitroso-N-Methyl-4-Aminobutyric Acid (NMBA) as well as nitrosamines with short alkyl or aryl side chains. N-nitroso amides (Nitrosamides and N-nitroso amines), also belongs to this class [31]. N-nitroso amidines are another class of derivatives. Furthermore, it was demonstrated that 112 drugs were nitrosatable in the presence of nitrite, a combination that may have genotoxic and carcinogenic consequences [32].

The nitrosation assay method as a strategy for risk analysis

Obtaining sufficient knowledge about the chemistry of the by-products to function as a suitable advisor is the most significant hurdle to risk assessment [33, 34]. Unexpected chemical reactions that produce trace amounts of unknown impurities at ppm and especially ppb levels are often unclear and challenging to understand, even with well-known impurity guidelines like ICH Q3A (R2) and ICH Q3B (R2). Therefore, it is improbable that risk assessments will address every threat. More concerning is that not all instances of the contaminants are identified by the hazardous contaminant quality check in completed API and medicinal product formulations [35].

Additionally, the International Agency for Research on Cancer (IARC) monograph suggests searching for the likelihood of N-nitrosation in any pharmaceutical by performing a Nitrosation Assay Procedure (NAP) test on relevant synthesis reagents, intermediary substances, or final medicinal compounds. The criterion for the test is that the test sample needs to react at substantial concentrations of nitrite in acidic solution during a given test duration. The identification and obtained yield are studied using mass spectrometry and other analytical techniques. This method detects related small alkyl and aryl nitrosamine production and potential risk sources for related N-nitroso compound formation [36].

How to avoid nitrosamine formation in drug products?

The quantity of nitrosamine in a pharmaceutical product might vary depending on numerous factors. Anywhere in the process chain from the synthesis of API to the manufacturing of pharmaceutical products to the storage of the finished product nitrosamines may occur. Depending on the site of the right circumstances for their development, they may form at one or more stages. Most obviously, as previously shown, decreasing the amount of the susceptible amine and the nitrosating agent will result in a reduction in the nitrosamine load due to the law of mass action. If the amine affects the API itself reducing it is not possible. It is possible to decrease the quantity of nitrosating agents or susceptible amines by changing the contaminated materials resource [3].

If the Market Authorization Holder (MAH) has control over the API synthesis, the nitrosamine that has formed or the contaminant can be eliminated by further or more purification processes. Additionally, the nitrosamine alone could be broken down using reductive, oxidative, electrophilic, nucleophilic, and radical chemistry. On the other hand, significant modifications to the API synthesis are difficult and time-consuming for recognized commercial techniques and even techniques that are currently under development, thus switching suppliers might not be possible due to a lack of substitutes [37].

Increasing pH, reducing water content or particle size, or using inhibitors may be ways to decrease the development of nitrosamines in therapeutic products. Furthermore, none of these actions can be taken as a quick fix for late-stage or commercially available products. Any formulation changes also must be carefully evaluated against unintended side effects that go beyond nitrosamines such as altered stability, manufacturability, and physicochemical properties. If technically possible, it might be useful to change process parameters or unit procedures that affect the exposure to temperature and moisture [38, 39].

Control strategy for nitrosamine impurities

Control measures should be implemented if nitrosamine is found in an API above the LOQ. The analytical method used should be sensitive with LOQ of ≤0.03 parts per million (ppm) [40]. For high-dose API, LOD and LOQ of the method should be kept as low as achievable. If multiple nitrosamines are present, then the validated analytical method with a LOQ below 0.03 ppm is necessary. The effective control strategy should include setting specification limits to ensure nitrosamine levels stay below the Acceptable Daily Intake (ADI) limit. In cases where nitrosamine levels are detected above the LOQ then each batch should be evaluated and batches with nitrosamine levels exceeding the ADI should not be released. It is crucial to inform the FDA about the presence of nitrosamines in marketed products. As per the clinical outcomes and the risk of product discontinuation, the FDA may agree to allow batches with nitrosamine levels exceeding the ADI to be used temporarily, provided the impurity levels are reduced or eliminated. For example, manufacturers distributing Rifampin MNP or CPNP above the acceptable intake limits were permitted to distribute the product until they reduced or eliminated the impurities with MNP and CPNP levels were below5 ppm and 14 ppm respectively [32].

For finished products, the FDA recommends a three-step risk assessment procedure. Manufacturers of drug products should cooperate with API producers to pinpoint potential synthetic routes and processing conditions that might result in nitrosamine creation. The risk assessment should also consider the API degradation pathway which was showed by ranitidine, can produce nitrosamines during manufacturing or storage [41, 42]. If no risk is found, no further action is necessary. However, if there is a risk of nitrosamine presence, confirmatory testing using validated analytical methods should be conducted. If nitrosamines are detected in the final product, the underlying cause needs to be investigated and manufacturing processes need be changed to mitigate or eliminate nitrosamine impurities. Before using any incoming components such as batches of high-risk API, drug product manufacturers should analyse representative samples of all raw materials. They should continue with the testing of API batches until they have confirmed that the API supplier can consistently produce API without exceeding acceptable nitrosamine levels. Additionally, it is important to assess whether nitrites are present in manufacturing processes using high-risk API.

Evaluation of developing nitrosamines during a drug product's shelf life is also important. If nitrosamine contamination is caused by other sources, the contamination source must be eliminated.

A strategy for maintaining nitrosamine levels below the ADI limit would be necessary if there was a nitrosamine impurity present above the LOQ. The control plan should specify limits for the

observed nitrosamine and fig. out whether the structure, synthetic route or manufacturing method of the pharmaceutical product or API is the cause of addition of nitrosamine. Every batch must be sent for testing. Unless the FDA has agreed to temporarily allow the distribution of such products based on the severe impact of drug product shortage on patients, any drug product batch found to contain levels of nitrosamine impurities at or above the recommended ADI should not be released for distribution by the drug product manufacturer [43].

Drug product recalls due to nitrosamine impurities

Irbesartan

In October 2021**,** due to an increased levels of N-nitroso irbesartan in irbesartan (used in hypertension), Lupin Pharmaceuticals voluntarily recalled few batches of the drugs from the market. The company said that it would stop selling the medications in January 2021.

Losartan

Several tablets of antihypertensive drug such as Losartan potassium and Losartan potassium/hydrochlorothiazide were recalled by Torrent Pharmaceuticals in 2019 due to nitrosamine contamination. Torrent Pharmaceuticals claimed the issue was linked to API from Hetero Labs Ltd. In 1995, Merck received permission for the angiotensin II inhibitor-Losartan potassium, which was marketed under the Cozaar.

Metformin

In 2020**,** N-nitrosodimethylamine was discovered by FDA in Metformin-anti diabetic drug. Apotex, Amneal, Teva Pharmaceuticals, Marksans Pharma, and Viona Pharmaceuticals are among others whose businesses got affected by the recalls. In recent years, the independent lab Valisure has also been involved in the identification of contaminated batch of Metformin. In connection with their findings, the business also gave a citizen petition to the FDA.

Nizatidine

Mylan launched a nationwide recall of three batches of Nizatidine from Solara Active Pharma Sciences in January 2020 due to the presence of n-nitroso dimethylamine in the API. For the same reason, Amneal Pharmaceuticals voluntarily recalled massive quantities of nizatidine oral solution in April.

Ranitidine

In 2019, The FDA found that increased quantities of nitrosamines, including n-nitroso dimethylamine were present in few batches of ranitidine (Zantac), an over-the-counter drug used to treat heartburn. After the lab Valisure's discovery of n-nitroso dimethylamine in the medication, the FDA eventually asked manufacturers of Ranitidine to remove the product from distribution.

Rifampin

FDA also reported in 2020 that nitrosamine contaminants were present in many batches of Rifampin and Rifapentine drug products. Alleviate shortages, the EPA so approved many drugs with high concentrations of MNP or CPNP.

Sitagliptin

Merck and Co. (NYSE: MRK) said in August that nitrosamine was found in samples of its sitagliptin-containing medications Januvia, Janumet, and Steglujan. The FDA announced that sitagliptin with nitroso-STG-19 (NTTP) levels over the suggested limit may be temporarily distributed. The agency told that it would decide whether to issue massive quantities of sitagliptin medications with elevated nitroso-STG-19 (NTTP) levels on a case-by-case basis. Merck stated that to make sure that their medications follow the FDA temporary nitroso-STG-19 (NTTP) limitations, it has changed its quality control system.

Valsartan

It is a well-known angiotensin II receptor blocker, was one of the first medications affected by the 2018 nitrosamine recalls. Increased concentrations of nitrosamine contaminants, as n-nitroso dimethylamine and N-Nitrosodiethylamine also present in generic forms of ARBs medications. The source of the problem is an API that was used in certain generic valsartan-based medications and was produced in China by Zhejiang Huahai Pharmaceutical Co. Ltd. (ZHP).

Varenicline

Following the discovery of the nitrosamine N-nitroso-varenicline, Pfizer stopped shipping Chantix (varenicline) to other countries in June 2021. A few months later, the business extended the recall. A proposed lawsuit concerning the presence of N-nitroso-varenicline in some batches of the smoking cessation aid was dismissed by a federal judge in February 2022. The FDA declared in May 2022 that it was certain that recently manufactured varenicline in the United States met or surpassed its N-nitroso-varenicline impurity limit of 37 ng or less daily [43].

Challenges in risk assessment

The challenges in nitrosamine risk assessment are multifaceted and encompass various technical, regulatory, and operational hurdles. Analytical interference poses a significant obstacle as the specificity of nitrosamine detection can be compromised by the presence of other chemicals in medicinal products leading to reduced accuracy. Additionally, the diverse chemical structures of nitrosamines make it challenging to develop universal analytical techniques capable of recognizing all variations. The stability of nitrosamines further complicates detection efforts requiring meticulous handling to prevent degradation during sample preparation and storage [44]. Furthermore, the lack of high-quality reference standards hinders exact quantification impeding the development and validation of reliable analytical methods. Real-time monitoring of nitrosamine levels during manufacturing is hindered by operational and technical complexities delaying prompt intervention. Pharmaceutical formulation complexity with a combination of excipients, active substances, and more compounds, presents further difficulties in nitrosamine detection causing robust separation and detection techniques. Meeting stringent regulatory requirements adds another layer of complexity requiring continuous monitoring and adaptation to evolving standards [45]. Moreover, achieving global harmonization in nitrosamine testing standards and procedures is challenging due to variations in regional regulations posing disparities in testing and compliance efforts. Addressing these challenges requires collaborative efforts among stakeholders to develop innovative solutions and harmonize regulatory frameworks [46].

Emerging technologies

Emerging technologies offer promising solutions to address the challenge of nitrosamine impurities in drug substances. Automation and robotics play a pivotal role in enhancing the effectiveness and reliability of analytical processes by minimizing human errors and contamination risks during sample preparation and analysis. Real-time monitoring systems enables continuous surveillance of industrial processes swiftly showing nitrosamine production and allowing for prompt corrective actions to prevent tainted batches from being produced. Machine learning and Artificial Intelligence (AI) further enhances nitrosamine detection by improving pattern recognition and data analysis capabilities. Predictive modelling eased by AI algorithms helps to find potential circumstances leading to nitrosamine production during drug manufacturing enabling initiative-taking mitigation strategies. By using these emerging technologies, the pharmaceutical industry can bolster its efforts to ensure the safety and quality of drug substances offering a comprehensive approach to addressing nitrosamine impurities [46].

Recent regulatory updates on nitrosamine impurities/Nitrosamine drug substances-related impurities (NDSRIS)

Initially, nitrosamines were found to have n-nitroso dimethylamine in Sartan API in the middle of 2018, resulting in the market recall of specific batches. International regulatory bodies from all over the world which includes the USFDA, EMA, Health Sciences Authority (HAS-Singapore), Pharmaceutical and Medical Devices Agency

(PMDA-Japan), Therapeutic Goods Administration (TGA-Australia) and Health Canada (HC-Canada) worked together to inform API manufacturers regarding the harm of nitrosamine to the patients [47]. They proposed different rules, guidelines, and recommendations to examine their manufacturing procedure and start risk analysis concerning the production of nitrosamines [48].

The primary aim was to assess raw materials, intermediates, reagents, and solvents used in production of API to determine the presence of susceptible amines. The market had to remove Metformin extended-release, Ranitidine and Nizatidine products in 2020 after it was discovered that they formed nitrosamines. The storage conditions of Ranitidine products were discovered to be the main reason for the elevated levels of nitrosamines [48].

Revision from EMA/409815/2020 Rev.18 (Effective from October 02, 2023)

This revision clarified the roles of MAH to control, report, and mitigate the analysis of Nitrosamine impurities throughout the product life cycle by using the already set procedures. Q and A 3 ('call for review, 'when and how should MAHs report steps 1 and 2 to competent authorities) has been updated. The highlighted points below represent modifications [49].

With the Call for Review (Steps 1, 2, and 3) deadlines for medications containing both biologically and chemically manufactured active ingredients have passed. It must be a top priority for those MAHs that have not yet notified any detected nitrosamine impurities along with any revisions to previous reports to the appropriate competent authority.

The answer templates and available channels for communication that were previously established should be used by MAHand they are reminded of their roles to guarantee the effectiveness, safety, and quality of their medications as well as to follow the EU Network's guidelines on nitrosamines [50].

Update from EMA/409815/2020 Rev.19 (effective from 12 October 2023)

What limitations apply to nitrosamines in pharmaceutical products? is the revised Question and Answers (QandA) 10 (which was previously Appendix 2 and Appendix 3). These have been modified, transformed, and become separate documents from the main document. N-nitrosamine Carcinogenic Potency Classification Approach Appendix 2 to QandA (EMA/451665/2023). Appendix 3 to QandA on Enhanced Ames Test Conditions for N-nitrosamines, EMA/451666/2023 [51].

Update from EMA/517258/2023: (20 November 2023, Appendix-1)

Appendix-1 of EMA/517258/2023 was amended on November 20, 2023. Further, NDSRIs that were not there before were added along with the associated AIs (in ng/d) [51].

Update from EMA/409815/2020 Rev.20 (15th January 2024)

Question 3: In Appendix 1, nitrosamine impurities that are considered non-mutagenic according to *in vitro* mutagenicity investigations are not subject to step 2 confirmatory testing requirements; instead, they need to be controlled as per ICHQ3A (R2) and ICH Q3B(R2) guidelines.

Question 9: The required sensitivity for the technique of analysis should be derived from the suitable tolerable intake which can be established using the methods described in Q and A 10. The MAH/applicant bears the duty of developing the analytical method effectively and ensuring the necessary sensitivity.

Question 10: Nitrosamine impurities should be regulated as per ICH Q3A (R2) guidelines for products that are intended only for advanced cancer as defined in the terms of the ICH S9 guideline. Furthermore, any contaminants in Appendix 1 that are shown to be non-mutagenic based on negative findings from a carefully executed *in vivo* mutagenicity research are subject to restrictions under ICH Q3A (R2) and Q3B(R2). The Enhanced Ames Test (EAT) must be followed by all Ames tests started after August 2023 for them to be approved. Ames assays started before August 2023 can be approved on an individual basis and evaluated under the guidelines of the Enhanced Ames test protocol, but they must be submitted on January 31, 2024, at the latest [51, 52].

Update from Health Canada: October 20, 2023, health Canada, guidance on nitrosamine impurities in drug products

The revised version involves updates to Appendix-1 (Guidance concerning nitrosamine impurities and risk assessments for Post NOC [Post Notice of Compliance] Changes of new drug products containing chemically synthesized, semi-synthetic Drug substance) and Appendix-2. Sections 2, 3, 4, 10, 12, 13, and 15 have also been updated. Updated NDSRIs and new nitrosamine impurities (about rivaroxaban, cinacalcet, and alogliptin) are available. The N-nitroso-sertraline (from 100 to 1500* ng/d) and N-nitroso-varenicline (from 37 to 400 ng/d) Acceptable Intakes (AI) limits have been loosened. Based on a negative *in vitro* bacterial mutagenicity test, the limit is set [8].

The deadline for completing Step 3, or "changes to the MHAs, for pharmaceutical products comprising chemically and semi-synthetically manufactured Drug substances has been extended from October 1st, 2023, to August 1st, 2025. For biological and radiopharmaceutical products, the due date for completing Step 3, or "changes to the market authorization has been moved up from November 30, 2023, to August 1, 2025. The Carcinogenic Potency Categorization Approach (CPCA) has supplanted the concept of applying the Total Threshold Concentration (TTC) of 18 ng/d limit for Nitrosamine impurities/NDSRIs (for which an AI limit has not been set up) [51-56].

Impact of recent developments related to nitrosamines on the pharmaceutical industry

The FDA and other global regulatory bodies are focusing on nitrosamines found in angiotensin II receptor blockers like Valsartan, Losartan, and others. These chemicals have been detected at trace levels prompting recalls of certain batches especially of Valsartan and Losartan due to their popularity. Additionally, histamine-receptor inhibitors like Ranitidine and Nizatidine were recalled over concerns of n-nitroso dimethylamine contamination. Diabetes medications like Pioglitazone and Metformin have also been scrutinized for n-nitroso dimethylamine content by various agencies, including the FDA. While few Metformin and Metformin-extended-release batches have showed low N-nitroso dimethylamine levels leading to recalls for which the FDA has taken steps to address these issues.

The agency has been actively addressing concerns about nitrosamines in drug including recalling specific batches, developing sensitive testing methods for nitrosamines, and setting daily Acceptable limits for these impurities. For example, FDA has set a maximum allowable daily exposure limit of 96ng/d for n-nitroso dimethylamine and 26.5 ng/d for NDEA. The safety limit for NMBA, a newly discovered impurity, has also been set at 96 ng/d.

The discovery of nitrosamine impurities in medications like Tartans and Ranitidine has led to increased scrutiny and resources being devoted to testing and remediation efforts. This has caused concern among consumers, businesses, and regulatory agencies as it has affected the perceived safety of commonly used drugs. Given that most affected medications are generics, there are concerns about the impact on the Generic Pharmaceutical Industries which accounts for 90% of prescriptions filled in the United States by volume [57].

The focus on addressing nitrosamine impurities may divert resources and attention away from efforts to provide affordable and efficient medications. The generic medications sector is proactively putting forth a science-driven choice to thoroughly retrospectively analyse every drug product that is approved for the potential inclusion of nitrosamines. By actively investigating the cause of nitrosamines in drug products to reduce risk the proposal uses a risk-based approach in evaluating nitrosamines in medicines. With this strategy products that might include nitrosamine would be evaluated to meet the safety requirements outlined by the FDA and other international agencies [8].

Future perspectives

When handling excipients that can involve nitrites and/or nitrates at ppm levels, it is advisable to take preventative measures such as evaluating the materials being used for nitrite promptly upon receipt or arranging for their manufacturers to do a nitrite test as a release test. Every time a raw material is found to be contaminated with nitrosamines or their precursors during the ongoing evaluations of all Generis products it should be agreed that the manufacturer will investigate the sources of the contamination and either remove them entirely or control them to acceptable levels. If this is not possible finding a new supplier who meets these specifications must be the next step [53].

If the manufacturers of API and excipients were under pressure from the medicine authorities such as publishing a mandate for them to send all information required by the MAHs would be helpful in controlling/eliminating nitrosamines. By this measure time would be saved and the process for collecting all the data needed for the risk assessments could be made easier [54].

One must remain up to speed with published studies and new guidelines so that the risk assessment can be updated considering any future discoveries of new reasons for finding nitrosamines in pharmaceutical products. A further step toward addressing the issue of nitrosamine impurities in general, would be to extend to veterinary medications to assess the risk of nitrosamine impurity present in human pharmaceuticals. This extension may occur in the future because previously shown N-nitroso compounds including nitrosamines have been examined in animal species and have caused cancer in each of them [56].

CONCLUSION

In summary, this review aims to empower researchers, analysts, and regulatory professionals with a comprehensive understanding of nitrosamine impurities in drug substances. It highlights on how the nitrosamine contamination sources like API, excipients, manufacturing procedures and storage environments of products sheds light on the problems complexity. By achieving knowledge of nitrosamine synthesis from various sources the review seeks to foster collaboration and promote the development of initiative-taking strategies that will contribute to the creation of safer and more reliable pharmaceutical products in alignment with global regulatory expectations. The review also covers analytical techniques ranging from conventional to innovative approaches offering industry experts and researchers a comprehensive guidance on how to efficiently identify and quantify nitrosamine pollutants. The ongoing vigilance and commitment of the pharmaceutical industry to address nitrosamine-related challenges will play a pivotal role in supporting public trust and safeguarding patient well-being.

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CONFLICTS OF INTERESTS

The authors declare no conflict of interest.

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