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Development and Validation of a Sensitive LC-MS/MS Method for Quantifying N-Nitroso-Ketamine in Ketamine Hydrochloride Injections

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Method Article

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Abstract

N-Nitrosamine drug substance–related impurities (NDSRIs) are important quality aspects in medicines due to their mutagenesis and carcinogenic potential, requiring strict toxicological assessment and sensitive analytical monitoring. This research examines the toxicology and analysis of N-nitroso-ketamine (NNK), a nitrosamine impurity generated by ketamine hydrochloride nitrosation. To classify toxic substances, the FDA-aligned Carcinogenic Potency Categorization Approach (CPCA) was used, evaluating structural factors such α-hydrogen availability, steric hindrance, and metabolic activation. Ketamine is a nitrosatable secondary amine, but its NNK structure is sterically protected around the α-carbon, limiting cytochrome P450-mediated α-hydroxylation and reducing DNA-reactive intermediates. These structural factors placed NNK in CPCA Category 5, allowing 1500 ng/day of intake.

A highly sensitive and selective LC-MS/MS method was developed and validated for trace-level NNK detection in ketamine hydrochloride drug substance and injectable formulations. The chromatographic separation was done on an Acquity HSS T3 column utilizing gradient settings and electrospray ionization in multiple reaction monitoring mode (m/z 267.0 → 207.0). The method met regulatory expectations with strong linearity ($r > 0.999$), accuracy (95-104%), precision (RSD ≤ 5%), and low quantification limit (∼139 ppb). This paper proposes a thorough and structure-guided approach to pharmaceutical NDSRI monitoring and management.

1 | Introduction

Nitrosamines are a family of N-nitroso compounds bearing the functional group of N-N = O, which are notorious for their powerful mutagenic and carcinogenic properties. Their toxicological importance is mainly owing to metabolic activation pathways leading to electrophilic intermediates which can interact with nucleophilic sites in DNA, resulting in genetic damage and carcinogenesis [1–3]. The recent unexpected detection of nitrosamine impurities in pharmaceutical products has been subject to increasing regulatory scrutiny and has prompted global health authorities, such as the United States Food and Drug Administration (FDA), European Medicines Agency (EMA) and World Health Organization (WHO) to develop comprehensive risk assessment and control strategies [1–4].

Nitrosamine impurities can be introduced at many stages of the pharmaceutical product lifecycle including active pharmaceutical ingredient (API) manufacture, formulation, storage and contact with excipients or packaging materials. Such pollutants may be produced by chemical interactions of nitrosating agents such as nitrite or nitrous acid and amine-containing sensitive compounds, especially under acidic or oxidative circumstances [5–8]. It has been demonstrated that trace levels of nitrite impurities in excipients, process water or container closure systems may make a significant contribution to nitrosamine production, especially in aqueous or parenteral formulations [10–12]. The chemical basis for nitrosamine production is well documented and involves the creation of reactive nitrosating species under particular conditions. Ketamine nitrosation is controlled by the acidic circumstances and a competition between reactivity of the secondary amine and availability of nitrosating species formed

from nitrite. In aqueous systems nitrite can serve as a precursor for reactive intermediates such as nitrous acid (HNO_2), which is in equilibrium with nitrosating agents such as N_2O_3 and NO^+. These electrophiles react with secondary amines to give N-nitrosamines. However, the extent of this reaction is highly dependent on nitrite levels, pH (often optimal between 3–5) and the presence of competing degradation or oxidation pathways. The absence of detectable NNK in the current formulation indicates that the generation of reactive nitrosating species is limited even in an acidic environment. Historically nitrosamines in pharmaceuticals have been simple low molecular weight compounds not connected to the API. In recent years, the interest in nitrosamines drug substance-related impurities (NDSRIs) chemically connected to the API or its intermediates has increased. The nitrosation of secondary or tertiary amine functionalities present in the drug molecule frequently generates impurities that are compound specific nitrosamines with variable structures and toxicological profiles [9–11]. The creation of NDSRIs is controlled by several criteria, including the availability of nitrosating agents, reaction conditions including pH and temperature, and the chemical reactivity of the API [9, 10]. The nitrosamine impurity risks have been systematically approached and controlled by regulatory agencies. A Carcinogenic Potency Categorization Approach (CPCA) has been established by the FDA to categorize nitrosamines by structural characteristics and projected carcinogenic activity. The key parameters considered by this model are the presence of α-hydrogen, steric accessibility and electronic effects on metabolic activation and is used to determine acceptable intake (AI) limits for NDSRIs [4, 12]. These limitations are reviewed on a regular basis, in the light of new toxicological and analytical evidence.

Ketamine hydrochloride is a dissociative anesthetic compound of cyclohexanone and N-methyl-D-aspartate (NMDA) receptor antagonist. Ketamine is widely used in clinical practice for anesthesia, analgesia and psychiatric reasons such as treatment-resistant depression [22–24]. Ketamine structurally has a secondary amine functional group that can act as a possible site for nitrosation. Under suitable conditions, for example in acidic environments and in the presence of nitrite, this functional group can be nitrosated to form N-nitroso-ketamine (NNK), a potential NDSRI of regulatory importance. Ketamine is administered parenterally and has a rather high maximum daily dose, which may result in exposure to minuscule levels of such pollutants, warranting thorough research.

Nitrosamines are carcinogenic mainly due to their metabolic activation via cytochrome P450-mediated α-hydroxylation, giving rise to unstable intermediates that breakdown to electrophilic molecules that can alkylate DNA, particularly at the O6 site of guanine [2, 5]. However, the extent of activation is very susceptible to structural parameters such as accessibility of the alpha-hydrogen atoms and steric hindrance around the nitrosamine functional group. These structural characteristics are crucial for the risk assessment performed according to CPCA and are important in setting the acceptable consumption limits for NDSRIs [12].

NDSRIs are difficult to detect and quantify analytically due to their presence at trace levels and low molar absorptivity in ultraviolet detection methods. Thus, conventional analytical techniques are generally not adequate for precise quantification in complicated pharmaceutical matrices. In contrast, LC-MS/MS has become the standard approach for nitrosamine analysis, offering great sensitivity and selectivity through

the use of multiple reaction monitoring (MRM) of precursor to product ion transitions [13–16, 28]. Recent improvements in LC-MS/MS technologies have permitted the detection of nitrosamines at sub-ppm and ng/L levels in complex dosage formulations such as parenteral formulations [17–19].

Despite an increased regulatory inspection, there is a lack of extensive study concerning the synthesis, toxicological evaluation and analytical quantification of N-nitroso-ketamine in ketamine hydrochloride pharmaceutical substances and injectable forms. The effect of structural constraints to its carcinogenic potential is not discussed in detail and sensitive analytical procedures for its identification are not developed.

Therefore, the present study is proposed to (i) evaluate the structural tendency of ketamine hydrochloride to generate N-nitroso-ketamine in the presence of nitrosating agents, (ii) conduct a CPCA based toxicological assessment to determine its carcinogenic potency and acceptable intake limit, and (iii) develop and validate a robust LC-MS/MS method for its quantification in pharmaceutical matrices. This integrated approach gives a holistic framework for risk appraisal, analytical management and regulatory compliance of NDSRIs in pharmaceutical development. Such integrative reviews are currently lacking for ketamine-based formulations, highlighting an important gap in the current research.

2 | Materials and Methods

2.1 | Instruments

Instrument / Column Model / Make Country of Origin (a) All the thorough initial separation investigations have been done using High Performance Liquid Chromatography (HPLC) system with Sciex 5500 Liquid Chromatography Mass Tandem Spectrometry (LC-MS/MS) system, Ontario, Canada. (b) Standard and sample weighing has been done using Micro Balance (Model: XPR2U), Mettler Toledo, Giessen, Germany. (c) All the solution preparation has been done using UC 200 sonicator, Cole-Parmer, USA. (d) Separation chemistry has been done using acquity HSS T3 2.5 µ, 4.6 X 100 mm, Waters Technologies, USA.

2.2 | Solvents and Materials

The solvents utilized in the study were: HPLC grade water was purchased from Merck, United States of America (USA). LCMS grade solvents (acetonitrile and methanol) were purchased from Merck. Formic acid (LC-MS grade) was received from Thermo Fisher Scientific, USA. The components, including the Ketamine Hydrochloride (KH) chemical substance and N-Nitroso-ketamine standard were purchased from LGC (Toronto Research Chemicals), Canada and the KH injection utilized for the investigations were made in-house (see Fig. 1, Potential Reaction Mechanism). Ketamine hydrochloride is a Schedule III prohibited substance as defined by the U.S. Drug Enforcement Administration (DEA), and was handled, stored, and used in accordance with applicable regulatory standards and institutional controlled substance protocols. All measurements were traceable to calibrated reference standards and verified analytical methods.

2.3 | Chromatographic Conditions

Liquid chromatography analysis was performed using an Acquity HSS T3 2.5 µm, 4.6 X 100 mm at 40°C. The mobile phases employed for the investigation include Mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile. In all the trials done in this investigation, the flow rate was kept constant at 0.9 mL/min. The overall run duration was 22.0 min, and the gradient elution process was shown in Table 1. Solutions were injected in volumes of 20 µL.

Table 1
Gradient program

Flow rate 0.9 mL/Min		
Time in Minutes	**% Mobile Phase A**	**% Mobile Phase B**
0.0	90	10
12.0	5	95
15.0	5	95
15.1	90	10
22.0	90	10

2.4 | MS/MS Parameters

The NNK was measured by MS/MS using the Multiple Reaction Monitoring (MRM) method with the Electrospray Ionization (ESI) technology. The curtain and collision gas were nitrogen gas. The curtain gas flow was set at 35 L/h as per the experimental trials and the collision gas was set as medium flow according to standard instrument specifications. Ionization spray (IS) was set at 5500 volts and ionization temperature (TEM) was kept at 400°C according to the ionization pattern and fragmentation. Gas 1 (GS1) is a nebulizer gas that creates small droplets that desolvate quickly in the ion source and operates at a flow rate of 65 L/h. In the ESI source, a heating gas Gas 2 (GS2) is used to evaporate the solvent and the ions. The flow rate of the gas is 60 L/h. The declustering potential and entry potential were set at 10V/10V. The parent ion and product ions of the NNK impurity were characterized with m/z values of 267.0 and 207.0, respectively. The daughter ion collision energy (CE) and collision exit potentials (CXP) were chosen as m/z 207 – 25/10.

2.5 | Structural, Carcinogenic Category Evaluation and Specification Limit

Ketamine hydrochloride (KH) has a single secondary amine functional group, which is a well-known structural alarm for nitrosamine production under nitrosating circumstances. Secondary amines can be electrophilically nitrosated in the presence of nitrite under acidic conditions to yield the matching N-nitrosamines as shown in Fig. 1.

Based on the chemical structure of KH, the presence of a single secondary amine nitrogen allows the production of one possible nitrosamine drug substance-related impurity (NDSRI), namely N-nitroso-

ketamine (NNK). Carcinogenic potential of NNK was evaluated utilizing the Carcinogenic Potency Categorization Approach (CPCA) established by the U.S. FDA. The framework is based on structural aspects influencing metabolic activation and DNA reactivity to set acceptable intake (AI) limits, CPCA potency assessment in Table 2.

Table 2
CPCA potency assessment NDSRIs

Name	α-Hydrogen (A)	Deactivation (B)	Activation (C)	Total (A + B+C)	Category	AI (ng/day)
NNK	+ 1(0,3)	-2	+ 1	0	5	1500

A: α-hydrogen availability score; B: deactivating structural features; C: activating structural features. Total score = A + B + C, used for CPCA potency classification.

The low overall potency score reflects the dominance of steric and structural constraints that limit metabolic activation via α-hydroxylation

The availability and accessibility of α-hydrogen atoms next to the nitrosamine functional group is a critical determinant in this evaluation, because they are needed for cytochrome P450-mediated α-hydroxylation, which leads to the formation of DNA-reactive intermediates (see Fig. 2).

Despite the presence of α-carbon environments with hydrogen atoms in the precursor structure (ketamine), structural change to NNK leads to a sterically restricted nitrosamine moiety in which the α-carbon neighboring the N–N = O group is embedded in a substituted cyclohexanone framework. This steric hindrance largely reduces the enzymatic accessibility and restricts the possibility of α-hydroxylation. Thus, although α-hydrogen atoms are theoretically present, the overall structural context reduces the potential for metabolic activation. NNK's bulky cyclohexanone scaffold imposes steric hindrance that limits the accessibility of α-hydrogens and reduces the probability of metabolic activation, in contrast to the high carcinogenic potency of low molecular weight dialkylnitrosamines such as NDMA and NDEA that are unhindered at the α-hydroxylation step.

On the basis of these considerations and CPCA judgment criteria, NNK is classed as a Category 5 nitrosamine (which is the lowest level of carcinogenic concern). Therefore, an AI limit of 1500 ng/day is established in accordance with FDA guidance [4]. The maximum daily dose of KH is about 1074 mg. Thus, a restriction of 1500/1074 or 1.397 ng/mg or 1397 ppb (1 ng/mg = 1000 ppb) is set as a limit.

2.6 | Sample Preparation

Transferred 1.0 mL of drug product sample/10 mg of drug substances into a 10 mL volumetric flask, added about 7 mL of water, sonicated for 5 minutes at room temperature, then diluted to a final volume of 10 mL with water and thoroughly mixed. The final concentration of KH in the sample is approximately 1.0 mg/mL.

2.7 Reference Solutions

Initially, impurity stock solution-1 was prepared as follows: about 9.3 mg of impurity standard was weighed and transferred separately into a 100 mL volumetric flask, 70 mL of acetonitrile was added to the flask, sonicated for dissolution, then diluted to the mark with acetonitrile and thoroughly mixed. Impurity stock solution-2 was prepared by taking 0.1mL of standard stock solution-1 into a single 100 mL volumetric flask, diluting to volume with water, and mixing vigorously. A common standard solution was prepared by taking 3 mL of impurity stock solution-2 into a single 200 mL volumetric flask, diluting to volume with water, and mixing vigorously. In this solution, impurity was contained at a concentration of 1390 ppb. A solution of 139.0 ppb was prepared by diluting 1 mL of the standard solution to a final volume of 10 mL with water to formulate the LOQ (Limit of Quantification) solution. 5 mL of LOQ solution is diluted to 10 mL with water to prepare the LOD (Limit of Detection) solution, resulting in an approximate LOD concentration of 69.5 ppb.

2.8 | Spiked Sample Solutions

The spiked samples, ranging from the limit of quantification 10% to 200% of the specification limit of 1397 ppb, were prepared within the sample matrix as detailed below: (a) LOQ solution - a 10 mg of KH equivalent sample powder taken into a 10 mL volumetric flask, added 7 mL of measured volume of water, 1.0 mL of impurity standard solution and sonicated for 5 minutes at room temperature, diluted to volume up to 10 mL with water and mixed well, the resultant concentration in final solution is about 139.0 ppb, (b) 100% spiked solution - a 20 mg of K equivalent sample powder taken into a 20 mL volumetric flask, added 15 mL of measured volume of water, 0.3mL of impurity standard stock solution-2 and sonicated for 5 minutes at room temperature, diluted to volume up to 20 mL with water and mixed well, the resultant concentration in final solution are about 1397 ppb, (c) A 200% spiked solution - a 20 mg of KH equivalent sample powder taken into a 20 mL volumetric flask, added 15 mL of measured volume of water, 0.6mL of impurity standard stock solution-2 and sonicated for 5 minutes at room temperature, diluted to volume up to 20 mL with water and mixed well, the resultant concentration in final solution are about 2780 ppb. Subsequently, all solutions were filtered through a 0.22-micron Nylon filter, and a clear solution was collected in a vial for analysis.

2.9 | Dynamic range (Linearity) Solutions

The linear calibration solutions have been prepared from the LOQ to 200% of the specified concentration. serial dilutions were made from the Impurity stock solution-2 to produce linearity solutions as follows: for LOQ – 0.3 mL diluted to 200 mL, for 33% – 1.0 mL diluted to 200 mL, for 66% – 2.0 mL diluted to 200 mL, for 100% – 3.0 mL diluted to 200 mL, for 150% – 4.5 mL diluted to 200 mL, and for 200% – 6.0 mL diluted to 200 mL, using water and mixed thoroughly. The final concentrations of all solutions are as follows, LOQ- 139.0 ppb, 33%-463 ppb, 66%-927 ppb, 100%- 1390 ppb, 150%-2085 ppb, and 200%- 2779 ppb.

2.10 | Formulation Composition and evaluation

The qualitative and quantitative compositions of Ketamine Hydrochloride Injection include 10 mg of Ketamine base (equivalent to 11.53 Ketamine Hydrochloride) as an active drug substance, 0.10 mg/mL of benzethonium chloride as preservative in water for injection, and it is formulated as an acidic (pH 3.5–5.5) [25]. Although acidic conditions can facilitate nitrosation in the presence of nitrite, the actual risk of NNK formation is governed by the availability of nitrosating species and trace nitrite levels rather than pH alone.

2.11 | Method Validation

The developed LC-MS/MS method for quantifying NDSRI impurities in KH was validated using international regulatory guidelines for parameters such as system suitability, system precision, specificity, method-sensitivity, precision, accuracy, linearity, robustness, and solution stability [26, 27]. Specificity was confirmed by preparing blank and placebo (drug product matrix without Ketamine) solutions according to the sample preparation protocol using a placebo powder. LOD and LOQ solutions were prepared and injected in order to check the signal to noise ratios (S/N) of impurity to evaluate the method sensitivity. Linearity was established from the LOQ to 200% of the standard by preparing the solutions as previously mentioned. The method was validated for accuracy at the LOQ, 100% and 200% of the specification values. The solutions were prepared as stated in the section on spiking sample solutions. The method precision and intermediate precision investigations were conducted by preparing six spiked samples at the 100% specification level. Robustness of the approach showed a change of ± 0.2 mL/minute from real flow and ± 20°C from the given MS/MS temperature. The stability of the analytical solution was assessed at various time intervals, including the initial 24 hours, for both standard and samples (containing contaminants at a defined concentration of 1395 ppb) at ambient temperature.

3 | Results and Discussion

3.1 | Method development:

3.1.1 | Analytical Strategy

The analytical objective was to develop a highly selective and ultra-sensitive LC-MS/MS technique capable of detecting and quantify NDSRI impurity NNK in KH Injection at trace levels in accordance with current regulatory expectations. Due to the extremely low permitted intake levels for N-nitrosamines, technique sensitivity in the low parts-per-billion (ppb) range was required.

Method development was focused on: (i) chromatographic separation of the N-nitrosamines from the high concentration of the sample matrix, (ii) optimization of the detection strategy (PDA versus MS/MS ionization) and fragmentation parameters, and (iii) implementation of a selective acquisition strategy to reduce matrix interference and increase instrument robustness.

3.1.2 | Optimization of Chromatography

The first step in developing the approach was the optimization of chromatographic parameters to achieve satisfactory baseline separation between KH and NNK. The chromatographic parameters were optimized systematically, including mobile phase composition, gradient program, flow rate, column temperature, injection volume and detection wavelength.

Volatile mobile phase additives were preferred to allow for compatibility with mass spectrometric detection. Formic acid was chosen because it improves protonation efficiency under positive electrospray ionization (ESI+) conditions and preserves the integrity of the chromatographic peaks. Mobile Phase A was 0.1% (v/v) formic acid in LC-MS grade water. Mobile phase B was acetonitrile containing 0.1% (v/v) formic acid. Methanol was initially used as an organic modifier, however acetonitrile provided superior chromatographic efficiency, sharper peak profiles, lower viscosity related backpressure and an improved MS response. Addition of formic acid to the aqueous phase provided stable baseline performance and reduced ionization variability during gradient transitions.

Finally, separation was performed on an Acquity HSS T3 column (2.5 µm, 4.6 × 100 mm) with a mobile phase of solvent A: 0.1% formic acid in water and solvent B: 0.1% (v/v) formic acid in acetonitrile, column temperature 40°C. UV absorbance was monitored at 200 nm during initial feasibility studies. Gradient parameters have been optimized to separate the nitrosamine impurities from the parent API sample and placebo matrix and from each other with minimum total run time. Special care was paid to the late eluting NNK impurity to avoid peak tailing and carryover; Under optimum circumstances NNK was separated with retention time of 9.0 min.

The optimized strategy gave an appropriate response. For the peak identification, the standard solution was injected at 30000 ppb with progressively increasing injection volumes 100µL. However, the NNK impurity concentration at the AI specification limits was less than the measurable range of UV detection. Hence, it was essential to develop a more sensitive and selective analytical detection technique.

3.1.3 | Reasons for not using Photodiode Array (PDA) Detection

PDA detection was explored as potential analytical methodologies in the initial feasibility trials. However, PDA detection was not adequate for quantitative measurement of the trace amount of the target nitrosamine impurity.

N-nitrosamines are in general weak UV absorbers, predominantly because of n→π* electronic transitions of N–N = O functional groups. These transitions are in the low UV range and have relatively low molar absorptivity. In the present development, NNK exhibited a weak UV max response at 200 nm and significant spectrum overlaps with K and related matrix components.

A significant drawback of PDA detection in this situation is the lack of sensitivity. Regulatory guidance demands quantification of N-nitrosamines at trace levels equivalent to ng/day exposure limits. PDA detection failed to detect and quantify this impurity to the required ppb amounts under optimum

chromatographic parameters. The baseline noise was also higher at low UV wavelengths due to absorption by the mobile phase and co-eluting species from the matrix. KH is also present at several orders of magnitude higher concentration than the nitrosamine impurity. However, this difference in concentration enhances the possibility of obscuring the impurity peaks, spectral interference and integration mistakes in UV detection.

On the other hand, LC-MS/MS operated in Multiple Reaction Monitoring (MS/MS) mode provides a higher analytical selectivity by the combination of chromatographic retention and impurity-specific precursor-to-product ion transitions. This orthogonal selectivity significantly reduces background interference, allowing for greater sensitivity for trace level quantification. Thus, PDA detection was not considered further in technique development, and tandem mass spectrometry was selected as the primary detection platform.

3.1.4 | Mass Spectrometric Optimization

Quantification of N-nitroso-ketamine (NNK) was carried out using a triple quadrupole LC-MS/MS system operating in positive electrospray ionization (ESI+) mode, incorporating multiple reaction monitoring (MRM) for sensitive and selective detection of trace-level nitrosamine impurities.

Mass spectrometric parameters were improved by direct infusion and flow injection study of NNK reference solutions. Under the optimum ionization circumstances, the most stable and abundant precursor ion was found as protonated molecular ion [M + H] + at m/z 267.0. Source parameters comprising ion spray voltage (5500 V), temperature (400°C) and gas settings (CUR: 35 psi; GS1: 65 psi; GS2: 60 psi) were tuned for improving ionization efficiency and desolvation.

Product ion scans showed a major fragment ion at m/z 207.0. The transition m/z 267.0 → 207.0 corresponds to a neutral loss of 60 Da, attributed to cleavage of the N–N = O link and subsequent rearrangement to generate a stable cyclohexanone-derived fragment. Compound dependent parameters were tuned as follows: DP 50 V, CE 25 eV, EP 10 V and CXP 10 V. Collision gas (CAD) was set at 8 pressures. A dwell time of 600 ms provided sufficient data capture over the chromatographic peak.

Multiple transitions were used to achieve confirmatory identification in accordance with regulatory standards for NDSRIs.

3.1.5 | Flow Diversion Strategy

A timed flow diversion method was used to avoid contamination of the mass spectrometer by the high concentration API. 0.00-0.5 min → diverted to waste 0.5–21.5 min → directed to MS Finally 21.5–22.0 min → diverted to waste. This strategy lowered matrix loading minimized ion suppression and increased the long-term instrument stability.

The method development strategy included optimization of chromatographic parameters, selective LC-MS/MS detection, and strategic flow diversion for trace-level measurement of NNK. Systematic

evaluation of PDA detection showed poor sensitivity and selectivity for regulatory compliant nitrosamine analysis and was excluded.

3.2 | Method Validation

The validation statistics for the approach clearly show that the method gives better performance and accuracy for the set system suitability parameters, for example the percent relative standard deviation (%RSD) of the standard solution. The % RSD was calculated from six replicate injections and is within the permissible amount i.e. it should not be more than 5.0% and the S/N ratio of the LOQ solution should be greater than 10. Furthermore, it exhibits good sensitivity and complies with the minimum requirement from regulatory authorities that any method should have sufficient sensitivity to reliably quantify a 10% specification value. The S/N of NNK contamination was greater above the regulatory limits 3 and 10 for LOD and LOQ solutions respectively. The specificity study results showed that the blank and placebo matrices did not interfere with the retention periods of impurity Fig. 3 typical MS/MS Chromatograms.

The method was validated for linear dynamic range from the boundary of the limits of quantification (LOQ) to 200% of the target range. Figure 4 shows the linear graph with concentration versus response, the concentration is on the X-axis, and the responses are on the Y-axis. The data show excellent correlation with regression coefficient of ≥ 0.999. The good correlation between concentration and detector response is shown.

The accuracy statistics for LOQ, 100% and 200% levels showed recovery range of 95%-104% which is in close agreement with actual spiking concentration. The robustness data shows that the method is robust to the fluctuations in flow and temperature with system suitability requirements of % RSD for the standard solution within the permissible limit of no more than 5.0% for six replicate injections in all the circumstances. The solutions of standards and spiked samples were stable for 24 hours at room temperature; the loss in potency of the standard and impurity in spiked samples was within the acceptance requirement of not more than 5.0%. The approach validation data are summarized in Tables 3.

Table 3
Method Validation Data

S.No.	Parameter	Details	NNK	Limit
1	**System Suitability**	% RSD of six replicates	4.0	NMT 5.0%
2	**LOD**	Concentration in ppb	69.5	--
		S/N ratio	14	NLT 3
	LOQ	Concentration in ppb	139.0	--
		S/N ratio	16	NLT 10
3	**LOQ Precision**	% RSD of six replicates	3.0	NMT 10.0%
4	**Linearity**	Range	139 ppb-2779 ppb	--
		Slope	163.1925	--
		Correlation coefficient(r)	0.999	NLT0.995
5	**Accuracy**	LOQ Recovery (n = 3)	104%	Between 90 to 110%
		100% Recovery (n = 3)	96%	
		200% Recovery (n = 3)	95%	
6	**Method Precision**	% RSD	5.0	NMT 10.0%
7	**Intermediate Precision**	% RSD	2.0	
8	**Robustness**	Flow (-); % RSD	2.0	NMT 5.0%
		Flow (+); %RSD	2.0	
		MS Temp (-); % RSD	3.0	
		MS Temp (+); %RSD	3.0	
9	**Solution Stability**	Standard - %Difference	7.4	NMT 10.0%
		Sample - % Difference	2.0	NMT 10.0%

MS Temp- Mass temperature; Solution Stability – the represented data is for 24 hours in room temperature; NLT- Not less than; NMT- Not more than

3.3 | Formulation Mitigation

An improved sensitive analytical platform based on LC–MS/MS was established and a complete stress testing and compatibility research was conducted to identify the likelihood of N-nitroso-ketamine (NNK) formation in the medicinal product. The study aimed to investigate the influence of process related variables, excipient interactions and container closing elements under worst case typical conditions.

No substantial rise of NNK was observed after exposure of the formulation to high temperatures (60°C for 30 days) indicating that thermal stress did not synthesize nitrosamines in the investigated framework. This suggests that the formulation does not contain sufficient reactive nitrosating species or suitable conditions for the initiation of nitrosation reactions even under accelerated degradation conditions.

Furthermore, the role of the quality of the raw material was investigated in depth. Batches of medicinal products manufactured using low-nitrite, high-purity starting materials showed consistent NNK levels at or below the limit of quantification of the assay, highlighting the importance of controlling trace sources of nitrite. It is important to note that all the formulations studied were in the acidic pH range where NNK was not detected which suggests that the formulation environment does not favor the development of nitrosamines under the measured conditions.

In summary, the results demonstrate that the integrated approach of formulation, raw material control and manufacturing design is an effective strategy to mitigate the risk of NNK formation during storage and is supportive of a sound control strategy that is consistent with current regulatory expectations for nitrosamine impurities. Three batches were tested and NNK levels were consistently below the limit of quantification (139 ppb) demonstrating that nitrosamine synthesis was successfully controlled under manufacturing and storage conditions.

3.4 | Risk Assessment of NNK Formation in Formulation and Process

In addition to the experimental evaluation, a systematic risk assessment was performed to identify probable factors affecting the development of N-nitroso-ketamine (NNK) during manufacture and storage. Figure 5 illustrated with their possible impact and mitigating options.

The developed LC-MS/MS technique was further confirmed reliable by analytical variability across validation parameters. While no formal evaluation of uncertainty was undertaken, the combined contributions from precision, accuracy and calibration linearity indicate a high level of confidence in quantifying at trace levels. The low %RSD values and uniform recovery at concentration levels prove technique robustness and reproducibility in compliance with quality assurance criteria for trace level impurity analysis.

4 | Conclusions

This study proposes an integrated methodology based on the combination of structure-based toxicological assessment and ultra-trace LC–MS/MS quantification for the evaluation of N-nitroso-ketamine in ketamine hydrochloride formulations. Although ketamine is in principle susceptible to nitrosation, there are structural restrictions that strongly diminish its metabolic activation capacity in the case of NNK, in favor of the interpretation of low-potency nitrosamine within CPCA framework. The proposed analytical approach is consistent with current regulatory requirements, including FDA guidelines on NDSRIs and ICH M7(R2) recommendations for mutagenic impurities.

The new method displays great sensitivity and compliance with regulations and is capable of being reliably used for detection at trace levels. Process and formulation evaluation further demonstrate effective control of nitrosamine generation, further supporting robustness of the pharmaceutical design. Taken together, the results provide a scientifically supported and regulatory compliant approach for the monitoring and control of NDSRIs in parenteral medicinal formulations. The integrated approach of structure-based toxicological evaluation and empirically validated impurity control strategies provides a comprehensive strategy compatible with current regulatory standards for nitrosamine risk management. The proposed paradigm could be applicable to additional medicinal items with secondary amines for a proactive risk evaluation for nitrosamines.

Declarations

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Author contributions

Ramamohana Reddy Maddike: Conceptualization; Methodology; Investigation; Formal analysis; Data curation; Validation; Software; Resources; Writing – original draft.

K.Suresh Babu: Supervision; Project administration.

Both authors read and approved of the final manuscript.

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Competing interests

The authors declare no competing interests.

Data Availability Statement

The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request.

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Figures

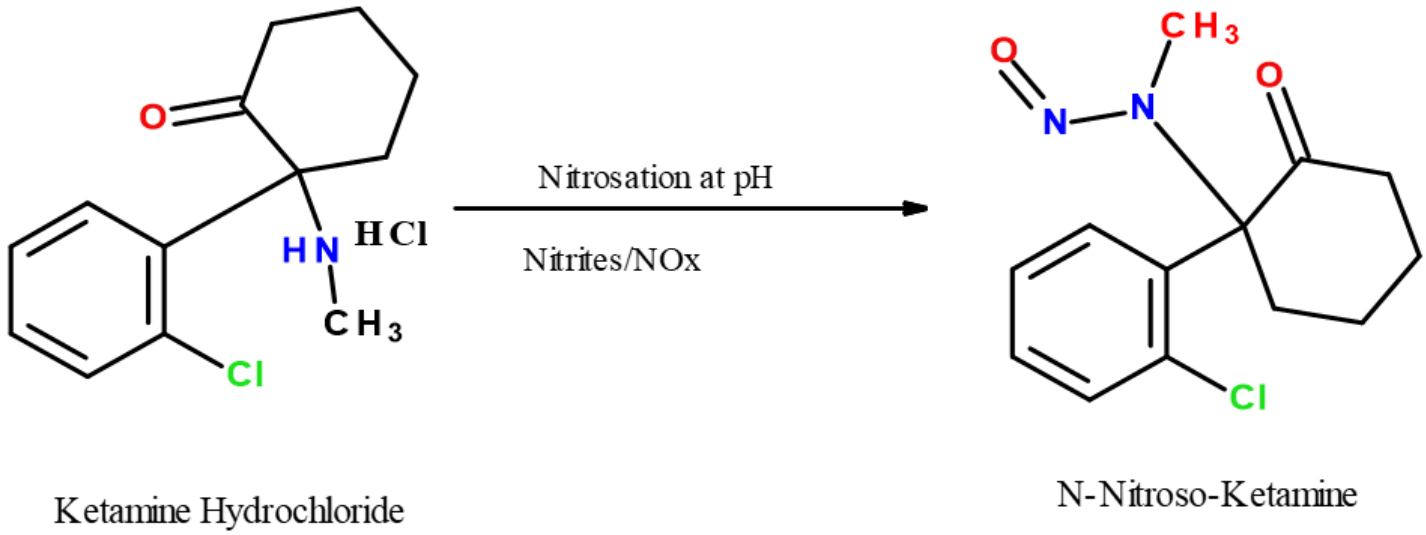


Figure 1

Potential Reaction Mechanism of NNK formation

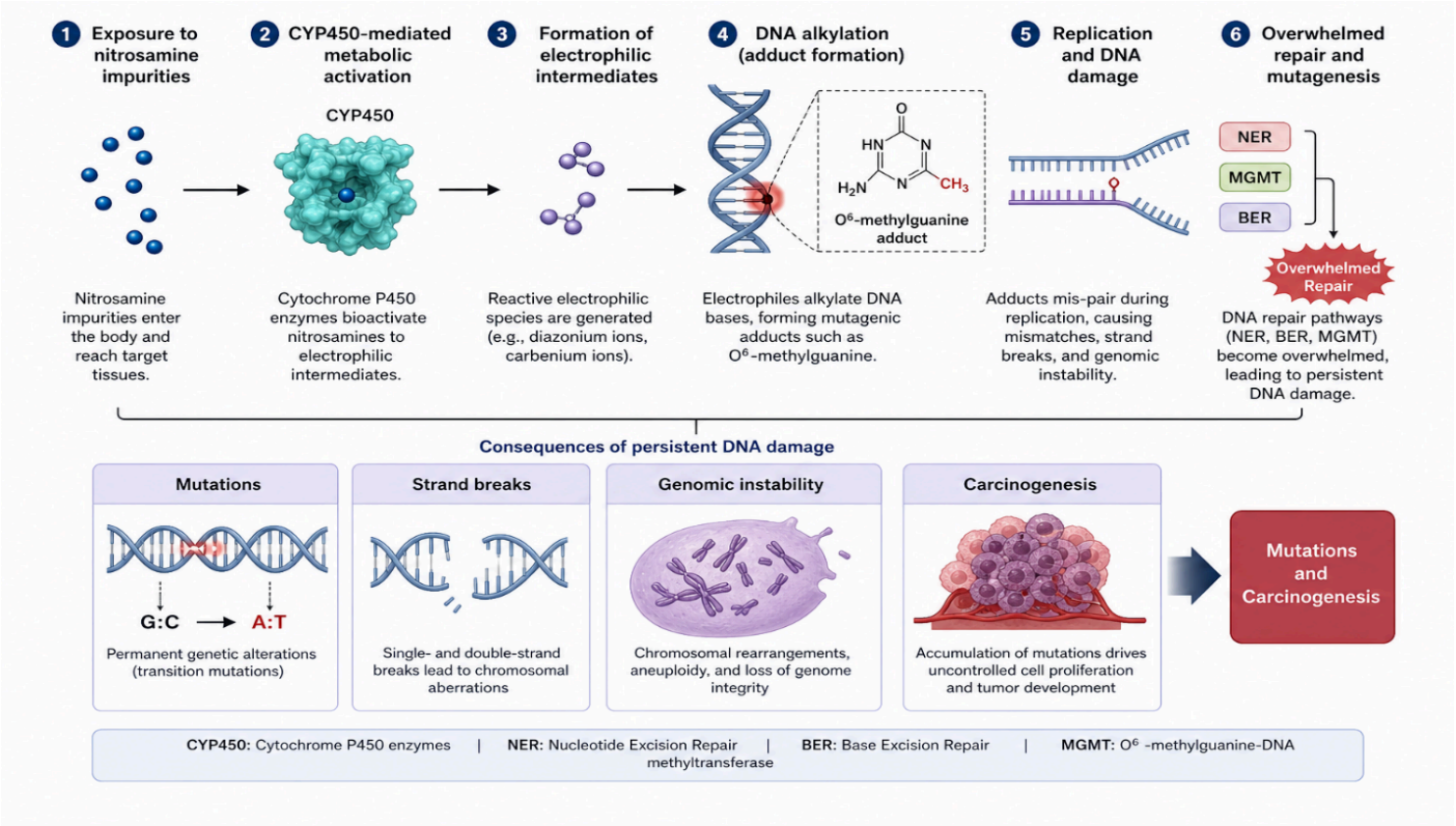


Figure 2

Metabolic Activation of Nitrosamine Impurities Leading to DNA Damage and Carcinogenesis

(Nitrosamine impurities undergo CYP450-mediated metabolic activation to form electrophilic intermediates that alkylate DNA bases, producing mutagenic adducts such as O6-methylguanine. These adducts mis-pair during replication, cause strand breaks, overwhelm repair pathways, and ultimately lead to mutations and carcinogenesis).

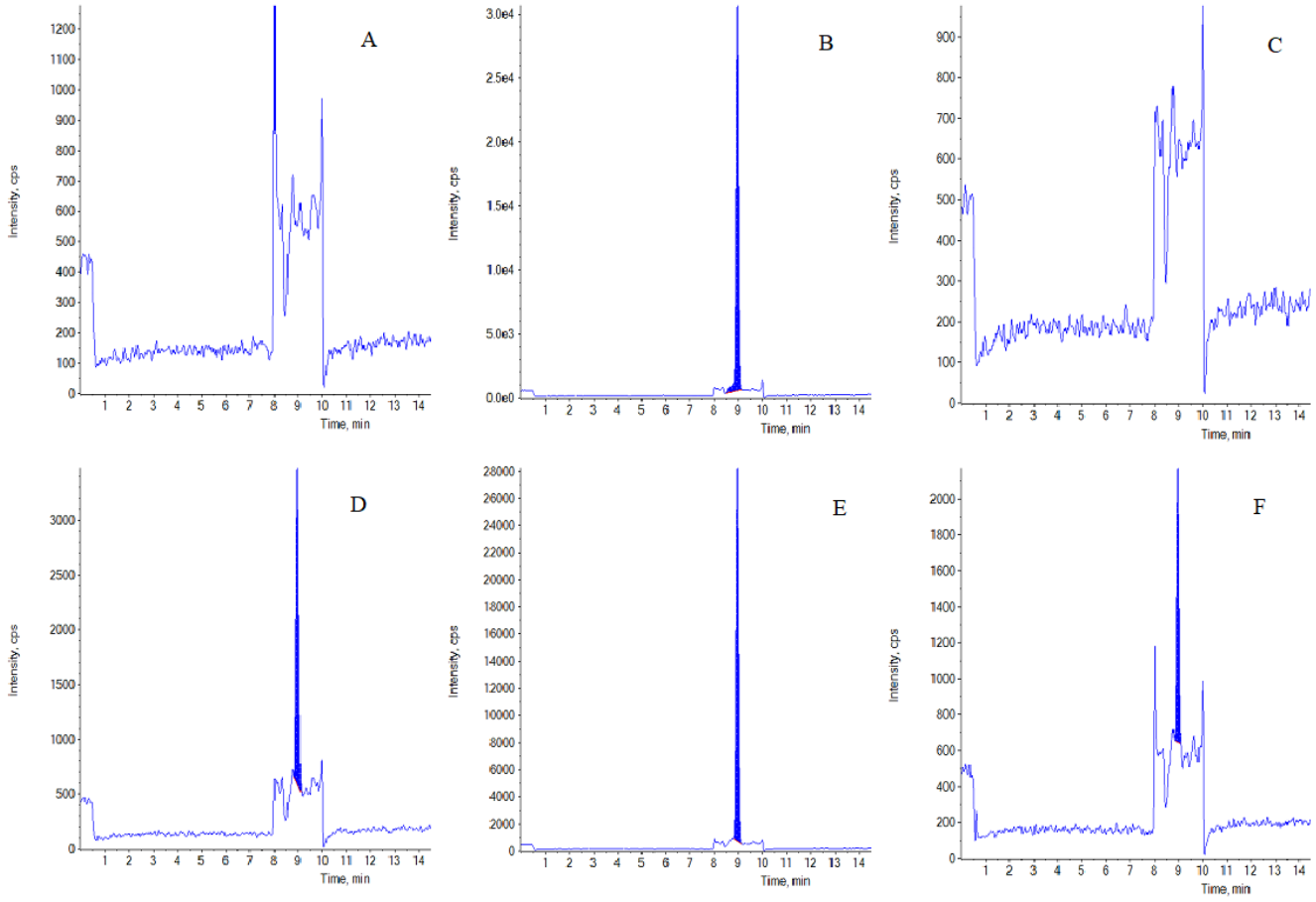


Figure 3

MS/MS Chromatograms of NNK: (A) Blank, (B) Standard, (C) Placebo, (D) LOQ, (E) Spiked sample, (F) Control Sample

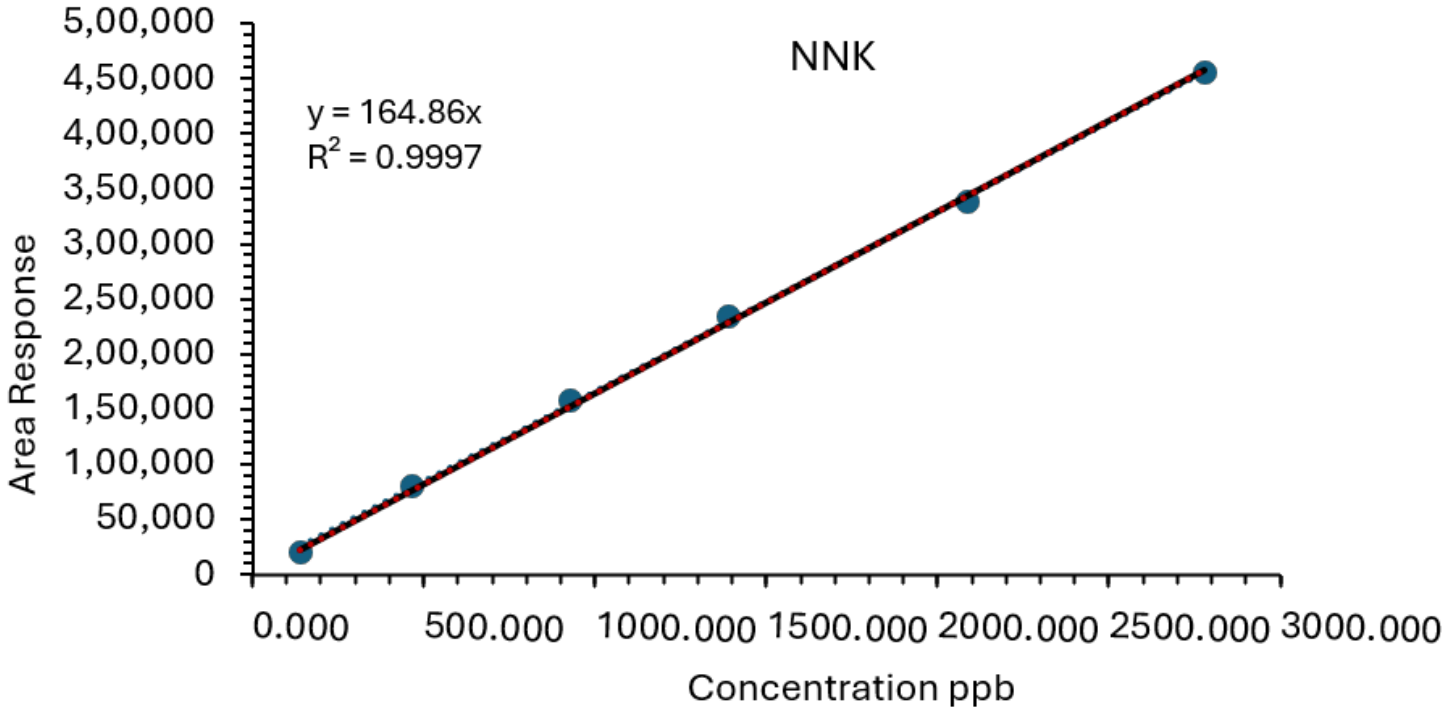


Figure 4

Linear Graph of NNK

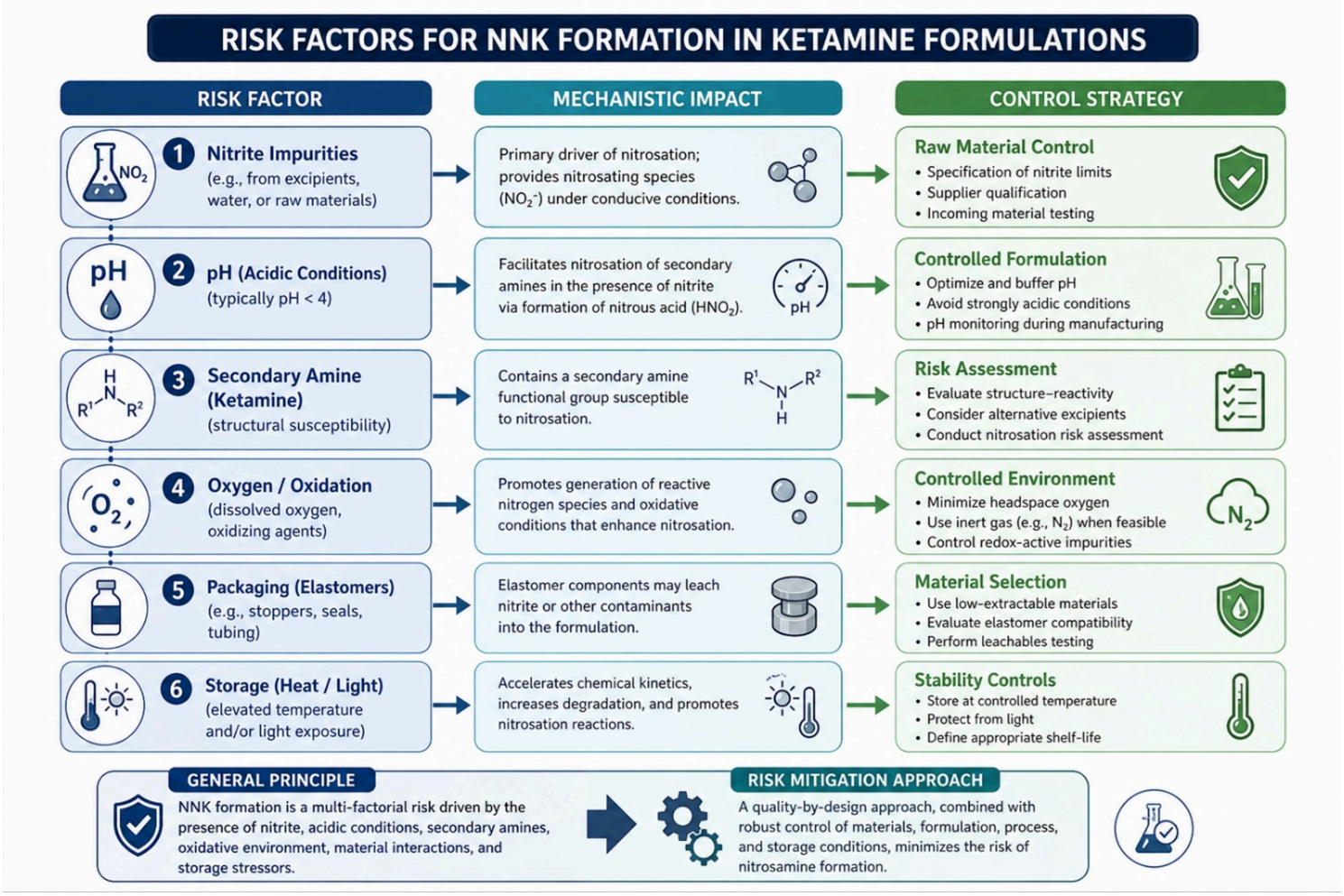


Figure 5

Risk Factors for NNK Formation in Ketamine Formulations

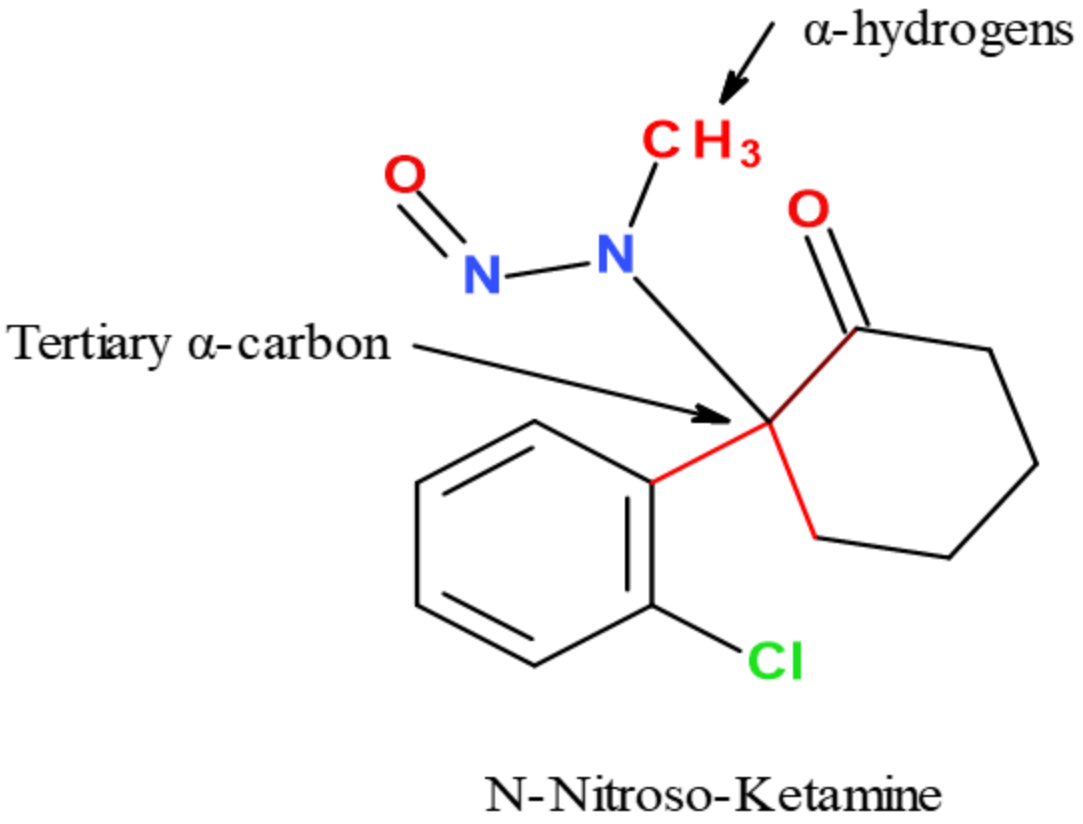


Figure 6

Unnumbered image in the Materials and Methods section

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