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DETERMINATION OF NITROSAMINES IN VARIOUS PHARMACEUTICALS AT VARIABLE TEMPERATURES

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Abstract: Nitrosamines have been classified as potent genotoxic agents for humans by the International Agency for Research on Cancer. Our study aimed to determine the levels of nitrosamine impurities that could be formed in the content of drugs under different temperature conditions during their shelf life using chromatographic analysis. Eleven drugs in pharmacies were subjected to long-term exposure at two different temperatures. Twelve nitrosamine impurities of all samples were performed using LC-MS/MS. When the impurity levels of the analyzed drugs were examined, no nitrosamine impurity was detected in any drugs. Our study revealed that if no impurity was detected under storage conditions, there was no impurity formation even when the temperature was increased. When impurity formation is effectively prevented during the manufacturing stage, the risk of impurity occurrence during the shelf-life of drugs belonging to the same group is estimated to be low.

Keywords: Drug, Impurity, LC-MS/MS, Nitrosamine, Toxicology

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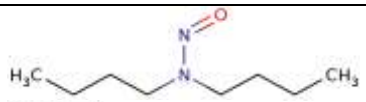
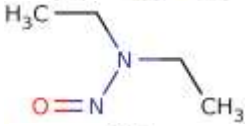
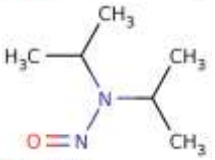
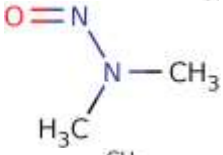
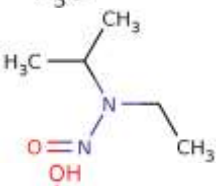
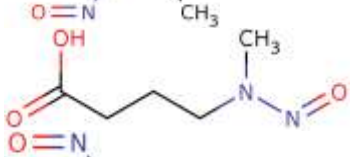
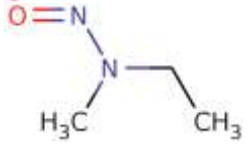
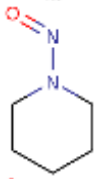
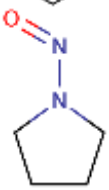
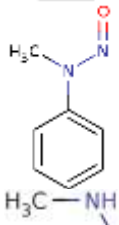
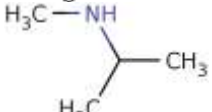
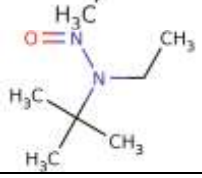
1. Introduction

Pharmaceutical impurities encompass undesired chemicals that can arise during the synthesis or production of pharmaceuticals, as well as under varying storage conditions such as temperature and humidity or as a result of interactions with excipients or contaminations (Kao et al., 2022). Among these impurities, nitrosamines (NAs) have garnered significant attention. NAs, characterized by the chemical structure "$R_2N–N = O$" is often formed by the interaction of a nitrosating agent (Sung et al., 2010; Bharate, 2021). Notably, NAs are potent genotoxic agents that can impact various living organisms and have been classified as potential human carcinogens by the International Agency for Research on Cancer (IARC) (Lapo, 2021). The emergence of NAs as a public health concern can be traced back to detecting impurities in angiotensin II receptor blocker drugs containing valsartan. In July 2018, the U.S. Food and Drug Administration (USFDA) reported the presence of N-nitroso dimethylamine (NDMA), one of the NA species, in valsartan-containing drugs (Lapo, 2021). Subsequently, NDMA was also identified in another angiotensin II receptor blockers like losartan and irbesartan, as well as in histamine II blockers such as ranitidine and nizatidine, and the anti-hyperglycemic drug metformin. Extensive studies have confirmed the mutagenic carcinogenicity of NDMA in various animal species, leading to its classification as "reasonably anticipated to be human carcinogens" by the IARC, World Health Organization (WHO), and the National Toxicology Program, U.S. Department of Health and Human Services (Tuesuwan and Vongsutilers, 2021). NA impurities can arise in drugs containing tetrazole, secondary, and tertiary amine structures (Figure 1).

Several NAs, including NDMA, N-nitroso-N-methyl-4-aminobutyric acid (NMBA), N-nitroso diethylamine (NDEA), N-nitroso diisopropylamine (NDIPA), N-nitroso ethyl isopropylamine (NEIPA), N-nitroso dibutylamine (NDBA), N-nitroso ethyl methylamine (NMEA), N-nitroso pyrrolidine (NPyR), and N-nitroso piperidine (NPIP) (Table 1), may be encountered as impurities in drug groups with this molecular structure (Li et al., 2021; Hu et al., 2021). Assessment reports released by the USFDA and European Medicines Agency (EMA) enounce that NDMA, NDEA, NMBA, NDBA, N-methyl-N-nitrosoaniline (NMPhA) are the most commonly detected impurities in specific drugs (Chidella et al., 2021; USFDA, 2021; EMA, 2021). Consequently, detecting NAs has become a crucial issue in drugs prone to impurities due to their susceptible production processes and materials.

Table 1. Twelve nitrosamine impurities reportedly identified in pharmaceuticals

Impurity	Structure	SMILES	MW (g/mol)	Log P	AI (ng/day)
NDBA		CCCCN(CCCC)N=O	158.25	2.57	26.5
NDEA		CCN(CC)N=O	102.14	1.79	26.5
NDIPA		CC(C)N(C(C)C)N=O	130.19	1.79	26.5
NDMA		CN(C)N=O	74.08	0.23	96.0
NEIPA		CCN(C(C)C)N=O	116.16	1.40	26.5
NMBA		CN(CCCC(=O)O)N=O	146.15	0.46	96.0
NMEA		CCN(C)N=O	88.11	0.62	-
NPIP		C1CCN(CC1)N=O	114.15	1.15	-
NpyR		C1CCN(C1)N=O	100.12	0.76	-
NMPhA		CN(C1=CC=CC=C1)N=O	136.15	1.80	-
NMIPA		CC(C)NC	73.14	0.61	-
N-tert-Butyl-N-ethylnitrosamine		CCN(C(C)(C)C)N=O	130.19	1.79	-

NDBA= N-nitrosodibutylamine, NDEA= N-Nitrosodiethylamine, NDIPA= N-nitrosodiisopropylamine, NDMA= N-Nitrosodimethylamine, NEIPA= N-nitrosoethylisopropylamine, NMBA= N-nitroso-N-methyl-4-aminobutyric acid, NMEA= N-nitrosoethylmethylamine, NPIP= N-nitrosopiperidine, NPyR= N-nitrosopyrrolidine, NMPhA= N-methyl-N-nitrosoaniline, NMIPA= N-Nitroso-N-methyl-2-propanamine, and N-tert-Butyl-N- ethylnitrosamine, MW= molecular weight, Log P= partition coefficient, AI= acceptable intake limit.

Guidance on controlling those impurities in medicinal and pharmaceutical products has been established by regulatory authorities like the USFDA and EMA due to the presence of different NA impurities (Chidella et al., 2021). These guidelines provide insights into the conditions that can contribute to the occurrence of NA impurities in pharmaceutical products. NA impurities can arise during active pharmaceutical ingredients (APIs) production through specific raw materials, starting materials, and intermediates. For instance, APIs may contain NAs due to using contaminated raw materials. Furthermore, the materials used in the production process, including solvents, reagents, and catalysts, can also play a role in NA formation. Amide solvents, for instance, are known to degrade into secondary amines, which are recognized as sources of NAs. Using sodium nitrite ($NaNO_2$) or other nitrites in the presence of secondary or tertiary amines presents a potential mechanism for NA formation (Schlingemann et al., 2023). Hu et al. (2021) reported that the formation of NAs can be attributed to side reactions during drug synthesis, breakdown of unstable drug compounds, contamination during production, and storage and packaging conditions. NA impurity levels in drugs can increase during production, especially under low pH and high-temperature conditions (Hu et al., 2021). According to a 2020 report by the USFDA, degradation occurring during the manufacturing of the finished metformin products led to high levels of NDMA (Tuesuwan et al., 2021; USFDA, 2021). Given the potential occurrence of NAs during production, it is imperative for pharmaceutical companies to take corrective actions to minimize the risk of NA formation in their products. Consequently, pharmaceutical companies have expanded their quality control analysis of NAs, encompassing assessments of raw materials, finished products, and residuals from the production process. Pharmaceutical products are released into the market following risk assessments, which include the analysis of NA impurities as part of the manufacturing process.

However, current quality control practices are primarily based on the analysis data of drugs before they are released to the market. Nonetheless, it is paramount to assess the impurity levels that may arise under different conditions during the shelf life of drugs, such as varying temperature and humidity conditions, after they have been launched to the market. Limited research exists on the potential risk of impurities posed by impurities that may develop due to exposure to high temperatures during the shelf life of drugs. While the NA impurities in the sartan group of drugs represent the tip of the iceberg, it is known that NA impurity formation is possible in other drug groups, mainly those containing tetrazole, secondary, and tertiary amine groups (Figure 1). Therefore, our study aims to determine the levels of NA impurities that may form under different temperature conditions during the shelf life of drug formulations using chromatographic methods. For this purpose, 11 drugs (valsartan, losartan, pioglitazone, escitalopram, rifampicin, fluoxetine, imipramine HCl, acyclovir, famotidine, metformin HCl, and venlafaxine) available in pharmacies, specifically those containing tetrazole, secondary, and tertiary amine groups (Figure 1), were subjected to prolonged exposure at two different temperatures (room temperature and 50°C). Subsequently, potential NA impurities were analyzed for all samples using an LC-MS/MS instrument.

2. Materials and Methods

2.1. Chemicals and Pharmaceuticals

The reference standard solutions of the 12 NAs (NDBA, NDEA, NDIPA, NDMA, NEIPA, NMBA, NMEA, NPIP, NpyR, NMPhA, NMIPA and N-tert-Butyl-N- ethyl nitrosamine in MeOH) in a concentration of 1000 µg/mL, and NDMA-D6 (isotope-labeled NDMA standard; as internal standard (IS)) at a concentration of 1 μgmL^{-1}, were purchased from Toronto Research Chemicals (Toronto, Canada). Methanol and acetonitrile of LC-MS grade and formic acid (> 98% purity) were purchased from Merck (Darmstadt, Germany). All drugs to be analyzed, namely valsartan, losartan, pioglitazone, escitalopram, rifampicin, fluoxetine, imipramine HCl, acyclovir, famotidine, metformin HCl, and venlafaxine were purchased from local pharmacies (Table 2).

2.2. Preparation of Calibration Standard Solutions and Quality Controls

The method employed for analyzing NA impurities in pharmaceuticals is directly utilized from the study by Mavis et al. (2023) (Mavis et al., 2023). Reference standard solutions at 1000 µg/mL concentration were diluted with methanol to prepare a working mix solution (WS1) at a concentration of 1 μgmL^{-1}. WS1 was diluted with deionized water to obtain six working mix solutions (WS2) at different concentrations (0.25, 2.5, 10, 25, 50, and 250 $ngmL^{-1}$). All solutions were stored at -20 °C. To calibration standards, each WS2 solution was diluted in a ratio of 1/5. One hundred microliters of each WS2 solution were taken, and 50 µL IS (1 μgmL^{-1}) and 350 µL deionized water were added to it. The concentrations of the obtained calibration standards were 0.05, 0.5, 2, 5, 10, 50 $ngmL^{-1}$. Furthermore, quality control samples (QCs) were prepared by adding calculated volumes of the WS2 to blank powders of the corresponding drug tablets at concentrations of 4 and 20 $ngmL^{-1}$.

2.3. Chromatographic Condition

Samples were analyzed by an Agilent Ultivo triple quadrupole LC/MS (6465B, Agilent Technologies, Santa Clara, CA, USA) equipped with an APCI source (Agilent Technologies, Santa Clara, CA, USA). Positive chemical ionization in dMRM mode was selected for MS/MS detection of the NAs. The analytical column (Poroshell HPH C18, 4.6 Å~ 150 mm 2.7 µm (P/N 693975–702, Agilent Technologies, Santa Clara, CA, USA)) was maintained at temperature of 20 °C, while the autosampler temperature was set at 8 °C. Mobile phase A (pH 3) consisted of 0.2% formic acid in deionized water,

and phase B consisted of methanol, with a flow rate of 0.6 mL/min. The initial gradient of 18% B was held for 1.0 minutes, followed by a linear increase to 78% B over 6.0 min. Subsequently, the gradient was converted to 100% B and maintained for 5.0 min. Finally, the gradient was returned to the initial condition over 5.0 minutes. The total running time was 17.0 min., and the sample injection volume was 20 µL. The mass spectrometer settings were as follows the drying gas temperature was set to 300 °C, the drying gas flow rate was 6 L/min, the nebulizer pressure was 55 psi, the vaporizer temperature was 350 °C, the corona current was 4 µA, and the capillary voltage was 3000 V. The quantification of the analytes was conducted by constructing calibration curves based on the concentrations of the calibrators, considering the matrix effect by adjusting for the yields of the corresponding IS (NDMA-D6).

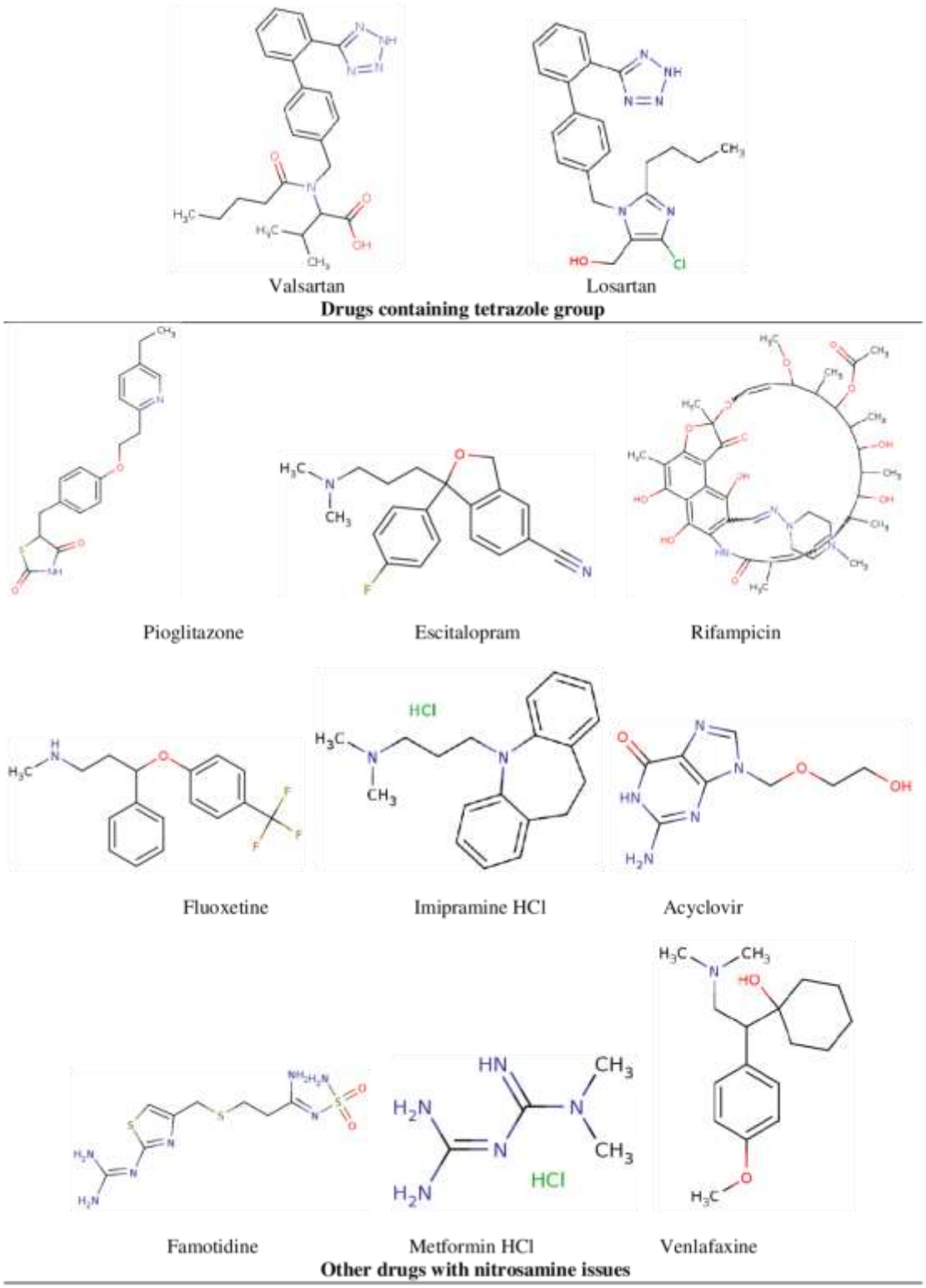


Figure 1. Chemical structures of the eleven pharmaceuticals with potential NA impurities in our study.

Table 2. Drugs and packaging shapes used in the study

Drug	DS (mg)	Formulation
Valsartan/Amlodipine	160 /5	Film tablet
Losartan potassium	50	Film tablet
Pioglitazone	30	Tablet
Essitalopram	10	Film tablet
Rifampicin	300	Capsule
Fluoxetin	20	Capsule
Imipramine HCl	25	Film tablet
Acyclovir	800	Tablet
Famotidine	40	Film tablet
Metformin HCl	500	XR* tablet
Venlafaxine	37.5	Micropellet

DS= dose strength, * extended-release.

2.4. Sample Treatment Procedure

A mandatory degradation study was conducted using stability chambers to investigate the impact of temperature on the formation of NA impurities in the drug packaging of the listed drugs in Table 2. The study involved subjecting the drugs to high temperatures without opening the packaging. The temperature ranges for drug stability, including drugs like ranitidine and similar drug groups, were reported to be 40-50°C (Hao et al., 2023). Workgroups were determined considering the shelf life and the upper limit temperatures for drug stability. The drugs were kept in their original packaging within stability chambers at different temperatures and time intervals, resulting in four groups.

As the drug packaging remained unopened, the drugs were not exposed to any oxygen entry. The first group was kept at 25°C/75% relative humidity (RH) for one week, adhering to the temperature conditions specified for shelf life. The second group was kept at 25°C/75% RH for four weeks following the shelf-life temperature conditions. The third group was subjected to the upper limit temperature for drug stability, which was 50°C/75% RH, for one week. Similarly, the fourth group was exposed to the upper limit temperature of 50°C/75% RH but four weeks.

To extract the analytes, tablet powder equivalent to 50 mg of all drugs was weighed and placed in a glass tube. Subsequently, 250 µL of IS was added to the tube and vortexed for 5 seconds. Next, 2250 µL of an extraction solution composed of deionized water and acetonitrile (80:20, v/v) with a pH of 4.0 adjusted using formic acid was added to the tube. The mixture was agitated at room temperature for 30 minutes. Following the extraction step, the suspension was centrifuged at 3600 Å~ g for 5 minutes and filtered through a 0.45 µm regenerated cellulose membrane prior to injection.

3. Results

Considering the potential carcinogenic effects of NAs, both the USFDA and EMA have issued guidelines outlining chromatographic analysis methods for the detection of these impurities in drug products. Additionally, various validated chromatographic analysis methods have been reported in the literature. In our study, we directly utilized the LC-APCI-MS/MS method developed by Mavis et al. in their 2023 publication, which was originally designed for the detection of multiple NA impurities in valsartan and irbesartan drugs (Mavis et al., 2023). We applied this method to analyze the impurities present in our drug samples. In our study, a comprehensive analysis of 12 NAs in drug samples was conducted using LC-APCI-MS/MS. A dynamic multiple reaction monitoring (mrm) method with a runtime of 17 minutes was performed, utilizing a Poroshell HPH C18 (4.6 × 150 mm, 2.7 µm) column in a gradient system mobile phase flow (Mobile phase A: 0.2% formic acid, Mobile phase B: Methanol) to detect and quantify the 12 NAs (Figure 2).

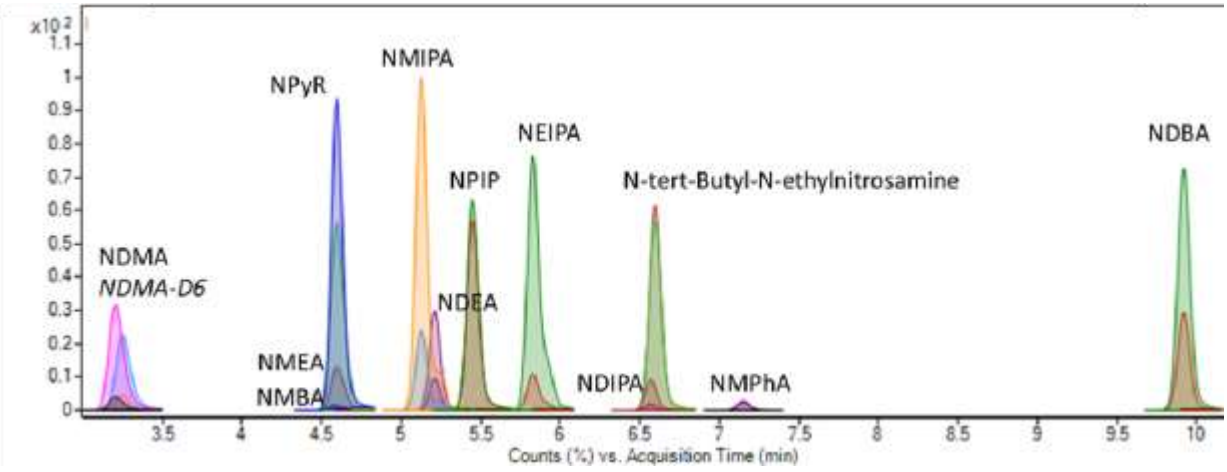


Figure 2. Chromatogram of the 12 NA impurities included in the study. NDBA= N-nitrosodibutylamine, NDEA= N-Nitrosodiethylamine, NDIPA= N-nitrosodiisopropylamine, NDMA= N-Nitrosodimethylamine, NEIPA= N-nitrosoethylisopropylamine, NMBA= N-nitroso-N-methyl-4-aminobutyric acid, NMEA= N-nitrosoethylmethylamine, NPIP= N-nitrosopiperidine, NPyR= N-nitrosopyrrolidine, NMPhA= N-methyl-N-nitrosoaniline, NMIPA= N-Nitroso-N-methyl-2-propanamine, and N-tert-Butyl-N- ethylnitrosamine, NDMA-D6= isotope-labeled NDMA standard (as internal standard).

The determined level in our study had a calibration curve range of 0.05–50 ngmL^{-1} for all NAs. The analysis's limit of quantitation (LOQ) was determined to be 0.05 ngmL^{-1} for all 12 NAs. The results presented in Table 3 indicate that the calibration curves exhibited strong linearity, with a coefficient of determination (R^2) exceeding 0.997 for all NAs (Figures 3-5).

Table 3. Calibration results for 12 NA impurities

Impurity	Precursor/product ion (m/z)	RT (min)	R^2 Value	Calibration Range (ngmL^{-1})	LOQ (ngmL^{-1})
NDBA	159.0/57.0	9.93	0.998	0.05-50	0.05
NDEA	103.1/75.5	5.22	0.999		
NDIPA	131.1/43.1	6.62	0.998		
NDMA	75.0/43.0	3.28	0.998		
NEIPA	117.0/74.9	5.84	0.999		
NMBA	147.0/44.1	4.60	0.999		
NMEA	89.1/61.0	4.61	0.998		
NPIP	115.1/41.0	5.46	0.999		
NpyR	101.1/55.0	4.61	0.998		
NMPhA	137.0/107.0	7.16	0.999		
NMIPA	103.1/60.9	5.14	0.999		
N-tert-Butyl-N-ethylnitrosamine	131.0/75.0	6.59	0.997		

RT= retention time, R^2= coefficient of determination (R squared); LOQ= limit of quantification

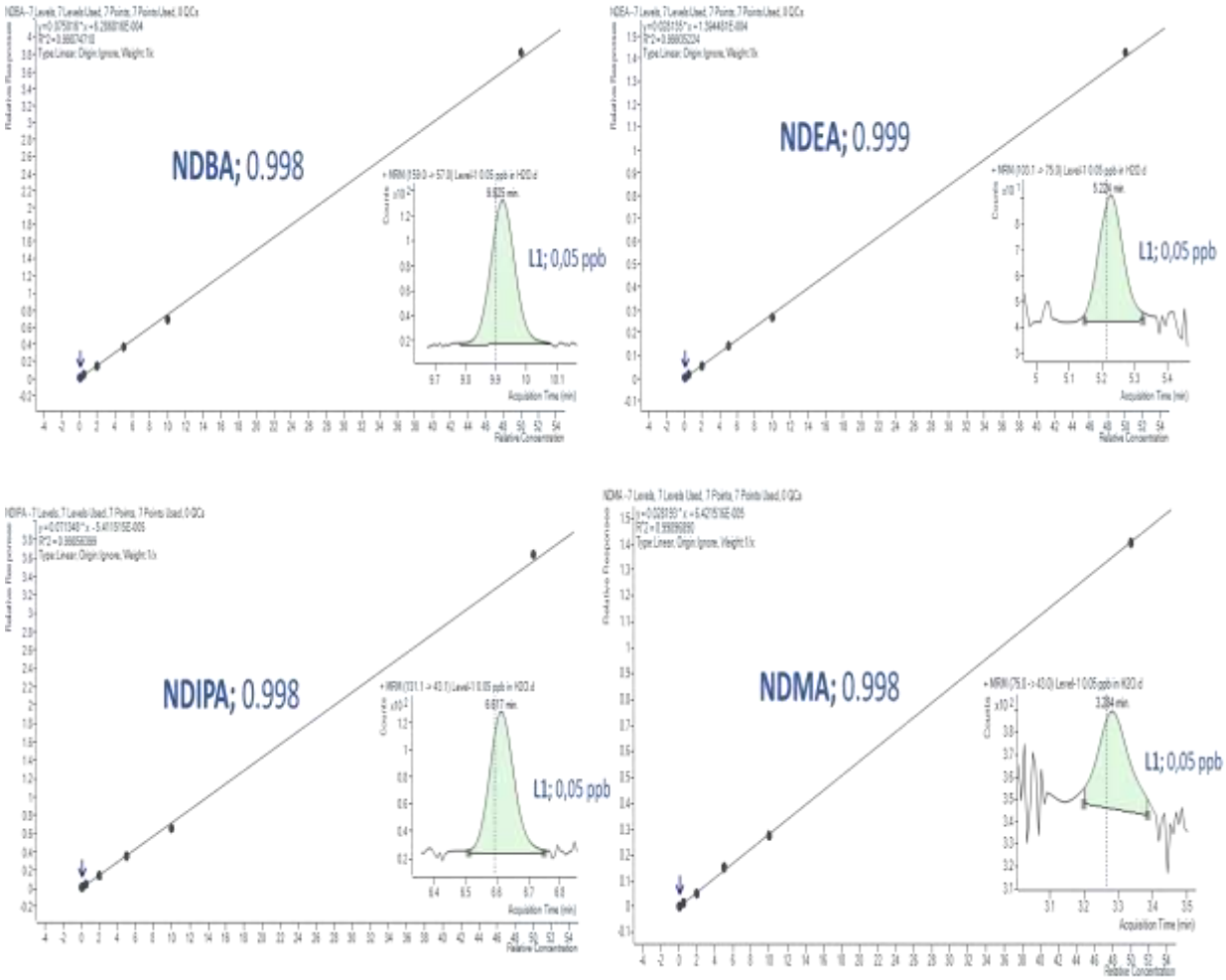


Figure 3. Calibration curves of the NDBA, NDEA, NDIPA and NDMA impurities included in the study. NDBA= N-nitrosodibutylamine (R2= 0.998, retention time (RT, min)= 9.93= precursor/product ion (m/z)= 159.0/57.0), NDEA= N-Nitrosodiethylamine (R2= 0.999, retention time (RT, min)= 5.22= precursor/product ion (m/z)= 103.1/75.5), NDIPA= N-nitrosodiisopropylamine (R2= 0.998, retention time (RT, min)= 6.62= precursor/product ion (m/z)= 131.1/43.1), NDMA= N-Nitrosodimethylamine (R2= 0.998, retention time (RT, min)= 3.28= precursor/product ion (m/z)= 75.0/43.0).

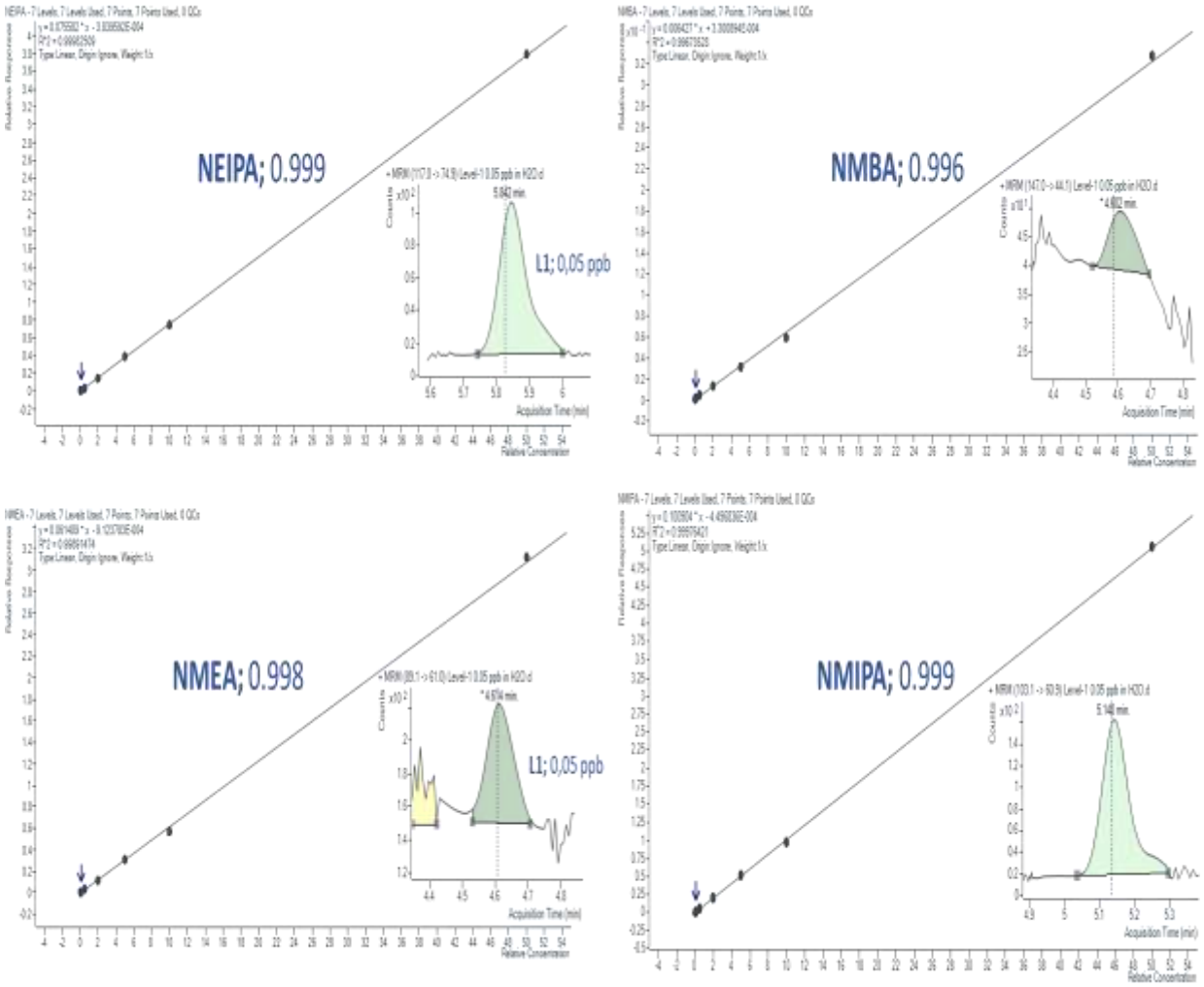


Figure 4. Calibration curves of the NEIPA, NMBA, NMEA and NMIPA impurities included in the study. NEIPA= N-nitrosoethylisopropylamine (R2= 0.999, retention time (RT, min)= 5.84= precursor/product ion (m/z)= 117.0/74.9), NMBA= N-nitroso-N-methyl-4-aminobutyric acid (R2= 0.999, retention time (RT, min)= 4.60= precursor/product ion (m/z)= 147.0/44.1), NMEA= N-nitrosoethylmethylamine (R2= 0.998, retention time (RT, min)= 4.61= precursor/product ion (m/z)= 89.1/61.0), NMIPA= N-Nitroso-N-methyl-2-propanamine (R2= 0.999, retention time (RT, min)= 5.14= precursor/product ion (m/z)= 103.1/60.9).

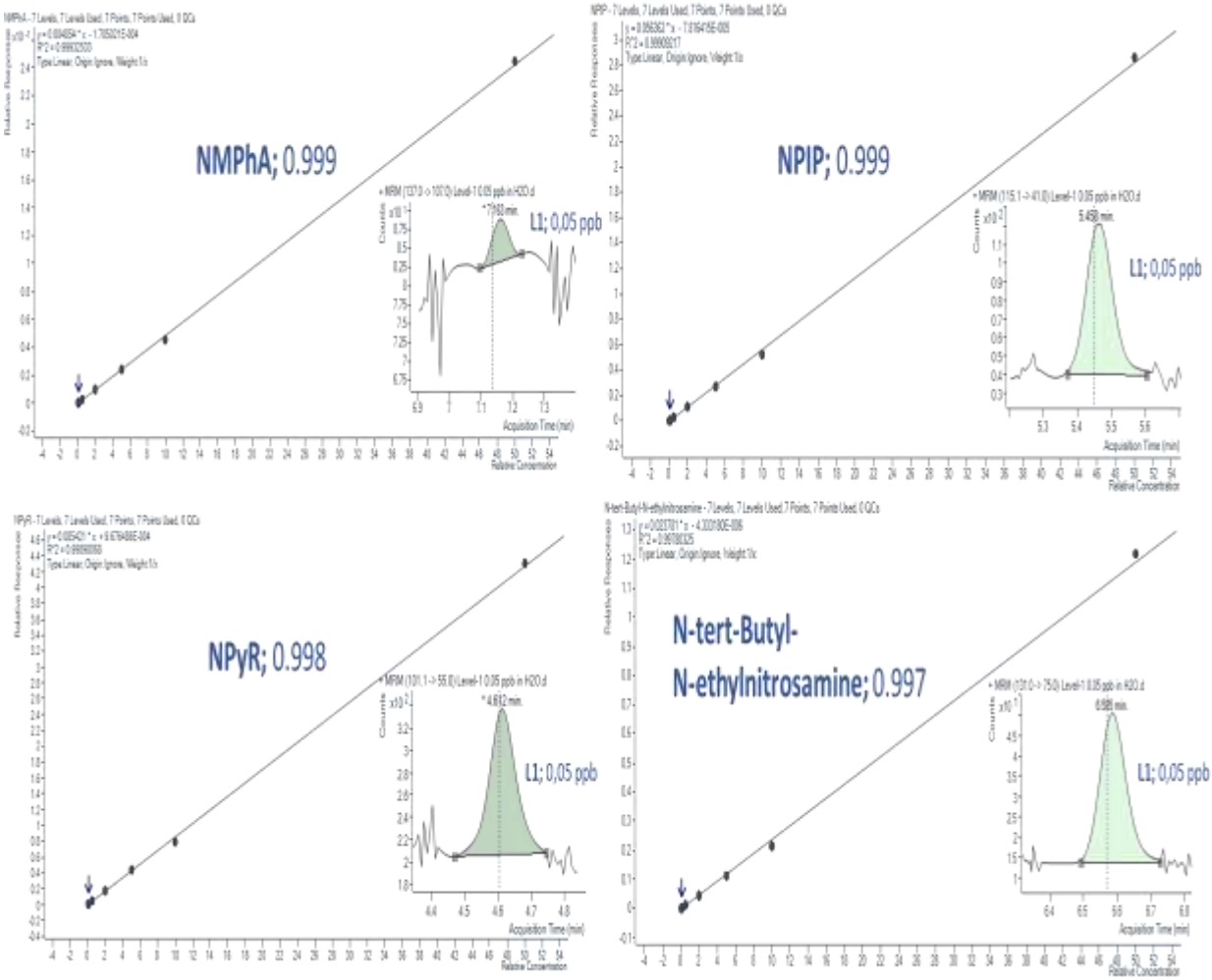


Figure 5. Calibration curves of the NMPhA, NPIP, NPyR and N-tert-Butyl-N- ethylnitrosamine impurities included in the study. NMPhA= N-methyl-N-nitrosoaniline (R2= 0.999, retention time (RT, min)= 7.16= precursor/product ion (m/z)= 137.0/107.0), NPIP= N-nitrosopiperidine (R2= 0.999, retention time (RT, min)= 5.46= precursor/product ion (m/z)= 115.1/41.0), NPyR= N-nitrosopyrrolidine (R2= 0.998, retention time (RT, min)= 4.61= precursor/product ion (m/z)= 101.1/55.0), and N-tert-Butyl-N- ethylnitrosamine (R2= 0.997, retention time (RT, min)= 6.59= precursor/product ion (m/z)= 131.0/75.0).

Our study presents the NA analysis conducted on 11 commercially available drugs (valsartan, losartan, pioglitazone, escitalopram, rifampicin, fluoxetine, imipramine HCl, acyclovir, famotidine, metformin HCl, and venlafaxine) listed in Table 2 in the materials and methods section. These drugs were kept unopened and subjected to various time intervals and temperature conditions, reflecting real-world scenarios. The analysis results of NA impurities in 11 drugs, stored at various time intervals and temperature conditions, are presented in Table 4. When the 11 drugs were analyzed for NA impurities after being stored for one week and four weeks under specific storage conditions, no impurities were detected within the calibration range of 0.05-50 $ngmL^{-1}$. Furthermore, when the NA impurity results of the 11 drugs stored for one week and four weeks at 50°C and 75% humidity stability chambers were examined (as shown in Table 4), it was observed that no impurities developed at this temperature and storage duration.

Table 4. NA impurity results of 11 drug samples stored at different temperatures

Impurity	Valsartan/ Amlodipine	Losartan	Pioglitazone	Escitalopram	Rifampicin	Fluoxetine	Imipramine HCl	Acyclovir	Famotidine	Metformin HCl	Venlafaxine
Room temperature; 25 ℃ / 75% humidity (one week and four weeks)											
NDBA	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
NDEA	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
NDIPA	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
NDMA	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
NEIPA	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
NMBA	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
NMEA	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
NPIP	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
NpyR	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
NMPhA	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
NMIPA	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
N-tert-Butyl-N-ethylnitrosamine	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
50 ℃/ 75% humidity (one week and four weeks)											
NDBA	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
NDEA	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
NDIPA	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
NDMA	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
NEIPA	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
NMBA	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
NMEA	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
NPIP	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
NpyR	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
NMPhA	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
NMIPA	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
N-tert-Butyl-N-ethylnitrosamine	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d

NDBA= N-nitrosodibutylamine, NDEA= N-Nitrosodiethylamine, NDIPA= N-nitrosodiisopropylamine, NDMA= N-Nitrosodimethylamine, NEIPA= N-nitrosoethylisopropylamine, NMBA= N-nitroso-N-methyl-4-aminobutyric acid, NMEA= N-nitrosoethylmethylamine, NPIP= N-nitrosopiperidine, NPyR= N-nitrosopyrrolidine, NMPhA= N-methyl-N-nitrosoaniline, NMIPA= N-Nitroso-N-methyl-2-propanamine, and N-tert-Butyl-N- ethylnitrosamine, n.d= not dedected.

4. Discussion

Numerous studies have focused on detecting and investigating N-nitrosamine precursors, particularly NDMA, in drugs. The IRAC has classified NDMA as a possible human carcinogen (group 2A) (Yoon et al., 2021). Following identifying NDMA in ranitidine-containing medications in September 2019, the USFDA and EMA established an acceptable daily intake limit of 96 ng or 0.32 ppm for ranitidine. However, manufacturers voluntarily recalled ranitidine worldwide when NDMA levels were found to exceed the USFDA's recommended limit by a factor of nine in numerous ranitidine-containing medicines (Yoon et al., 2021). The USFDA has temporarily set a tolerable limit of 96 ng/day in a tablet or capsule (Monajjemzadeh and Robertson, 2022). The EMA has set temporary tolerable limits of 26.5 ng/day or 96 ng/day for selected NA impurities, depending on their similarity to NDEA or NDMA (Thresher et al., 2020).

In response to the occurrence of NA contamination in sartans, regulatory bodies, and research organizations have made substantial endeavors to create analytical approaches utilizing different chromatographic analysis methods like GC-MS/MS, LC-MS/MS, and HPLC. These methods aim to accurately measure the levels of NAs in both the active APIs and pharmaceutical products (Tuesuwan and Vongsutilers, 2021). However, analyzing ppm-level contaminants like NAs in pharmaceuticals can lead to accurate results with rigorous quantitative analytical methods.

In a study by Schmidtsdorff and Schmidt, a supercritical fluid chromatography (SFC) method was developed to simultaneously determine NAs (NDMA, NDEA, NMEA, N-nitrosodi-n-propylamine (NDnPA), NDPA, NDBA, N-nitroso diphenylamine (NDPhA), NPyr, N-nitroso piperidine (NPip), NMor) at ppb levels in the sartan drugs valsartan and losartan (Schmidtsdorff and Schmidt, 2019). In another study by Xu et al. (2021) an LC-MS/MS method was developed to analyze NAs (N-nitroso dimethylamine (NDMA), N-nitroso diethylamine (NDEA), N-nitroso diisopropylamine (NDIPA), N-nitroso ethyl isopropylamine (NEIPA), N-nitroso dibutylamine

(NDBA), and N-nitroso methyl aminobutyric acid (NMBA)) as impurities in a valsartan sample. The calibration range for these NA impurities in valsartan was 0.05 – 3.6 ppm (Xu et al., 2021). Furthermore, Wohlfart et al. conducted a study in 2021 where they developed an LC-MS/MS method to quantify the amount of 4-methyl-1-nitrosopiperazine (MeNP) impurity in rifampicin. The calibration range for MeNP in rifampicin was established to be 0.7 to 5.1 ppm (Wohlfart et al., 2021). In a recent study by Wogel and Norvig, they developed an LC-MS/MS method utilizing solid phase extraction to detect 13 NAs (NDMA, NMEA, NPyr, NDEA, nitroso morpholine (NMor), ethyl-nitroso-(propane-2-yl)-amine (EIPNA), N-Nitrosodiisopropylamine (DIPNA), N-nitroso-di-n-propylamine (NDPA), N-Nitroso-N-methylaniline (NMAni), N-N-Nitrosomethyl-4-aminobutyric acid (NMBA), NDBA, N-nitroso diphenylamine (NDPhA)) that could potentially be present as impurities in pharmaceuticals. The method exhibited a remarkable detection limit of up to 0.025 ppb, demonstrating its high sensitivity and reliability in identifying trace levels of NAs (Vogel and Norwig, 2022). Similarly, Mavis et al. developed an LC-APCI-MS/MS method for the quantitative determination of 11 NAs (NMEA, NMIPA, NPIP, NPyR, NDMA NDEA, NMBA, DIPNA, NDBA, NMPhA, and EIPNA) in valsartan and irbesartan drugs. The method utilized had a calibration range of 0.5-50 $ngmL^{-1}$, allowing for precise quantification of the NA impurities (Mavis et al., 2023). These studies demonstrate the development of effective chromatographic analysis methods to accurately measure and quantify NAs in pharmaceuticals, addressing the need for reliable analytical techniques in detecting and monitoring these contaminants. Our study directly utilized the method of Mavis et al. to analyze NA impurities at ppb levels (Figure 3-5). It is essential to have a sensitive analytical method in impurity studies for accurate detection. Therefore, reporting impurity detection limits in ppm or ppb levels is customary in the literature. In our study, we detected and quantified NAs at low concentrations with a sensitive analysis method similar to the literature. (Table 3, Figure 2).

In our study, we utilized an LC-MS/MS method to investigate the presence of NA impurities in drugs and assess the impact of temperature on impurity formation during storage conditions. The literature contains several recent studies that are relevant to drug impurity analysis under storage conditions. A notable study by Abe et al. (2020) sheds light on the effect of high-temperature storage on ranitidine stability, particularly on NDMA formation. Samples were analyzed using LC-MS/MS. Following the drug stability guidelines outlined by the International Conference on Harmonization (ICH-Q1A), it was observed that NDMA levels significantly increased over 8 weeks from 0.19 to 116 ppm and from 2.89 to 18 ppm for tablet A and tablet B, respectively, when stored under accelerated conditions (40 °C with 75% RH) (Abe et al., 2020). The study demonstrated a correlation between temperature and NDMA formation, with higher temperatures increasing impurity levels. Another study conducted by Golob et al. (2023) investigated film-coated tablets containing crospovidone from two different manufacturers with varying levels of nitrite. The tablets were subjected to room temperature and accelerated stability temperature (40 °C/75% RH). NDMA and nitrite were detected at ppb levels, while DMA was detected at ppm levels in the drug product. The NDMA formation in the drug product was time, temperature, and nitrite-dependent, with a reduction in DMA and nitrite emphasized. The study highlighted that impurity formation increased over time and with high-temperature exposure in the presence of nitrite at room temperature. However, when there was no impurity formation at room temperature, impurity formation was not observed at the stability temperature (Golob et al., 2023).

In a study conducted by Hao et al. (2023) the impact of mandatory degradation tests on NA formation was investigated using a long-acting metformin drug (Hao et al., 2023). The analysis conditions included oxidation conditions at different temperatures and low pH conditions. Impurities were not detected in sample groups stored at different temperatures when stored under specific conditions (25°C/60% RH, 4 days) in an acidic environment. Even when these samples were subject to high temperatures (50°C/60% RH, 4 days), no impurities were observed. However, impurity formation was observed in samples stored in a peroxide environment under storage conditions, with high temperatures leading to increased impurity levels, consistent with observations in other conditions. Our study analyzed the drug samples at different time points and temperatures to assess the presence of NA impurities. Our findings indicate that none of the drugs analyzed (one week or four weeks) exhibited any detectable NA impurities under storage conditions of temperature and 75% humidity (Table 4). Additionally, when these drugs were subjected to high-temperature conditions of 50 °C and 75% humidity for either one week or four weeks, no NA impurities were formed (Table 4). The initial formation of NA impurities in pharmaceuticals mostly occurs through the reaction of nitrous acid with organic solvents during the API synthesis. Therefore, if impurities form during the API synthesis, these impurities can be detected using chromatographic methods under storage conditions. In our study, since no nitrosamine impurity was detected in the analyzed drugs, it can be concluded that the quality controls during the API synthesis of the drugs included in the analysis were conducted with precision.

Based on our study results, it can be concluded that for the selected drugs, if no NA impurities are present during the drug production steps, the temperature variations encountered during storage conditions do not contribute to impurity formation. Our findings suggest that the drugs analyzed in our study were not contaminated

during production, and NA formation did not occur. It is worth noting that our study data aligns with existing literature findings. However, while previous research has focused on a limited number of drugs and NA groups in terms of impurity analysis under storage conditions, our study expands upon this by analyzing a more extensive range of drugs and NA groups. The studies mentioned above offer valuable insights into the interplay between storage conditions, temperature, and the formation of NA impurities in pharmaceuticals. They underscore the significance of closely monitoring and comprehending the factors contributing to generating these impurities. Considering the findings from both the literature and our study, it becomes evident that controlling and monitoring stability-related factors and controlling impurities during production is crucial. This holistic approach is essential for mitigating some pharmaceuticals' health risks associated with NA impurities.

5. Conclusion

Nitrosamines are an essential source of impurities for public health. Pharmaceutical companies launch drugs to the market by performing various quality control analyses to prevent the formation of NA impurities. However, evaluating the level of NA impurity formation during the shelf life of drugs released to the market after passing quality control and risk assessment is vital to protect public health. Therefore, in our study, we specifically investigated the effect of the temperature parameter, which affects NA impurity formation, on pharmaceuticals available on the market. Our study revealed that for pharmaceuticals on the market that did not have nitrosamine impurities detected during production and passed quality control, nitrosamine impurities did not form even when storage conditions were changed (temperature was increased) for four weeks. This demonstrates the importance of simultaneous impurity quality audits during the manufacturing phase of pharmaceuticals and that the level of impurity formation during shelf life may be low for medicines that have passed quality audits. It is vital to continue to monitor and control NA impurities in pharmaceuticals to minimize health risks. As long as the Pharmaceutical Industry raises safety standards by prioritizing quality control measures throughout the manufacturing process and considering factors such as temperature, it is anticipated that the integrity of pharmaceutical products will not be compromised. Continued research and extensive studies are required to further improve our understanding of NA impurity formation and effectively address the public health concerns associated with these impurities.

Author Contributions

The percentage of the author(s) contributions is presented below. All authors reviewed and approved the final version of the manuscript.

%	F.C	A.A
C	100	
D	100	
DCP	80	20
DAI	60	40
L	70	30
W	60	40

C= concept, D= design, DCP= data collection and/or processing, DAI= data analysis and/or interpretation, L= literature search, W= writing.

Conflict of Interest

The authors declared that there is no conflict of interest.

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Ethical Approval/Informed Consent

Ethics committee approval was not required for this study because of there was no study on animals or humans.

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