

Synthesis and Structural Elucidation of Nitrosamine Impurities in Active Pharmaceutical Ingredients

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ABSTRACT

Nitrosamine impurities have become an important concern in the pharmaceutical industry because of their potential mutagenic and carcinogenic risks. The present study reports the synthesis, isolation, and structural characterization of three representative nitrosamine drug substance-related impurities (NDSRIs): N-nitroso tadalafil, N-nitroso ciprofloxacin, and N-nitroso ticagrelor. These impurities were synthesized through controlled nitrosation of the corresponding drug substances using sodium nitrite or tert-butyl nitrite under optimized reaction conditions. The crude products were purified by chromatographic techniques to obtain the desired nitrosamine impurities with suitable purity. Structural confirmation was carried out using high-performance liquid chromatography (HPLC), mass spectrometry (MS), and ¹H nuclear magnetic resonance (¹H NMR) spectroscopy. The analytical results verified the successful formation and identity of all three target compounds. The developed synthetic procedures and comprehensive characterization data provide valuable reference materials for impurity profiling, analytical method development, reference standard preparation, and toxicological evaluation. This work also contributes to regulatory investigations and risk assessment related to nitrosamine contamination in active pharmaceutical ingredients, supporting the development of safer pharmaceutical products.

Keywords: Impurity synthesis, Nitrosamine impurity, Impurity profiling, Nitrosation reaction, Tadalafil impurity, Ciprofloxacin impurity, Ticagrelor impurity, Carcinogen, Carcinogenic compounds.

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1. INTRODUCTION

Nitrosamines are a class of N-nitroso compounds that are typically formed when secondary amine reacts with nitrosating agents under suitable conditions. In recent years, the presence of nitrosamine impurities in pharmaceutical products has become a major concern due to their potential mutagenic and carcinogenic effects. The discovery of these impurities in several marketed medicines has led regulatory authorities, including the European Medicines Agency (EMA) and U.S. Food and Drug Administration (FDA), to establish stringent guidelines for the identification, risk assessment, and control of nitrosamine impurities in active pharmaceutical ingredients (APIs) and finished pharmaceutical products. Among these, nitrosamine drug substance-related impurities (NDSRIs) are of particular importance because they can be generated during API synthesis, manufacturing, storage, or packaging when amine-containing compounds are exposed to nitrosating agents. The availability of authentic impurity standards is essential for understanding their formation mechanisms, developing reliable analytical methods, and supporting toxicological evaluations. In this study, the N-nitroso derivatives of tadalafil, ciprofloxacin, and ticagrelor (Structure chemical name and appearance shown in table 02) were synthesized, isolated, and structurally characterized using chromatographic and spectroscopic techniques. The resulting analytical data provide valuable support for impurity identification, structural confirmation, reference standard development, and regulatory compliance during pharmaceutical research and quality control.

➤ Potential sources of nitrosamine formation

- Nitrite-contaminated excipients
- Recycled solvents
- Manufacturing under acidic conditions
- Long-term storage with trace nitrite contamination

➤ Acceptable Intake Limits

Nitrosamines are classified as potentially mutagenic and carcinogenic compounds; therefore, their presence in pharmaceutical products is strictly regulated. To protect patient safety, regulatory authorities have established health-based acceptable intake (AI) limits for these impurities (shown in Table 1). For several well-studied nitrosamines, compound-specific limits have been defined based on available toxicological data. However, for most nitrosamine drug substance-related impurities (NDSRIs), a default acceptable intake of 18 ng/day is recommended using the Threshold of Toxicological Concern (TTC) approach unless sufficient carcinogenicity data support an alternative limit.

Table 01: Nitrosamine Impurity Intake Limit

API Name	Impurity Name	Intake limit
Tadalafil	N-nitroso Tadalafil	18 ng/day
Ciprofloxacin	N-nitroso Ciprofloxacin	12,000 ng/day
Ticagrelor	N-Nitroso Ticagrelor	1500 ng/day

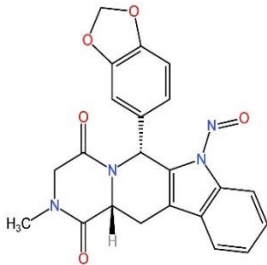
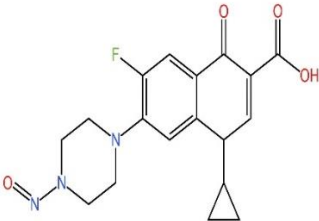
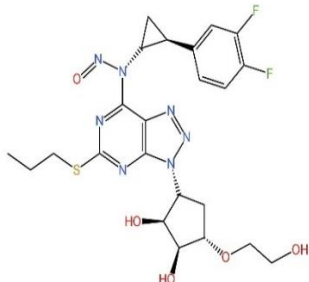
➤ Damage to the Human Body

Nitrosamines are considered potentially harmful because they are metabolically activated by cytochrome P450 enzymes to form highly reactive intermediates. These reactive species can interact with DNA and other cellular macromolecules, leading to DNA damage, oxidative stress, and genetic mutations. Long-term exposure to nitrosamines has been linked to an increased risk of cancer in several organs, including the liver, stomach, esophagus, pancreas, and urinary bladder, as demonstrated in experimental animal studies. Although nitrosamine impurities in pharmaceutical products are usually present at trace levels, continuous exposure above the recommended acceptable intake may increase the lifetime risk of cancer. Therefore, strict control of nitrosamine impurities is essential to ensure the safety and quality of pharmaceutical products.

➤ Degradation Possibilities of APIs

Nitrosamine impurities may also be formed during the degradation of APIs that contain nitrosatable amine groups. Factors such as acidic conditions, nitrite-containing excipients, residual oxidizing agents, and exposure to nitrogen oxides during manufacturing or storage can promote nitrosation reactions. In addition, environmental factors, including heat, moisture, and light, may accelerate degradation, leading to the formation of reactive intermediates that can subsequently generate nitrosamines. The APIs investigated in this study, namely tadalafil, ciprofloxacin, and ticagrelor, contain amine functionalities that are susceptible to nitrosation under favourable conditions. Therefore, careful control of formulation components, minimization of nitrite contamination, optimized manufacturing processes, and appropriate packaging and storage conditions are essential to reduce the risk of nitrosamine formation throughout the product lifecycle.

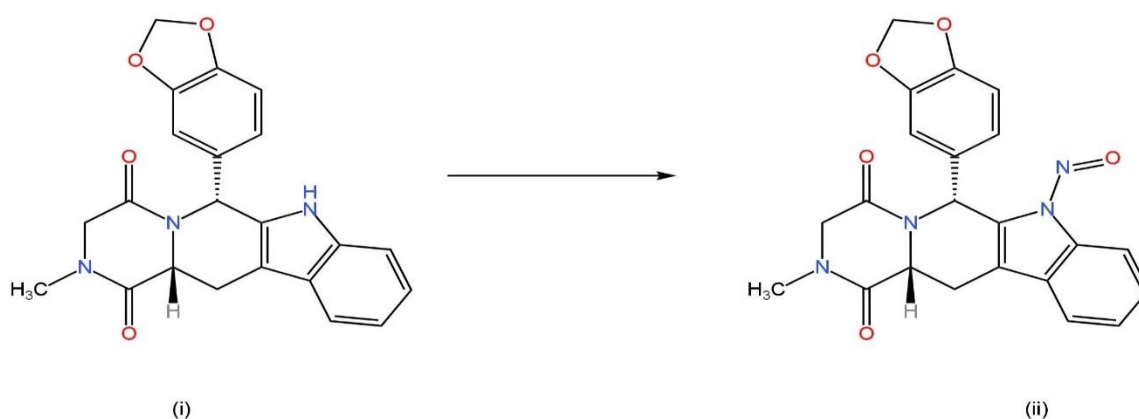
Table 02: Structure, Physical and Chemical properties of Nitrosamine Impurities

Name	N-Nitroso Tadalafil	N-Nitroso Ciprofloxacin	N-Nitroso Ticagrelor
Structure			

Chemical Name	2-(2H-1,3-benzodioxol-5-yl)-6-methyl-17-nitroso-3,6,17-triazatetracyclo[8.7.0.0 ^{3,8} .0 ^{11,16}]heptadeca-1(10),11,13,15-tetraene-4,7-dione	4-cyclopropyl-7-fluoro-6-(4-nitrosopiperazin-1-yl)-1-oxo-1,4-dihydronaphthalene-2-carboxylic acid	(1S,2S,5S)-3-(7-[(2S)-2-(3,4-difluorophenyl)cyclopropyl](nitroso)amino)-5-(propylsulfanyl)-3H-[1,2,3]triazolo[4,5-d]pyrimidin-3-yl)-5-(2-hydroxyethoxy)cyclopentane-1,2-diol
Molecular formula	C ₂₂ H ₁₈ N ₄ O ₅	C ₁₇ H ₁₇ FN ₄ O ₄	C ₂₃ H ₂₇ F ₂ N ₇ O ₅ S
Molecular weight	418.4 g/mol	360.3 g/mol	551.57 g/mol
Cas Number	NA	864443-44-7	2476859-55-7
SMILES	<chem>CN1CC(=O)N2[C@@H](C1=O)CC3=C([C@H]2C4=CC5=C(C=C4)OCO5)N(C6=CC=CC=C36)N=O</chem>	<chem>O=NN1CCN(C2=C(F)CC(C(C(C(O)=O)=CN3C4CC4)=O)C3=C2)CC1</chem>	<chem>CCCSC1=NC2=C(C(=N1)N([C@H]3C[C@H]3C4=CC(=C(C=C4)F)F)N=O)N=NN2[CH]5C[CH]([CH]([CH]5O)O)OCCO</chem>
Colour	Pale yellow	Off-White	Pale yellow
Appearance	Solid	Solid	Solid
Solubility	Dichloromethane, Ethyl Acetate, Chloroform, Methanol	Ethyl Acetate, Methanol	Dichloromethane, Ethyl Acetate, Dichloroethane, Chloroform, Methanol

Experimental:

- a) Synthesis of 2-(2H-1,3-benzodioxol-5-yl)-6-methyl-17-nitroso-3,6,17-triazatetracyclo[8.7.0.0^{3,8}.0^{11,16}]heptadeca-1(10),11,13,15-tetraene-4,7-dione:



Example 1.

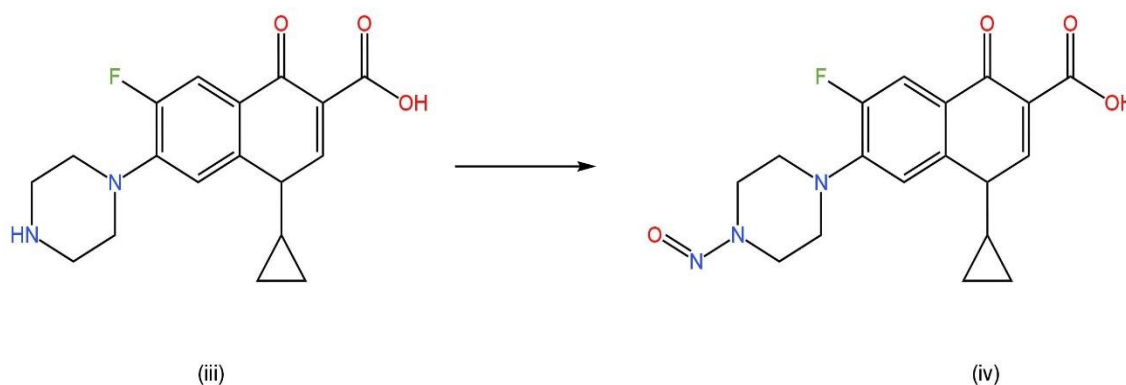
To the clear solution of (2R,8R)-2-(2H-1,3-benzodioxol-5-yl)-6-methyl-3,6,17-triazatetracyclo[8.7.0.0^{3,8}.0^{11,16}]heptadeca-1(10),11(16),12,14-tetraene-4,7-dione (i) (450mg, 1.1568 mmol) in 20ml dichloromethane, (154.73mg, 1.504 mmol) tert-butyl nitrite was added under stirring at room temperature. Stir reaction mixture overnight at room temperature and monitor reaction progress on Thin Layer Chromatography. (2R,8R)-

2-(2H-1,3-benzodioxol-5-yl)-6-methyl-3,6,17-triazatetracyclo[8.7.0.0^{3,8}.0^{11,16}]heptadeca-1(10),11(16),12,14-tetraene-4,7-dione disappears observed on TLC. Concentrate reaction mass under vacuum at room temperature. Purify desired product by column chromatography {eluent Dichloromethane: Ethyl Acetate (1:1)} yields 120mg (26.66%) pale yellow colored 2-(2H-1,3-benzodioxol-5-yl)-6-methyl-17-nitroso-3,6,17-triazatetracyclo[8.7.0.0^{3,8}.0^{11,16}]heptadeca-1(10),11,13,15-tetraene-4,7-dione. Purity by HPLC 94.21% isomeric mixture (Shown in Fig. 1).

Example 2.

To the clear solution of (2R,8R)-2-(2H-1,3-benzodioxol-5-yl)-6-methyl-3,6,17-triazatetracyclo[8.7.0.0^{3,8}.0^{11,16}]heptadeca-1(10),11(16),12,14-tetraene-4,7-dione(i) (450mg, 1.156 mmol) in 5 ml glacial acetic acid, (79.76mg, 1.156 mmol) sodium nitrite was added lot wise to reaction mass below 10°C, after addition of NaNO₂ take reaction mass to room temperature and monitor reaction progress on Thin Layer Chromatography, starting material disappears observed on TLC, adjust pH of reaction to 7-8 by Sodium Hydrogen Carbonate and Aqueous layer was extracted in Dichloromethane (20ml *3), mix organic layer and wash with water, anhydrous organic layer with Sodium sulphate and distill under reduced pressure at room temperature and purify desired material with column chromatography{eluent Dichloromethane: Ethyl Acetate (1:1)} yields 100mg (22.22%) pale yellow colored 2-(2H-1,3-benzodioxol-5-yl)-6-methyl-17-nitroso-3,6,17-triazatetracyclo[8.7.0.0^{3,8}.0^{11,16}]heptadeca-1(10),11,13,15-tetraene-4,7-dione.

- b) Synthesis of 4-cyclopropyl-7-fluoro-6-(4-nitrosopiperazin-1-yl)-1-oxo-1,4-dihydronaphthalene-2-carboxylic acid



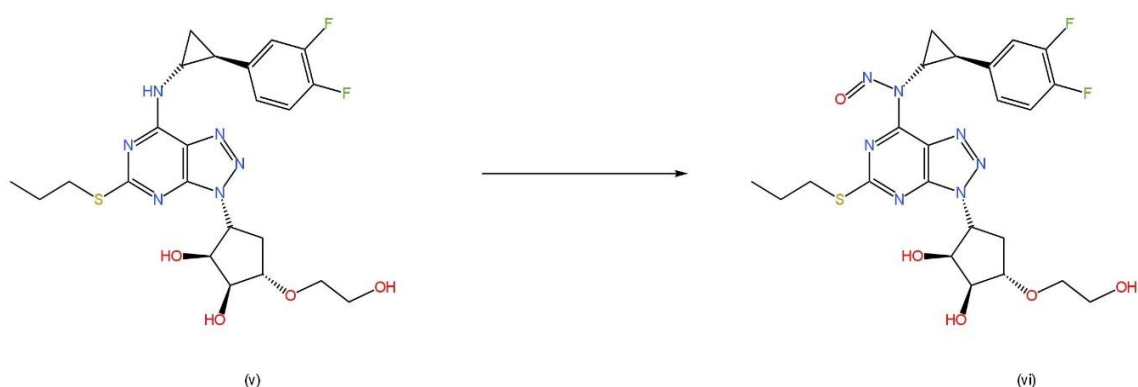
Example 3.

To the clear solution of (0.00135 mol) 4-cyclopropyl-7-fluoro-1-oxo-6-(piperazin-1-yl)-1,4-dihydronaphthalene-2-carboxylic acid hydrochloride (iii) in 30 ml Dichloromethane charge (0.17gm, 0.00164 mol) tert-butyl nitrite at room temperature. Stir reaction mass overnight at room temperature and monitor reaction progress on Thin Layer Chromatography {Ethyl Acetate: MDC (1:1)}. Solid formation observed in reaction mass, reaction completion observed on TLC, filter solid under vacuum and wash with Dichloromethane to obtain 4-cyclopropyl-7-fluoro-6-(4-nitrosopiperazin-1-yl)-1-oxo-1,4-dihydronaphthalene-2-carboxylic acid pale yellow to off-white solid yields 0.553 gm (50%), purity by HPLC 99.67% (Shown in Fig. 3).

Example 4.

To the clear solution of (0.00135 mol) 4-cyclopropyl-7-fluoro-1-oxo-6-(piperazin-1-yl)-1,4-dihydronaphthalene-2-carboxylic acid hydrochloride (iii) in Dil. HCl {(1:10) Conc. HCl: Water} charge 1 eq Sodium nitrite at 10-15°C and stir reaction mass at rt for 30 min, monitor reaction progress on TLC, after completion of reaction filter RM, resulting off-white to pale yellow solid of 4-cyclopropyl-7-fluoro-6-(4-nitrosopiperazin-1-yl)-1-oxo-1,4-dihydronaphthalene-2-carboxylic acid under vacuum and wash solid with 1 vol. water and dry solid at room temperature yields 0.48 gm.

- c) Synthesis of (1S,2S,5S)-3-(7-((2S)-2-(3,4-difluorophenyl)cyclopropyl) (nitroso)amino)-5-(propylsulfanyl)-3H-[1,2,3]triazolo[4,5-d]pyrimidin-3-yl)-5-(2-hydroxyethoxy)cyclopentane-1,2-diol



Example 5.

To the solution of (1gm, 0.00191 mol) (1S,2S,5S)-3-(7-((2S)-2-(3,4-difluorophenyl)cyclopropyl)amino)-5-(propylsulfanyl)-3H-[1,2,3]triazolo[4,5-d]pyrimidin-3-yl)-5-(2-hydroxyethoxy)cyclopentane-1,2-diol (v) in 30ml Dichloroethane charge (0.393gm, 0.00382 mol) tert-butyl nitrite stir reaction mass at reflux temperature till starting material disappears observed on thin layer chromatography. Distil reaction mass under vacuum. Purify resulting material by column chromatography {Eluent: Ethyl acetate} to yield 0.125 gm (1S,2S,5S)-3-(7-((2S)-2-(3,4-difluorophenyl)cyclopropyl)(nitroso)amino)-5-(propyl sulfanyl)-3H-[1,2,3]triazolo[4,5-d]pyrimidin-3-yl)-5-(2-hydroxyethoxy)cyclopentane-1,2-diol pale yellow solid purity by HPLC 93.38% (Shown in Fig. 5).

2. RESULTS

a) N-nitroso Tadalafil

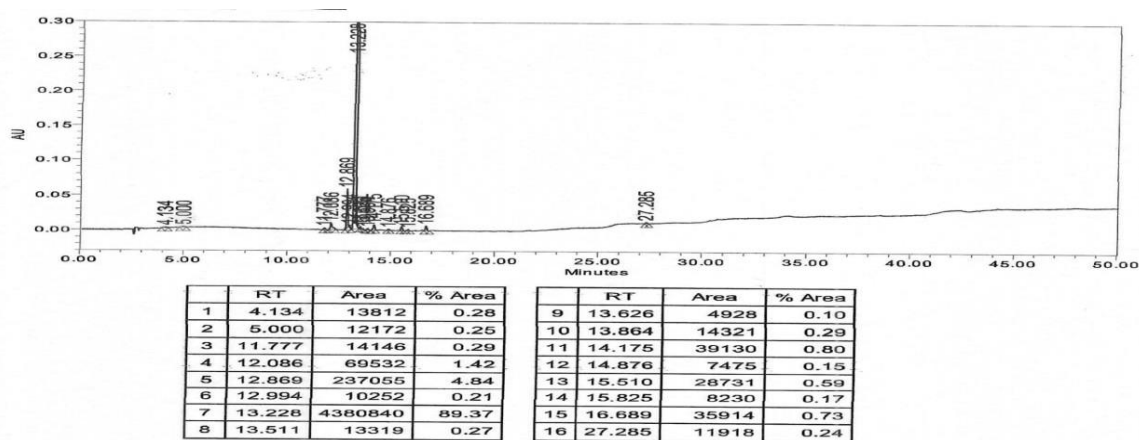
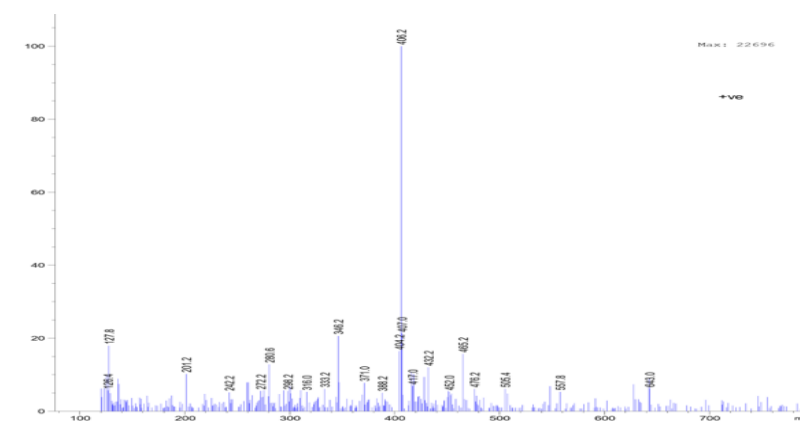


Figure 1. HPLC chromatogram of N-nitroso tadalafil



NMR

¹H NMR (400 MHz, DMSO-d₆, δ ppm)

11.53 (br s, 1H, NH), 7.96 (d, *J* ≈ 2.8 Hz, 1H, Ar-H), 7.61 (d, *J* ≈ 8.0 Hz, 1H, Ar-H), 7.43 (m, 2H, aromatic H), 7.33 (m, 2H, aromatic H), 7.08 (m, 1H, aromatic H), 6.18 (s, 1H, benzylic/heterocyclic CH), 5.76 (s, 1H, N-CH), 4.02 (d, *J* ≈ 3.2 Hz, 1H, CH), 3.48–3.28 (m, 4H, piperazinedione CH₂), 2.99 (m, 1H, CH), 2.53–2.45 (m, 2H, CH₂), 1.23 (m, 2H, aliphatic CH₂).

b) N-nitroso Ciprofloxacin

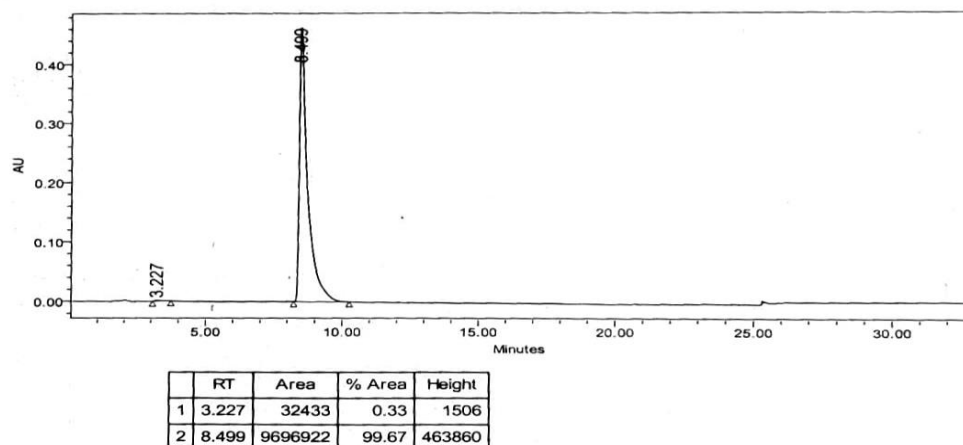


Figure 3. HPLC chromatogram of N-nitroso Ciprofloxacin

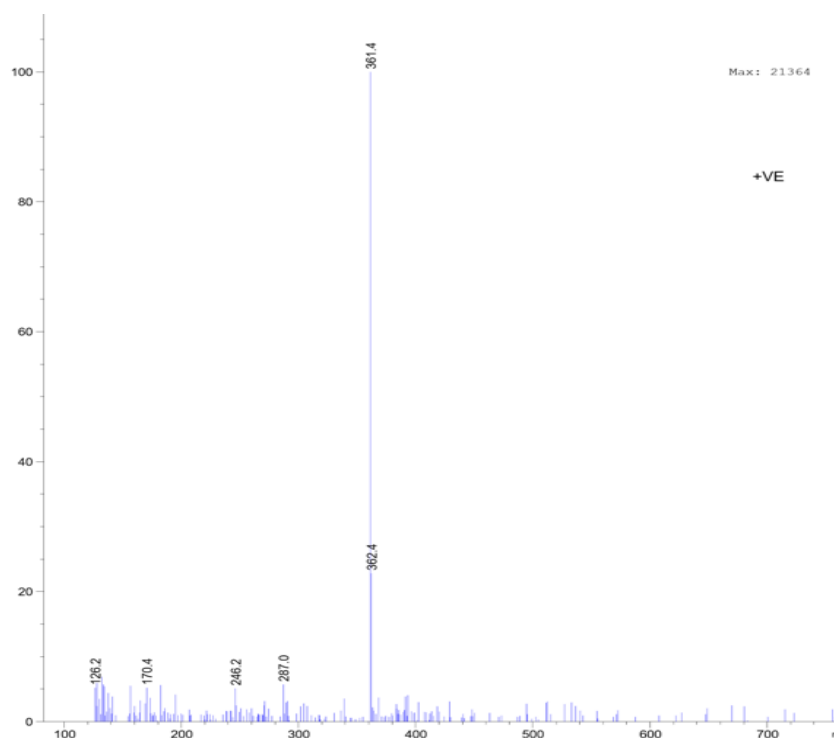


Figure 4. Mass spectrum of N-nitroso ciprofloxacin

NMR

¹H NMR (400 MHz, DMSO-d₆, δ ppm)

15.18 (br s, 1H, COOH), 8.68 (s, 1H, H-2 quinolone), 7.97 (d, *J* ≈ 13.2 Hz, 1H, aromatic H), 7.62 (d, *J* ≈ 7.5 Hz, 1H,

aromatic H), 4.47 (m, 2H, NCH₂), 3.97 (m, 2H, CH₂), 3.82 (m, 2H, piperazine CH₂), 3.63 (m, 2H, piperazine CH₂), 3.41 (br s, 1H, CH), 1.32 (m, 2H, cyclopropyl CH₂), 1.19 (m, 2H, cyclopropyl CH₂)

c) N-nitroso Ticagrelor

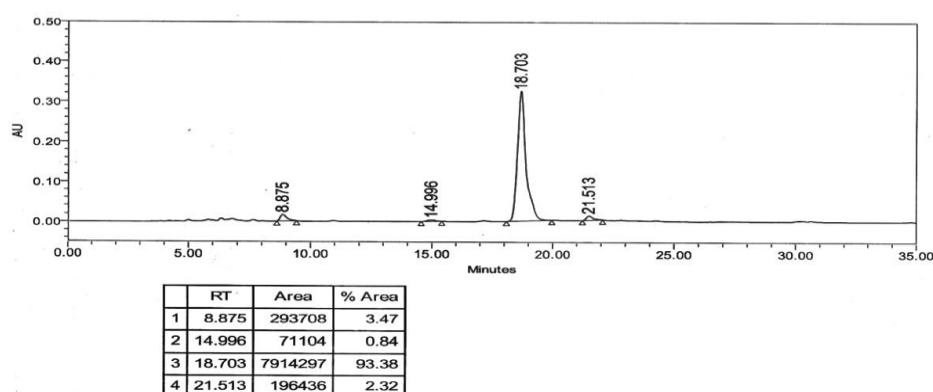


Figure 5. HPLC chromatogram of N-nitroso Ticagrelor

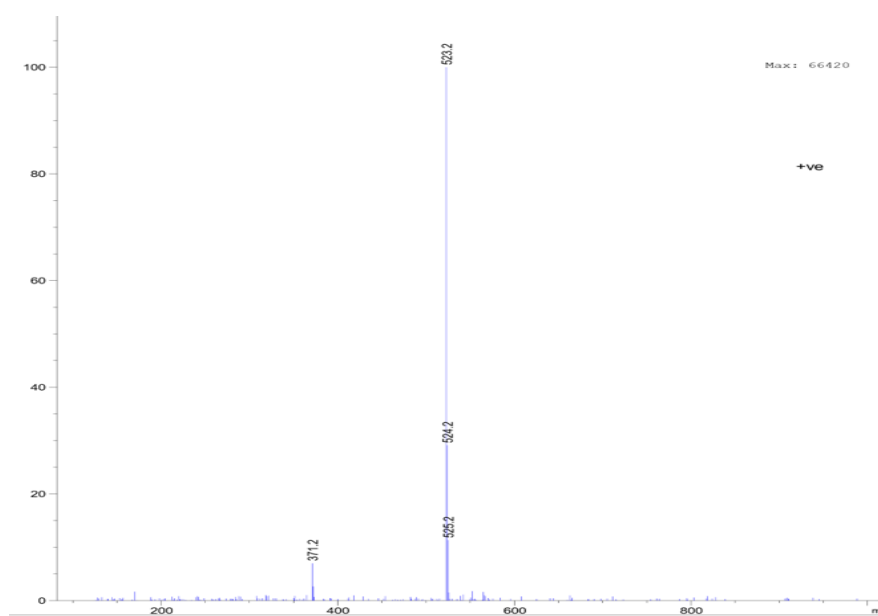


Figure 6. Mass spectrum of N-nitroso Ticagrelor

NMR

¹H NMR (400 MHz, DMSO-d₆, δ ppm):

10.05 (br s, 1H, OH/NH), 7.82 (d, J ≈ 9.6 Hz, 1H, aromatic H), 7.58 (d, J ≈ 7.2 Hz, 2H, aromatic H), 6.82 (d, J ≈ 8.1 Hz, 1H, heteroaromatic H), 5.16 (m, 1H, CH-O), 5.03 (m, 2H, CH-OH), 4.59 (m, 1H, oxygenated methine), 3.95 (m, 1H, CH-O), 3.76 (m, 1H, CH), 3.51 (s, 2H, N-CH₂), 3.15 (m, 2H, CH₂-N), 2.06 (s, 3H, CH₃CO), 1.72 (m, 3H, cyclopentyl CH), 1.00 (d, J ≈ 6.8 Hz, 3H, CH₃)

3. DISCUSSION

The present study successfully exhibited the synthesis and structural elucidation of three nitrosamine drug substance related impurities (NDSRIs), namely N-nitroso Tadalafil, N-nitroso Ciprofloxacin, N-nitroso Ticagrelor. These nitrosamine impurities were synthesized by selective nitrosation of secondary amine from active pharmaceutical ingredients using Sodium nitrite or tert-butyl nitrite under certain reaction condition.

Purity analysis done by HPLC and confirmed successful isolation of the target impurities. N-nitroso ciprofloxacin exhibited the highest chromatographic purity 99.67%, whereas N-nitroso tadalafil and N-nitroso ticagrelor showed chromatographic purity 94.21% and 93.38% respectively shown in Fig. 1,3,5. The results suggest that the optimized purification process successfully eliminated most reaction related impurities without affecting the integrity of the synthesized nitrosamine

products.

Further mass spectrometric analysis confirmed nitrosamine formation through the expected molecule ion peaks corresponding to each synthesized impurity. Figure 2, 4, and 6 shows the mass spectra of N-nitroso tadalafil, N-nitroso Cefadroxil, and N-nitroso Ticagrelor respectively. The observed increase in molecular mass with respect to parent APIs was consistent with incorporation of the ($-N=O$) nitroso functionality. The obtained mass data agreed well with the proposed molecular formula, and providing strong evidence for successful chemical formation.

Structural elucidation by ^1H NMR spectroscopy provided additional confirmation of the synthesized nitrosamine impurities. The chemical shifts, signal multiplicities, and proton integration was consistent with the proposed structures. Aromatic, aliphatic, and heterocyclic proton resonance remained unchanged with the parent compounds, whereas characteristic deshielding effects associated with nitrosamine formation were observed around the nitrogen bearing region. Therefore, the spectral data supported selective nitrosation without modification of the remaining molecule.

Nitrosamine impurity in drug substance has become an important focus of pharmaceutical quality control because they can form during API synthesis, degradation, formulation, storage or manufacturing when susceptible amine reacts with nitrosating agents. Therefore, availability of well characterized nitrosamine impurity standards is essential for analytical method development, impurity identification, chromatographic validation.

Overall, the synthetic procedures described in this work provide practical laboratory routes for preparation of nitrosamine reference materials. The analytical data generated through High Performance Liquid Chromatography (HPLC), Mass spectrometry, and ^1H NMR spectroscopy that can support impurity profiling, regulatory investigation and pharmaceutical quality assurance. The study also contributes the useful experimental information toward understanding the formation and identification of NDSRIs in amine containing active pharmaceutical ingredients.

4. CONCLUSION

This study describes the successful synthesis, isolation of N-Nitroso Tadalafil, N-Nitroso Ciprofloxacin, N-Nitroso Ticagrelor and structural elucidation of three pharmaceutically relevant nitrosamine impurities. Controlled nitrosation of corresponding drug substance was carried out using Sodium nitrite or tert-butyl nitrite. Nitrosamines were isolated in acceptable yields and purified by column chromatographic techniques. Their purity was confirmed by HPLC, and their structure were confirmed by Mass spectrometry and ^1H NMR spectroscopy.

The prepared impurities can be used as analytical reference standard for impurity identification, chromatographic method development, method validation, routine quality control. These reference materials are also valuable for degradation studies, forced degradation experiments, and investigation of nitrosamine formation pathways during manufacturing and Storage of active pharmaceutical ingredient.

Considering the increasing regulatory focus on nitrosamine risk assessment, the synthetic strategies and characterization data reported in this work provide practical support for pharmaceutical research, and regulatory compliance.

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