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Determination of Potential Genotoxic NDSRI (Nitrosamine Drug Substance Related Impurity) in Ticagrelor – Antiplatelet Medications: (Q) SAR Assessment, HPLC-ESI-MS/MS Method Development and Validation

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ABSTRACT

Regulatory agencies, including the FDA, have identified that commonly used amine-based Active Pharmaceutical Ingredient (API) synthesis processes may lead to the unintentional formation of nitrosamine impurities, which are classified as potentially genotoxic. Although N-nitrosamines are not deliberately introduced during drug manufacturing, they can form as impurities or degradation products under certain processing or storage conditions. The challenge in nitrosamine determination arises from their extremely low acceptable daily intake (ADI) limits, lack of strong chromophores, and the continuously expanding list of controlled nitrosamines, necessitating highly sensitive and selective analytical methods.

This study describes the development and validation of a highly sensitive Liquid Chromatography-Tandem Mass Spectrometry (LC–MS/MS) method for the trace-level quantification of N-nitroso-ticagrelor in ticagrelor API. Chromatographic separation was achieved on a Supelco Ascentis Express C18 column (10 cm × 4.6 mm × 2.7 µm) using a gradient elution with 0.1% v/v formic acid in water and methanol at a flow rate of 0.8 mL/min, with a total run time of 30 minutes. Detection was performed using a triple quadrupole mass spectrometer with electrospray ionization (ESI) in positive mode and multiple reaction monitoring (MRM).

The method was validated in accordance with ICH guidelines. The calibration curve demonstrated excellent linearity with a correlation coefficient (r) of 0.9922. The limits of detection (LOD) and quantification (LOQ), determined using the signal-to-noise approach, were 0.0031 ppm and 0.0093 ppm, respectively. Method recoveries & accuracy were within 70–130%, and all validation parameters met the predefined acceptance criteria. This validated method enables sensitive, accurate, and regulatory-compliant quantification of N-nitroso-ticagrelor, supporting risk assessment and quality control of ticagrelor API.

Keywords: *Ticagrelor, N-nitroso-ticagrelor, Method Validation, Drug Substances, LC-MS, ESI.*

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1. INTRODUCTION

Ticagrelor is known by its IUPAC name (1S,2S,3R,5S)-3-[7-[[(1R,2S)-2-(3,4-difluorophenyl)cyclopropyl]amino]-5-(propylthio)-3H-[1,2,3]triazolo[4,5-d]pyrimidin-3-yl]-5-(2-hydroxyethoxy)cyclopentane-1,2-diol and it is a P2Y12 platelet inhibitor used in patients with a history of myocardial infarction or who have acute coronary syndrome (ACS) to prevent future myocardial infarction, stroke, and cardiovascular death [1]. Ticagrelor, also known as AZD6140, was first described in the literature in 2003 [2-3]. Ticagrelor is an ADP derivative developed for its P2Y12 receptor antagonism [3]. It is marketed by AstraZeneca as Brilinta in the US [4] and as Brilique or Possia in the EU [5]. Ticagrelor received EMA

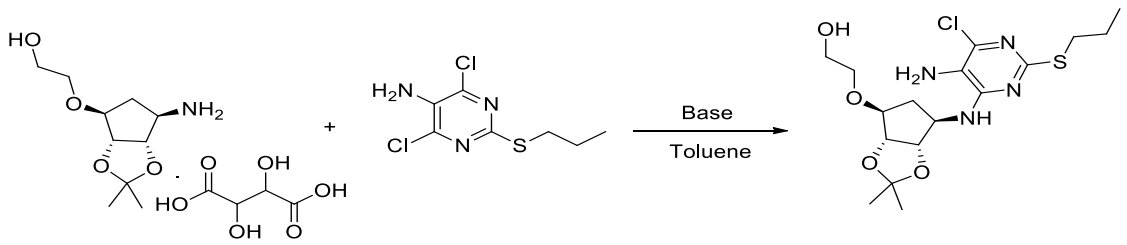
approval on December 3, 2010 [5], and FDA approval on July 20, 2011 [4]. It functions as a P2Y12 receptor antagonist [6]. The P2Y12 receptor couples with Gαi2 and other Gi proteins, which inhibit adenylyl cyclase. Gi-mediated signaling also activates PI3K, Akt, Rap1b, and potassium channels [7]. The downstream effects of these activities promote hemostasis and lead to platelet aggregation [7]. Antagonism of the P2Y12 receptor reduces the formation of occlusive thromboses, thereby decreasing the risk of myocardial infarction and ischemic stroke [7].

N-nitrosamines are a class of chemical compounds known for their carcinogenic properties, posing significant health risks to humans. These compounds have attracted attention in the pharmaceutical industry due to their accidental presence in certain medications, raising concerns about patient safety and regulatory standards. N-nitrosamines are not intentionally added to drugs but can form as impurities or breakdown products during manufacturing or storage under specific conditions [8-10]. The appearance of N-nitrosamines in drugs gained widespread concern in the industry and among regulators after several high-profile recalls of contaminated medications. Nitrosamine drug-related impurities (NDSRIs) are potentially potent carcinogens, with even low levels of exposure raising long-term health concerns. Pharmaceutical contamination with N-nitrosamines can occur through various mechanisms involving the presence of vulnerable amines and nitrosating agents under particular conditions, such as using contaminated raw materials during drug synthesis, manufacturing processes, or the degradation of drugs or excipients caused by high temperature, humidity, or light exposure [9]. In response, agencies like the U.S. Food and Drug Administration (FDA), the European Medicines Agency (EMA), and other global health authorities have established strict guidelines to control N-nitrosamines in drugs. These include risk assessments, setting acceptable limits for impurities, testing, monitoring pharmaceutical products, and implementing mitigation strategies by manufacturers [10]. Analytical techniques such as LC-MS [11-13], HPLC [14-19], and spectrophotometry [20-22] are employed to detect genotoxic impurities, organic impurities, and degradation products in APIs and formulations. These methods enable rapid and accurate detection of ticagrelor, supporting clinical research, quality control, and the identification of the medication in biological matrices and pharmaceutical products. The present work involves the development and validation of an analytical method for the identification and quantification of N-nitroso-ticagrelor in API in accordance with regulatory guidelines. Lower-level detection for NDSRI impurity analytical methods has various challenges facing the separation; the similar structure to the API or fragment makes it very difficult to develop a specific, lower detection, accurate, and stable method [27-37].

1.1 Root of synthesis (ROS) of Ticagrelor and N-nitroso-ticagrelor formation

1.1.1 ROS:

Stage-I: KSM to Crude GRE-I

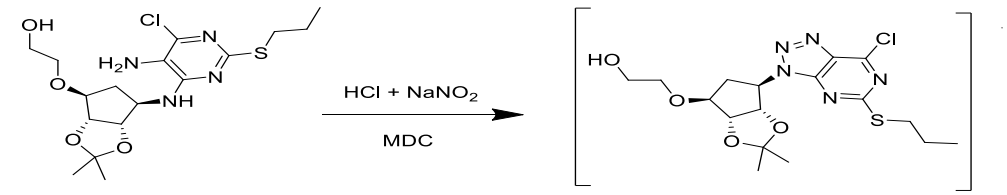


ZCL-18005
Molecular Weight: 367.35

ZCL-18006
Molecular Weight: 238.14

GRE-I
Molecular Weight: 418.94

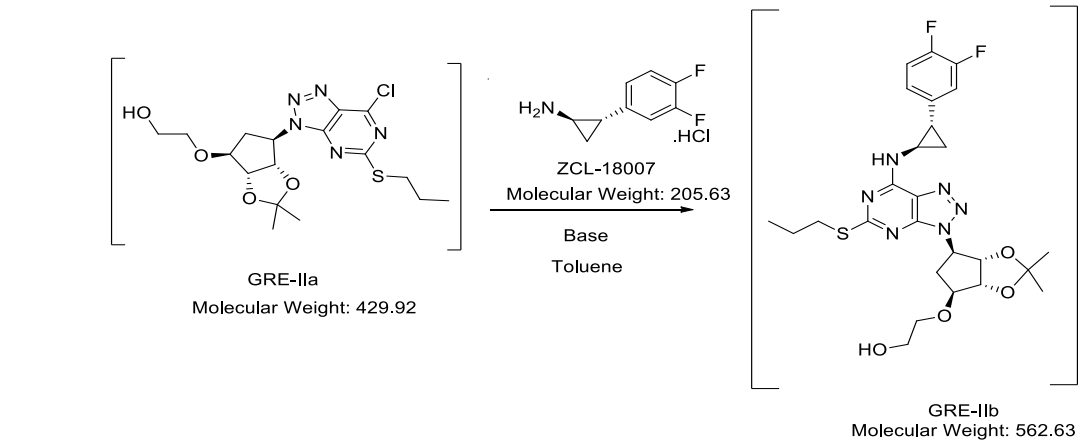
Stage-II: GRE-I to GRE-IIa



GRE-I
Molecular Weight: 418.94

GRE-IIa
Molecular Weight: 429.92

Stage-III: GRE-IIa to GRE-IIb



Stage-IV: GRE-IIb to Ticagrelor

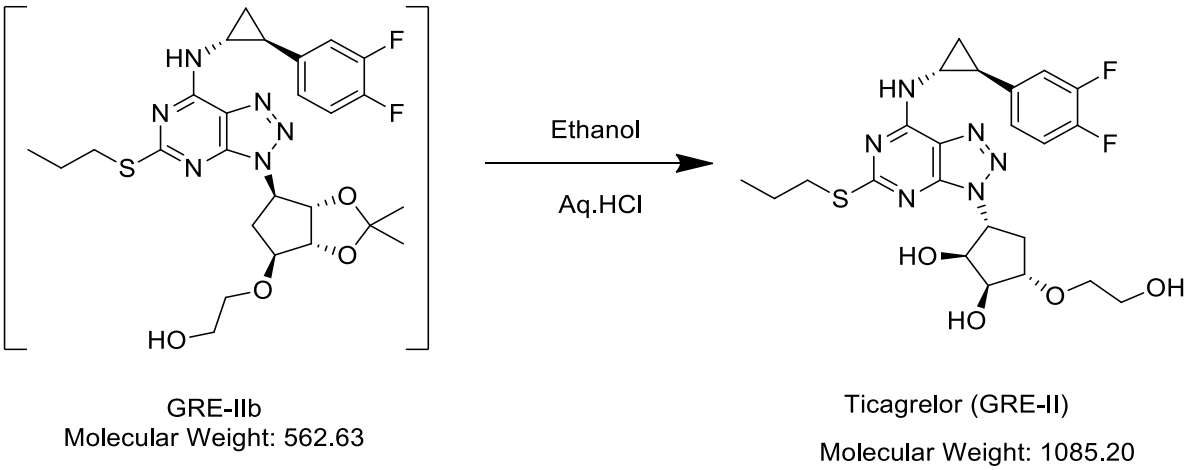


Fig. 1 Root of synthesis of ticagrelor

1.1.2 N-nitroso-ticagrelor formation:

Based on ROS evaluation (Figure 1), a secondary amine is present in the structure of ticagrelor, and $NaNO_2$ is used in the process, so the amine reacts with nitrite or nitrate, which is present at a residual level, leading to N-nitroso-ticagrelor formation [8-10, 24]. Figure 2 shown the formation of NDSRI.

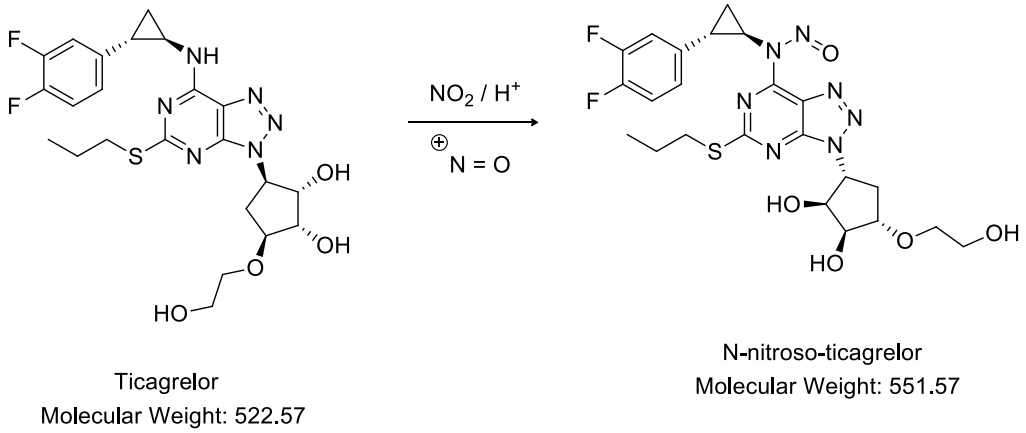


Fig. 2 Formation of N-nitroso-ticagrelor in ticagrelor.

1.2 QSAR Prediction and Limit establishment based on Acceptable intake (AI):

QSAR studies for N-nitroso-ticagrelor aim to predict the biological activity and potential toxicity of this compound based on its chemical structure. The QSAR analysis of N-nitroso-ticagrelor indicates positive predictions from Derek and Sarah, ICH M7 class 3, and an overall positive in silico result. The AI of 1500 ng/day and CPCA score was 5, as published by

EDQM [23]. Figures 3 and 4 display the ICH M7 prediction data. Table 1 is represent the limit calculation.

Table 1: Limit of N-nitroso-ticagrelor in ppm

Impurity Name	AI	CPCA	Maximum Daily Dose (MDD)	*Limit (ppm)	$ Limit consideration for Method validation
N-nitroso-ticagrelor	1500	5	180 mg/day	NMT 8.33	0.1 ppm

***Limit (ppm)** = AI/MDD (AI= Acceptable intake, MDD = maximum daily dose of an API (in mg)

Classification Table

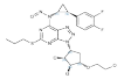
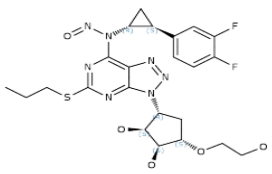
Structure	Reviewed	ICH M7 Class	Cohort of Concern	Derek Prediction	Sarah Prediction	Other QSAR Prediction	Experimental Data	Similarity to API	Overall In Silico	Comments
 N Nitroso Ticagrelor		Class 3	Yes (N-Nitroso compound)	■■■□	□□□□		Carc: Unspecified Ames: Unspecified	Alert(s) not found in API	Positive	Ticagrelor - AI = 1500 ng/day, based on designation as Potency Category = 5, as described in Appendix 1 (EMA/307633/2024 Rev.5 (01 July 2024)) to Q&A document (EMA/409815/2020 Rev.20 (15 January 2024)) available at https://www.ema.europa.eu/en/human-regulatory/post-authorisation/referral-procedures/nitrosamine-impurities, and in US Food & Drug Administration Recommended acceptable intake limits for Nitrosamine Drug Substance-Related Impurities (NDSRIs) Table 1 (5 March 2024) available at https://www.fda.gov/regulatory-information/search-fda-guidance-documents/updated-information-recommended-acceptable-intake-limits-nitrosamine-drug-substance-related.

Fig. 3 QSAR report of N-nitroso-ticagrelor

Classification Card - N Nitroso Ticagrelor



Name:	N Nitroso Ticagrelor
CAS RN®:	
ICH M7 Class:	Class 3 (Calculated Class)
Cohort of Concern:	Yes (N-Nitroso compound)
Derek Prediction:	PLAUSIBLE: Alert007 - Secondary N-nitrosamine or N-nitramine
Sarah Prediction:	EQUIVOCAL - --
Other QSAR Prediction:	
Similarity to API:	Alert(s) not found in API
Overall Carcinogenicity:	Unspecified
Overall Ames:	Unspecified
Overall In Silico:	Positive (Calculated Call)
User Carcinogenicity:	
User Ames:	
Lhasa Carcinogenicity:	Unspecified
Lhasa Ames:	Unspecified
Average Mol Mass:	551.57
Exact Mol Mass:	551.1762
Log Kp:	-4.51
Log P:	2.22
SMILES:	CCCSC4=NC(N([C@@H]1C[C@H]1C2=CC=C(F)C(F)=C2)N=O)=C5N=NN([C@@H]3C[C@H](OCCO)[C@@H](O)[C@H]3O)C5=N4
Comments:	Ticagrelor - AI = 1500 ng/day, based on designation as Potency Category = 5, as described in Appendix 1 (EMA/307633/2024 Rev.5 (01 July 2024)) to Q&A document (EMA/409815/2020 Rev.20 (15 January 2024)) available at https://www.ema.europa.eu/en/human-regulatory/post-authorisation/referral-procedures/nitrosamine-impurities, and in US Food & Drug Administration Recommended acceptable intake limits for Nitrosamine Drug Substance-Related Impurities (NDSRIs) Table 1 (5 March 2024) available at https://www.fda.gov/regulatory-information/search-fda-guidance-documents/updated-information-recommended-acceptable-intake-limits-nitrosamine-drug-substance-related.

Fig. 4 Classification card of N-nitroso-ticagrelor

2. MATERIAL AND METHOD

a. Materials, Chemicals, Instruments, and Column used

Ticagrelor and N-nitroso-ticagrelor (potency: 94.5%) standards were used in-house (Cohance Lifesciences Limited, India, formally ZCL chemicals). Impurity & samples, chemicals and reagents, and instrument, details are described in Tables 2 to 4, respectively.

Table 2: Impurity and sample details.

Sr. No.	Material Name	Lot. No./ A.R. No. / Batch. No.	Source	% Potency / Purity
1	N-nitroso-ticagrelor	APS/I/GRE-II/946/101 (RS)	ZCL Chemicals Ltd.	94.5
2	Ticagrelor	GRE3200119	ZCL Chemicals Ltd.	NA
3	Ticagrelor	GRE3200219	ZCL Chemicals Ltd.	NA
4	Ticagrelor	GRE3200319	ZCL Chemicals Ltd.	NA
5	Ticagrelor	GRE3200419	ZCL Chemicals Ltd.	NA

Table 3: Chemicals and Reagents

Sr. No.	Chemical/Reagent	Lot. No. / A.R. No. / Batch. No.	Make	Grade
1	Formic acid	173879	Fisher chemical	LCMS
2	Methanol	206054	Fisher chemical	LCMS
3	Water	NA	Milli Q	NA

Table 4: Instrument details

Sr. No.	Instruments	Make	Model
1.	LCMS	Shimadzu	LCMS-8040
2.	Balance	Mettler Toledo	XS205
3.	Ultrasonic Bath Sonicator	PCI analytics	9L250H/DTC

b. Chromatographic Method Conditions:

2.2.1 LC operating condition:

HPLC instruments with a PDA detector were used for method development and method validation. 1% formic acid in water as reservoir A and acetonitrile used as reservoir B in gradient mode with 30 minutes total run time and 0.8 mL/min flow rate are sufficient for the separation of ticagrelor and N-nitroso-ticagrelor. The Supelco Ascentis Express C18 analytical column with 4.6 mm (internal diameter), 10 cm (length), and 2.7 µm (particles) was found suitable in terms of peak shape and sensitivity. Column oven compartment temperature to be set to 40°C. Autosampler temperature maintained at 25°C (ambient). The injection volume of 50 µl is considered. Water and methanol in the ratio of 20:80 (v/v) were used as a diluent.

2.2.2 MS-MS operating condition:

Triple quadrupole LC-MS-8040 (Shimadzu) with ESI ion source was evaluated for method development and validation. Positive mode Multiple Reactions Monitoring (MRM) was selected for the identification and quantification of N-nitroso-ticagrelor. Table 5 represents the final method. APCI and ESI ion sources are introduced for the ionization, but ESI with positive MRM transition is found to be best for the identification and quantification of N-nitroso-ticagrelor.

2.3 Impurity standard and sample preparation:

2.3.1 0.004 ppm of N-nitroso-ticagrelor preparation:

The first 100 ppm stock of N-nitroso-ticagrelor impurity was made in methanol, and after using this solution, a 0.004 ppm standard solution was prepared in a mixture of water and methanol in the ratio of 20:80 (v/v). The external standard method is used for the quantification of NDSRI impurity.

2.3.2 Test solution preparation:

40000 ppm Ticagrelor API sample prepared in a 5 mL volumetric flask in the same diluent mixture.

2.4 Development and Method validation:

The method trial utilized reverse-phase LC-MS/MS because of its excellent resolution, sensitivity, and consistent reproducibility. The study aimed to develop a quantitative LC-MS/MS technique for measuring N-nitroso-ticagrelor. Initially, different columns, mobile phase compositions, and gradient program durations were screened to optimize the setup. Based on the initial findings, the experiments proceeded using a gradient program with a flow rate of 0.8 mL/min, as detailed in Table 5.

Table 5: Final optimized condition

Chromatography System parameter	
M. Phase A:	0.1 % formic acid in water
M. Phase B:	Methanol
Flow Rate:	0.8 ml/min
Injection Volume:	50 µl

Auto Sampler:	25 °C
Column Temperature:	40 °C
Run Time:	30 minutes
Analytical Column:	Supelco Ascentis express C18 (10cm x 4.6 mm x 2.7µ)
Gradient Program:	

Time (Minute)	Mobile phase-A (%)	Mobile phase-B (%)
0.01	90	10
3.0	75	25
8.0	60	40
15.0	40	60
20.0	15	85
23.0	15	85
23.1	90	10
30.0	90	10

MS/MS System parameter:

MRM

Precursor m/z	Product m/z	Dwell time (msec)	Q1 Pre Bias (V)	CE	Q3 Pre Bias (V)
552.05	371.20	100.0	-20	-20	-18
552.05	183.10	100.0	-20	-30	-19
552.05	141.00	100.0	-20	-41	-14

Interface (Ion source):	ESI
Nebulizing flow:	3.00 L/min.
DL temperature:	280 °C
Heat block temperature:	400 °C
Drying gas flow:	15 L/min
Interface voltage:	4.5 KV
Waste valve programming:	

Time (min)	Command	Value
0.01	FCV	0 (Waste)
15	FCV	1 (MS)
20.5	FCV	0 (Waste)

The analytical method validation was performed following the USP nitrosamine general chapter [24-25] and ICH Q2 [26]. Its appropriateness was confirmed by assessing various parameters, including system suitability, specificity, precision, limit of detection (LOD), limit of quantification (LOQ), accuracy, and linearity.

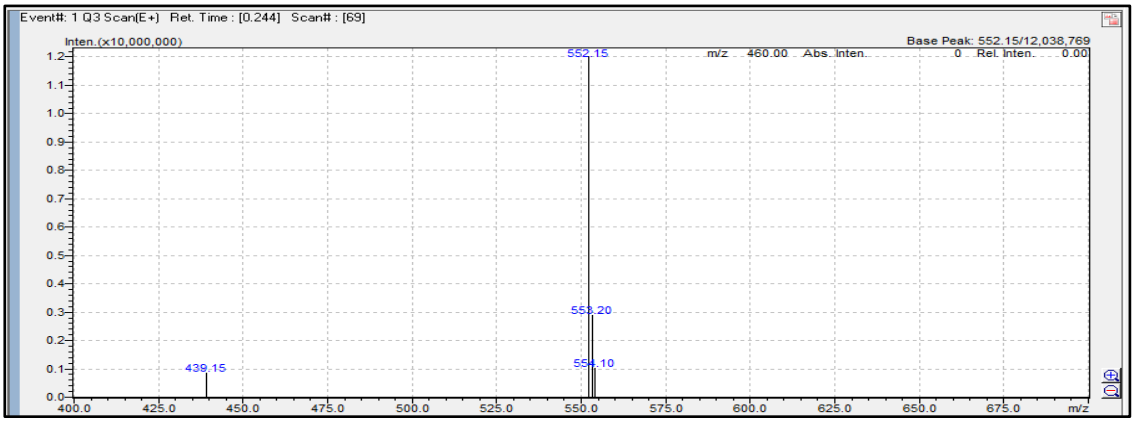


Fig 5: Mass spectra of N-nitroso-ticagrelor in positive mode

3. RESULTS AND DISCUSSION

The current work aimed to validate a method for quantifying N-nitroso-ticagrelor in ticagrelor. The validation was conducted by preparing a standard solution of 0.004 ppm mg/mL of N-nitroso-ticagrelor. The N-nitroso-ticagrelor was quantified with a limit of 0.1 ppm with respect to the sample solution (ticagrelor).

3.1 System Suitability:

The system suitability was assessed by a % RSD of six replicate standard solutions, % RSD observed 4.38 %.

3.2 Specificity (Interference from blank and impurities):

The specificity of this method was proven by injecting the blank solution, standard solution, sample solution (unspiked), and spiked sample solution (at specification level). The acceptance criteria set is that the analyte peak should be well separated from blank peaks. There is no interference observed in the blank and sample solution. Therefore, the method is considered specific. The MRM chromatograms of the blank, standard, sample solution, and spiked sample solution in Figure 6 and in Table 10 represent the % interference.

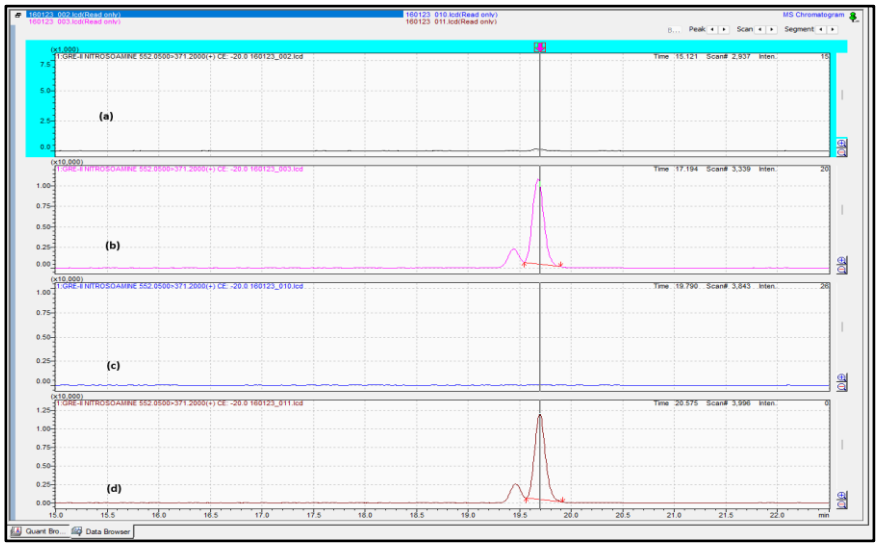


Fig 6: MRM overlay chromatogram of blank solution (a), standard solution (b), and Sample solution (c) and spiked sample solution (d).

3.3 LOD and LOQ Determination and Precision at LOQ Level Solution:

A known concentration solution was diluted from a prepared standard solution, and LOD and LOQ were determined by calculating the signal-to-noise ratio and found to be 107 and 123, respectively. The acceptance criteria set for S/N for LOD is NLT 3 and for LOQ is NLT 10. The LOQ precision observed was below 5% RSD of six replicate injections. The results of LOD & LOQ proved that the method is precise at the LOQ level. The MRM chromatogram of LOD & LOQ is represented in Figures 7 and 8. LOD and LOQ values are tabulated in Table 10.

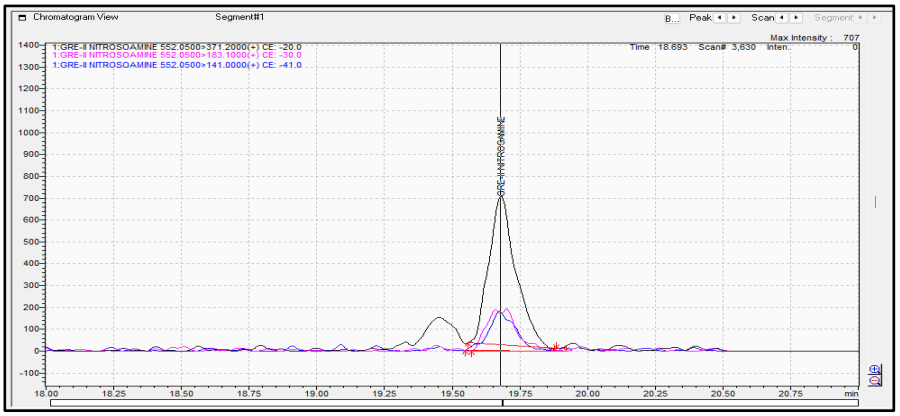


Fig7: MRM chromatogram of LOD solution

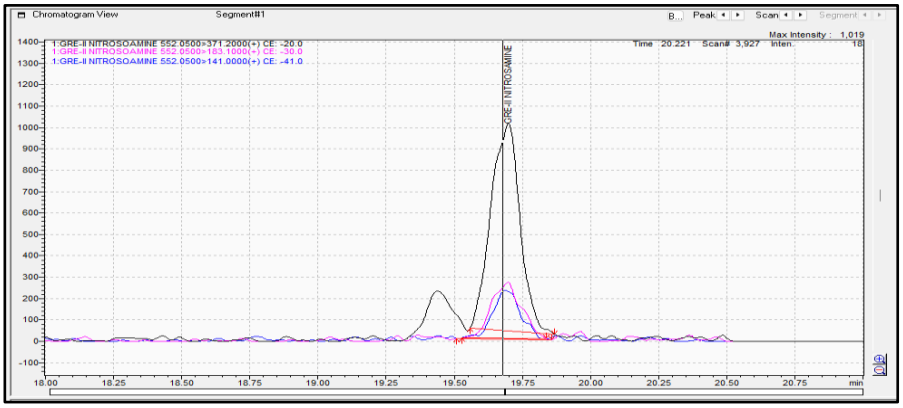


Fig 8: MRM chromatogram of LOQ solution

3.4 Linearity and Range:

Linearity was determined at six levels over the range of LOQ to 150% wrt sample conc. (0.009 ppm to 0.15 ppm). A standard stock solution of analyte peak was prepared and further diluted to attain concentration levels with respect to sample concentration and with respect to specification levels. Each linearity preparation was injected into a single. The area of each level and a graph of area versus concentration in ppm were plotted for N-nitroso-ticagrelor. The correlation coefficient, y-intercept, slope of the regression line, and residual sum of squares were calculated and recorded in Table 6. Figure 9 represents the linearity plot, and Figure 10 represents the overlay.

Table 6 Linearity of N-nitroso-ticagrelor

Linearity Level (%)	Concentration wrt test (ppm) (X-Axis)	Peak area (Y-Axis)
LOQ	0.0093	7560
50%	0.0465	31450
80%	0.0744	52875
100%	0.0930	64151
120%	0.1116	77857
150%	0.1395	110865
Slope of the regression line		**767897.5670**
y-intercept		**-3234.8035**
Residual sum of squares		**5039.1250**
Correlation Coefficient (r)		**0.9922**
r^2		**0.9844**

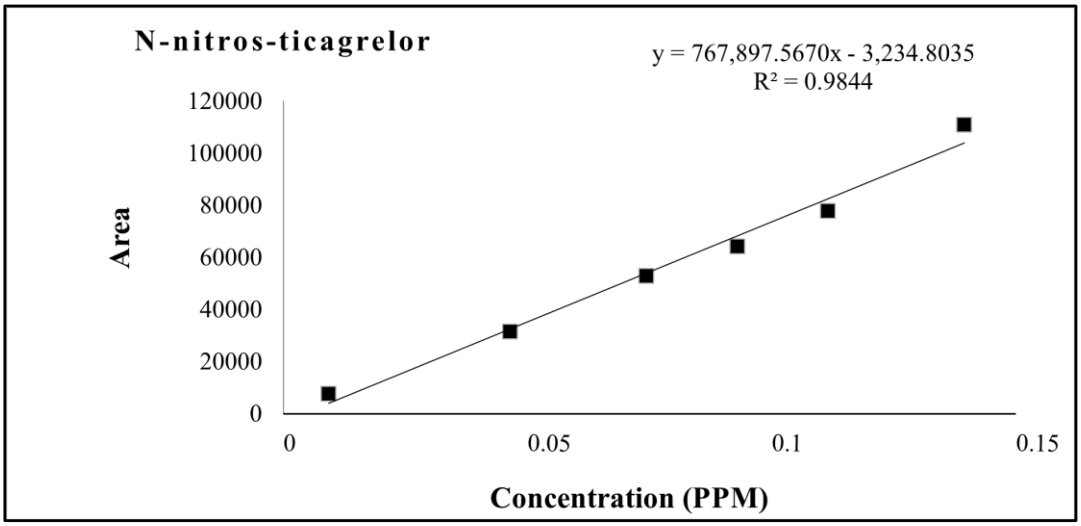


Fig 9 Liearity plot

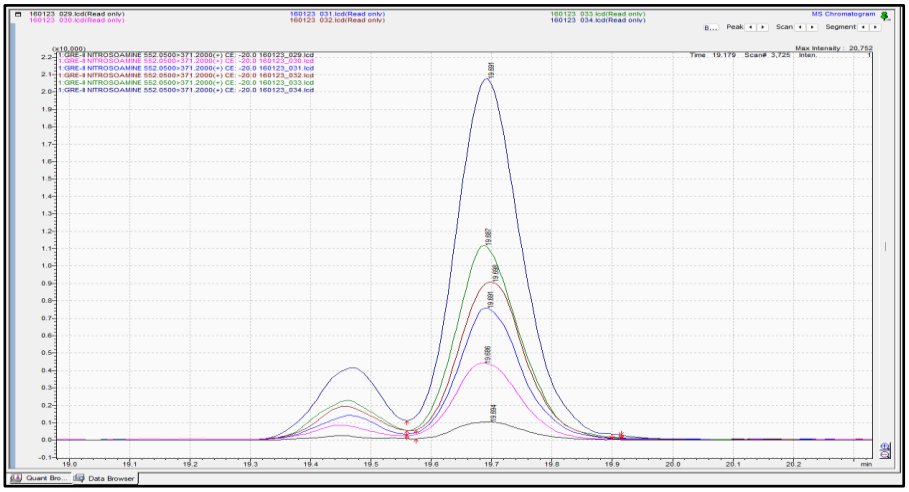


Fig. 10 Overlay MRM Chromatogram of LOQ to 150% Level

3.6 Accuracy (Recovery):

The accuracy of the analytical method for the analyte was established at three levels in the range of LOQ, 100%, and 150% with respect to the specification level of the analyte. Samples were spiked with N-nitroso-ticagrelor at LOQ, 50%, 100%, and 150% levels relative to the sample concentration. Analyze each spiked sample in triplicate. Calculate the percentage recovery and its %RSD for the spiked samples with respect to the standard solution. Three preparations of an accuracy sample shall be made at each concentration level as described. The acceptance criteria are set between 70.0% and 130.0% for all levels. The range of accuracy is 89.68% to 118.0%. The percentage recovery and %RSD values are presented in Table 7. The accuracy results across all concentration levels complied with the acceptance criteria, thereby demonstrating the reliability and accuracy of the method.

Table 7 Recovery of N-nitroso-ticagrelor

% Level	Preparation	Impurity (ppm) Added	Impurity (ppm) Found	Recovery (%)	Mean Recovery (%)	% RSD
LOQ	1	0.0100	0.0114	114.00	116.67	1.98
	2		0.0118	118.00		
	3		0.0118	118.00		
100%	1	0.1025	0.0976	95.22	92.46	2.82
	2		0.0944	92.10		
	3		0.0923	90.05		
150%	1	0.1550	0.1416	91.35	91.50	2.08
	2		0.1449	93.48		
	3		0.1390	89.68		

3.7 Method Precision (Repeatability):

The precision (method precision) was established by analyzing six samples spiked with impurities at the specification level with respect to sample concentration. The N-nitroso-ticagrelor impurity content was calculated in ppm for each individual preparation, and the mean and %RSD were determined and are presented in Table 8. The acceptance criterion for method precision, as per regulatory guidelines, is a recovery range of 70–130%. The %RSD obtained from six replicate preparations was 2.72%, confirming that the method demonstrates acceptable precision.

Table 8 % RSD of Method precision

Sample No.	Impurity found (ppm) N-nitroso-ticagrelor
Set-1	0.0976
Set-2	0.0944
Set-3	0.0923
Set-4	0.0920
Set-5	0.0973
Set-6	0.0924
Mean	**0.0943**
%RSD	**2.72**

3.8 Solution Stability:

Solution stability was evaluated by injecting both the sample solution and the spiked sample solution at the specified concentration level. The solutions were stored in an autosampler at 25 °C and reinjected after 7, 13, and 15 hours. The peak areas of N-nitroso-ticagrelor were compared with the initial values, and area ratios were calculated. As the area ratios at all time points remained within the acceptance range of 0.85–1.15, the solutions were considered stable for up to 15 hours.

Table 9 Spiked sample solution stability data

Time (HR: MIN)	Area of N-nitroso-ticagrelor	Area ratio
Initial	75006	NA
7:08	67636	0.90
13:16	75853	1.01
15:49	80968	1.08

3.8 Batch Analysis:

Four different commercial samples were analyzed using the validated method to quantify N-nitroso-ticagrelor. The impurity was not detected in any of the commercial API samples, remaining well within regulatory limits, thereby confirming that the API is safe for human use.

Table 10. Method validation summary

Parameter	Acceptance criteria	Results
System precision	% content (n = 6, % RSD < 20.0)	% RSD: 4.38
Specificity	Interference from blank and impurities	There is no interference between blank and other impurity peaks with the N-nitroso-ticagrelor peak.
LOD	S/N value (≥3)	107
	LOD in ppm	0.003 ppm
LOQ	S/N value (≥10)	123
	LOQ in ppm	0.009 ppm
Precision	% content (n = 6, % RSD < 20.0)	% RSD: 4.32
Linearity and Range	correlation coefficient (r) (>0.990)	(r): 0.9922 (r^2): 0.9844 Slope: 767897.5670 Intercept: -3234.8035
Accuracy	Between 70.0 % and 130.0 %.	LOQ: 116.67 / % RSD: 1.98% 100 %: 92.46 / % RSD: 2.82% 150 %: 91.50 / % RSD: 2.08%
Method Precision	% content (n = 6, % RSD < 20.0)	% RSD: 2.72
Solution Stability	The area ratio value at all-time intervals should be between 0.80 to 1.20.	Sample & Spiked solution stable up to 15hrs at 25 °C.

6. CONCLUSION

Based on ROS evaluations, it is strongly possible to generate NDSRI impurities due to the use of $NaNO_2$ in the manufacturing process of ticagrelor. Ticagrelor contains a secondary amine functional group, which under nitrosating conditions can react with nitrosating agents to form N-nitroso-ticagrelor, making it a potential N-nitrosamine drug substance-related impurity (NDSRI). This research presents a simple, rapid, and sensitive method for trace-level identification and quantification of N-nitroso-ticagrelor in API. The method was validated as per regulatory guidelines and found within the acceptance criteria. In accordance with regulatory guidelines, although the calculated identification threshold for the NDSRI impurity is 8.33 ppm, a more conservative specification limit of 0.1 ppm was established, based on a worst-case acceptable intake (AI) value of 18 ng/day. The developed method was validated according to ICH guidelines as cost-effective and time-efficient. All validation parameters were within the acceptability limits according to ICH criteria. The quantification of N-nitroso-ticagrelor impurity in the commercial API was found to comply with regulatory guidelines, leading to the conclusion that the API is safe for human consumption.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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REFERENCES:

[1] DrugBank. (2024). Ticagrelor. In *DrugBank Online*. Retrieved from https://go.drugbank.com/drugs/DB08816

[2] Jacobson, K. A., Costanzi, S., Ohno, M., Joshi, B. V., Besada, P., Xu, B., & Tchilibon, S. (2004). Molecular recognition at purine and pyrimidine nucleotide (P2) receptors. *Current topics in medicinal chemistry*, *4*(8), 805-819. https://doi.org/10.2174/1568026043450961

[3] Springthorpe B, Bailey A, Barton P, Birkinshaw TN, Bonnert RV, Brown RC, Chapman D, Dixon J, Guile SD, Humphries RG, Hunt SF, Ince F, Ingall AH, Kirk IP, Leeson PD, Leff P, Lewis RJ, Martin BP, McGinnity DF, Mortimore MP, Paine SW, Pairaudeau G, Patel A, Rigby AJ, Riley RJ, Teobald BJ, Tomlinson W, Webborn PJ, Willis PA: From ATP to AZD6140: the discovery of an orally active reversible P2Y12 receptor antagonist for the prevention of thrombosis. Bioorg Med Chem Lett. 2007 Nov 1;17(21):6013-8. Epub 2007 Aug 19. https://doi.org/10.1016/j.bmcl.2007.07.057

[4] U.S. Food and Drug Administration. (2011). *BRILINTA (ticagrelor) tablets, for oral use: Highlights of prescribing information*. Retrieved from label - Food and Drug Administration

[5] European Medicines Agency. (2024). *Brilique (ticagrelor): Summary of product characteristics*. Retrieved from Brilique, INN-ticagrelor

[6] Teng, R., Oliver, S., Hayes, M. A., & Butler, K. (2010). Absorption, distribution, metabolism, and excretion of ticagrelor in healthy subjects. *Drug Metabolism and Disposition*, *38*(9), 1514–1521. https://doi.org/10.1124/dmd.110.032250

[7] Dorsam, R. T., & Kunapuli, S. P. (2004). Central role of the P2Y12 receptor in platelet activation. *Journal of Clinical Investigation*, *113*(3), 340–345. https://doi.org/10.1172/JCI20986

[8] U.S. Food and Drug Administration. (2019). *Nitrosamine impurities in human drugs*. https://www.fda.gov/regulatory-information/search-fda-guidance-documents/control-nitrosamine-impurities-human-drugs

[9] Cioc, R. C., Joyce, C., Mayr, M., & Bream, R. N. (2023). Formation of N-nitrosamine drug substance related impurities in medicines: A regulatory perspective on risk factors and mitigation strategies. *Organic Process Research & Development*, *27*(7), 1736–1750. https://doi.org/10.1021/acs.oprd.3c00153

[10] European Medicines Agency. (2020). *Nitrosamine impurities*. https://www.ema.europa.eu/en/human-regulatory-overview/post-authorisation/referral-procedures-human-medicines/nitrosamine-impurities

[11] Moorthy, M. K., Ali, S. M., & Reddy, G. V. S. (2022). Development and validation of LC–QTOF–MS/MS method for the identification and determination of low levels of a genotoxic impurity, 4,6-dichloro-5-nitro-2-(propylthio) pyrimidine in ticagrelor API. *Biomedical Chromatography*, *36*(4), e5336. https://doi.org/10.1002/bmc.5336

[12] Shaik, M. A., Moorthy, M. K., Devanna, N., & Gopireddy, S. R. (2024). A novel LC-QTOF-MS/MS method for trace level identification and quantification of potential genotoxic impurity, 5-Nitro-2-(propylthio) pyrimidine-4,6-diol in ticagrelor drug substance. *Analytical Chemistry Letters*, *14*(2), 129–142. https://doi.org/10.1080/22297928.2024.2336018

[13] Kumar, N., Devineni, S. R., Gajjala, P. R., Gupta, D. K., Bhat, S., Kumar, R., & Kumar, P. (2016). Four process-related potential new impurities in ticagrelor: Identification, isolation, characterization using HPLC, LC/ESI–MSn, NMR and their synthesis. *Journal of Pharmaceutical and Biomedical Analysis, 120*, 248–260. https://doi.org/10.1016/j.jpba.2015.12.027

[14] Bueno, L. M., Manoel, J. W., Giordani, C. F. A., Mendez, A. S. L., Volpato, N. M., Schapoval, E. E. S., & Garcia, C. V. (2017). HPLC method for simultaneous analysis of ticagrelor and its organic impurities and identification of two major photodegradation products. *European Journal of Pharmaceutical Sciences, 97*, 22–29. https://doi.org/10.1016/j.ejps.2016.10.018

[15] Wingert, N. R., Ellwanger, J. B., Bueno, L. M., Gobetti, C., Garcia, C. V., Steppe, M., & Schapoval, E. E. (2018). Application of Quality by Design to optimize a stability-indicating LC method for the determination of ticagrelor and its impurities. *European Journal of Pharmaceutical Sciences, 118*, 208–215. https://doi.org/10.1016/j.ejps.2018.03.029

[16] Kelemen, H., Hancu, G., & Papp, L. A. (2019). Analytical methodologies for the determination of ticagrelor. *Biomedical Chromatography, 33*(7), e4528. https://doi.org/10.1002/bmc.4528

[17] Ambasana, M. A., Kapuriya, N. P., Mangtani, K. M., & Ladva, K. D. (2016). An improved assay method for the estimation of ticagrelor hydrochloride by reverse phase liquid chromatography. *International Journal of Pharmaceutical Sciences and Research, 7*(5), 2009–2014. https://doi.org/10.13040/IJPSR.0975-8232.7(5).2009-14

[18] Gobetti, C., Lazzaretti, P. R., Sebastian, L. M. A., & Garcia, V. C. (2014). Determination of the new antiplatelet agent ticagrelor in tablets by stability-indicating HPLC method. Current Pharmaceutical Analysis, 10, 279–283. https://doi.org/10.2174/1573412910666140610211656

[19] Joshy, E., Babu, A., D'Cruz, D., & Aneesh, T. P. (2016). Development and validation of RP- HPLC method for determination of ticagrelor in pharmaceutical dosage formulation. Der Pharmacia Lettre, 8, 206–212.

[20] Kumar, N. A., Swathi, P. R. N., Sharmila, D., Sharmila, S., & Pawar, A. K. M. (2016). A validated stability indicating method of UV-spectrophotometry for the estimation of ticagrelor in bulk & marketed formulation. Der Pharmacia Lettre, 8, 309–315.

[21] Oliveira, S. S., Bitencourt, S. A., Gobetti, C., Mendez, A. S. L., & Garcia, C. V. (2017). Assaying the antiplatelet ticagrelor by validated UV spectrophotometric method with performance equivalent to HPLC. *Current Pharmaceutical Analysis, 13*(5), 538–542. https://doi.org/10.2174/1573412913666170119150043

[22] Pandya, D., Pavel, M., Ghediya, R., Shah, A., & Khunt, R. (2016). UV–vis spectrophotometric assay determination of oral antiplatelet ticagrelor drug in pharmaceutical formulation: Application to content uniformity. Journal of Chemical and Pharmaceutical Research, 8, 316–321.

[23] European Medicines Agency. (2023). Appendix 1: Acceptable intakes established for N-nitrosamines. https://www.ema.europa.eu/en/documents/other/appendix-1-acceptable-intakes-established-n-nitrosamines_en.xlsx

[24] United States Pharmacopeial Convention. (2021). *General Chapter <1469> Nitrosamine impurities*. Rockville, MD: United States Pharmacopeial Convention. https://www.usp.org/sites/default/files/usp/document/stakeholder-forum/pnp/highlights-of-1469-nitrosamine-impurities.pdf

[25] United States Pharmacopeial Convention. (2023). *Validation of compendial procedures <1225>*. In *USP–NF*. Rockville, MD: United States Pharmacopeial Convention. https://doi.org/10.31003/USPNF_M99945_04_01

[26] International Council for Harmonisation. (2005). *ICH Q2(R1): Validation of analytical procedures: Text and methodology*. Geneva: ICH Secretariat. Retrieved from https://www.fda.gov/media/152208/download

[27] Shakleya, D., Asmelash, B., Alayoubi, A., Abrigo, N., Mohammad, A., Wang, J., ... & Keire, D. (2023). Bumetanide as a model NDSRI substrate: N-nitrosobumetanide impurity formation and its inhibition in bumetanide tablets. *Journal of Pharmaceutical Sciences*, *112*(12), 3075-3087.

[28] Nakka, S., Muchakayala, S. K., & Surya, S. B. M. (2024). A novel and eco-friendly UPLC-ESI-MS method for the quantification of Aceclofenac-NDSRI (Nitroso Drug Substance Related Impurity) from Aceclofenac drug substance and combination formulations. Sustainable Chemistry and Pharmacy, 38, 101495.

[29] Nakka, S., Katari, N. K., Muchakayala, S. K., Jonnalagadda, S. B., & Manabolu Surya, S. B. (2024). Synthesis and Trace-Level Quantification of Mutagenic and Cohort-of-Concern Ciprofloxacin Nitroso Drug Substance-Related Impurities (NDSRIs) and Other Nitroso Impurities Using UPLC-ESI-MS/MS─ Method Optimization Using I-Optimal Mixture Design. *ACS omega*, *9*(8), 8773-8788.

[30] Zheng, J., Radich, C. L., Gong, X., Liang, X., & Mowery, M. D. (2024). A practical HPLC-MS method for the analysis of nitrosamine drug substance related impurities using an inexpensive single quadrupole mass spectrometer. *Journal of Chromatography A*, *1736*, 465399.

[31] Nakka, S., Muchakayala, S. K., & Surya, S. B. M. (2025). A facile and eco-friendly simultaneous quantification LC-TQ-MS/MS approach for N-Nitroso Moxifloxacin and di-nitroso pyrrolopiperidine in Moxifloxacin tablets and eye drops. *Green Analytical Chemistry*, *12*, 100188.

[32] Bessa-Jambrina, S., Marlés-Torres, A., & Galán-Rodríguez, C. (2024). Ultra-high-performance liquid chromatography-tandem mass spectrometry analytical method for the determination of nitrosamine drug substance-related impurity in sitagliptin base and salts. *Separation Science Plus*, *7*(4), 2400003.

[33] Marlés-Torres, A., López-García, R., Bessa-Jambrina, S., & Galán-Rodríguez, C. (2023). Ultra-high-speed liquid chromatography combined with mass spectrometry detection analytical methods for the determination of nitrosamine drug substance-related impurities. *Journal of Separation Science*, *46*(13), 2300125.

[34] Bhimireddy, A., Kumar, J. S., Chittireddy, H. P. R., Shaik, M. R., Assal, M. E., & Khan, M. (2024). Safety and human health: The landscape of an effective UPLC-MS/MS method for the identification and quantification of N-Nitroso Hydrochlorothiazide impurity in Hydrochlorothiazide. *Journal of King Saud University-Science*, *36*(9), 103349.

[35] Birudukota, S., Mangalapu, B., Ramakrishna, R. A., & Halder, S. (2025). Development and application of an LC-MS/MS method for the detection of N-nitrosochlordiazepoxide as a potential genotoxic impurity. *Journal of Applied Pharmaceutical Science*, *15*(5), 242-253.

[36] Vadariya, S., Patel, J., Patel, H., & Hirpara, H. (2025). Development and Validation of Analytical Methods for the Quantification and Identification of N-Nitroso Trimetazidine, a Nitrosamine Impurity, in Drug Substance and Drug Product. *Development*, *23*, 2.

[37] Vadariya, S., Patel, J., Patel, H., Hirpara, H., & Patel, T. (2025). Development and Validation Of N-Nitroso Atomoxetine Impurity (Ndsri) Determination Method In Atomoxetine Hydrochloride With Uflc-Ms/Ms (Esi). South Eastern European Journal of Public Health, 2238–2248. https://doi.org/10.70135/seejph.vi.5447