Method Development And Validationfordetermination Of N-Nitroso Ranolazine In Ranolazine Prolonged Release (Pr) Tablets 750 MG BY LC-MS/MS

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ABSTRACT
This work offers a novel method for determining several genotoxic contaminants in ranolazine active pharmaceutical ingredient (API) at the same time with a single LC-MS/MS method. Although the majority of the literature currently in publication concentrates on the identification of specific impurities, our approach provides a thorough solution for concurrent analysis, increasing productivity and efficiency in pharmaceutical manufacturing processes. Critical parameters, such as sensitivity, repeatability, linearity, recovery, and robustness, were determined by thorough experimentation and adherence to ICH standards to evaluate the process's performance. For each of the five contaminants, the calculated Limit of Detection (LOD) and Limit of Quantification (LOQ) values show remarkable sensitivity and accuracy, enabling the detection of minuscule quantities. All things considered, our proven approach shows excellent dependability and appropriateness for regular production operations, supporting the development of strong safety standards in the pharmaceutical industry.

Keywords: genotoxic impurities, LC-MS/MS, pharmaceutical manufacture, validation of methods, sensitivity, reproducibility, limit of quantification, limit of detection, and safety protocols.

1. INTRODUCTION

The most prevalent sign of ischemic heart disease in developed nations is chronic stable angina, which is linked to a lower quality of life and a higher death rate. RS-43285; (6)-N (2,6-dimethyl-phenyl)-4[2-hydroxy-3(2-methoxy-phenoxy) propyl] 1-piperazine acetamide] is the piperazine derivative known as ranolazine, or Ranexa. For individuals with stable or poorly managed chronic angina pectoris who are not responding to other medications, ranolazine is used as a second-line treatment. [1]Patented in 1986 and given FDA approval in 2006, this non-hemodynamic anti-angina medication is prescribed in the US, Japan, and certain European nations. Nevertheless, ranolazine may also be helpful in treating a number of other cardiovascular pathologies, such as diabetes, metabolic disorders, post-operative, new-onset, paroxysmal, and chronic atrial fibrillation (AF), ventricular arrhythmias, revascularization, coronary artery disease, diastolic and microvascular dysfunction, and VA. Ranolazine's primary mode of action is to block late INa, which keeps the cell from going into sodium excess. Ranolazine thereby inhibits the reverse mode sodium-calcium exchange, which in turn avoids the diastolic build-up of calcium and may enhance both diastolic tone and coronary blood flow. Consequently, it has been demonstrated that ranolazine reduces post-ischemic contracture in isolated perfused rabbit hearts that have undergone ischemia and reperfusion. [2]
Pharmaceutical impurities are undesirable substances that are left behind after the active ingredients in a pharmaceutical are synthesised or obtained from sources such as catalysts, reagents, starting materials, intermediates, reagents, solvents, reaction by-products, and degradation products of the active pharmaceutical ingredient (API). Impurities with the potential to interact with genetic material and cause harm or modification to DNA are known as potential genotoxic impurities, or PGIs. These PGIs have been shown through toxicological evaluation to be genotoxic, with documented carcinogenicity. A number of regulatory bodies have published guidelines about regulatory matters pertaining to the presence of genotoxic impurities in new drug substances and products. These organisations include the United States Food and Drug Administration (USFDA) [3], the European Medicines Agency (EMEA) [3], and, most recently, the International Council of Harmonisation of Technical Requirements for Pharmaceuticals Use (ICH) [4]. A threshold exposure level of 1.5 µg/day was also determined, which is referred to as the threshold of toxicological concern. To determine if a pharmacological product is safe for human

consumption, it is crucial to measure these genotoxic contaminants at trace levels, which calls for the use of extremely sensitive analytical techniques like LC-MS/MS.

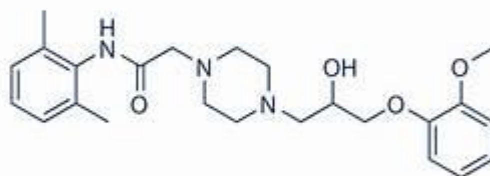


Fig 1: structure of ranolazine.

Chemical names : (±)-N- (2, 6-dimethylphenyl)-4-[2- hydroxy-3-(2-methoxyphenoxy) propyl]-1-piperazine acetamide
Molecular formula : $C_{24}H_{33}N_3O_4$
Molecular weight : 427.54
CAS No : 142387-99-3
Therapeutic category : Anti Angina
Indication : Chronic angina
The well-tolerated drug ranolazine, a piperazine derivative marketed under the brand name Ranexa, specifically blocks the late sodium current.[5] As a second-line treatment for chronic stable angina pectoris, ranolazine is currently approved in both the US and Europe (CSAP) Additionally, despite receiving maximum doses of amlodipine, one trial showed that ranolazine was an effective anti-anginal treatment for patients with stable coronary artery disease. Additionally, in people taking atenolol, amlodipine, or diltiazem concurrently, ranolazine reduces angina attacks and increases exercise tolerance. Heart ischemia is caused by calcium excess, which is indirectly avoided by ranolazine. [6]The current study aims to identify five possible genotoxic contaminants found at trace levels in the medication ranolazine. Many chromatographic methods that are stability indicators for the drug substance and formulation of ranolazine were described during an extensive literature review for analytical techniques for the identification of contaminants in ranolazine [7]. Numerous techniques for determining ranolazine in tablet and bulk dose form were published [8], and a few LC-MS/MS techniques were published for determining ranolazine in biological matrices. The concentration of a single genotoxic contaminant, 2-[(2-methoxy phenoxy) methyl] oxirane, in the drug ingredient ranolazine was determined using a single LC-MS/MS approach [9].
Regarding the LC-MS/MS approach for the simultaneous determination of five genotoxic contaminants in ranolazine, there were no published data available. However, the potential genotoxic impurities were acquired based on the findings of computational structural analysis for mutagenicity alerts. GTI concentration in ranolazine must be regulated at concentrations less than 0.5 ppm, taking into account the maximum permissible dosage. [10] This paper describes the development of a highly sensitive LCMSMS method for the detection of all five potentially genotoxic impurities in ranolazine, which are shown in Figure 2. These impurities are 2,6,-Dimethyl aniline, Related compound A, ((2,6-Dimethyl)amino carbonyl methyl) chloride, dichloro impurity, and chlorohydrin impurity. In compliance with ICH criteria, the method's specificity, linearity, reproducibility, accuracy, robustness, and limit of detection (LOD) and limit of quantification (LOQ) were all validated.
The structures of related molecule A (d), 2,6,-Dimethyl aniline (b), 2,6-Dimethyl)amino carbonyl methyl) chloride (c), dichloro impurity (f), chlorohydrin impurity (e), and ranolazine API (a) are shown in Figure 2.
John Shebin (2022) A piperazine derivative known as ranolazine (Ranexa) is prescribed as a second-line therapy for individuals with stable or poorly managed chronic angina who are not responding to other medications. Five known ranolazine impurities were synthesised separately, and their spectrum data (IR, NMR, and mass) were used to characterise each of the five impurities. The N-(2,6-dimethylphenyl)-2-(piperazin-1-yl) acetamide, 4-(2-methoxyphenoxy) butane-1,3-diol, 2,2'-(4,4'-(2-hydroxypropane-1,3-diyl) bis(piperazine-4,1-diyl)) bis(N-(2,6- dimethylphenyl) acetamide), 2-((2-methoxyphenoxy) methyl) oxirane and N-(2,6-dimethylphenyl)-2-(4-(2- hydroxy-3-(4-methoxyphenoxy) propyl) piperazin-1-yl)

acetamide were assigned as the structures of all five impurities, respectively. The synthesis and characterisation of these ranolazine impurities are described in the current work.[11]

Chidella (2021) A highly sensitive technology known as liquid chromatography-tandem mass spectrometry (LC-MS/MS) has been developed to determine five possible genotoxic contaminants in the active medicinal component ranolazine simultaneously. Using a Poroshell C18 PFP 150 × 3.0 mm 2.7 µ column, gradient elution, a flow rate of 0.4 ml/min, and an 18-minute run time, chromatographic separation was performed using 0.1% formic acid in water as mobile phase A and 0.1% formic acid in methanol as mobile phase B. Positive mode electrospray ionisation was used to optimise the conditions for mass spectrometry. For each of the five contaminants, the method exhibits good linearity between 0.05 and 5.0 ppm of the ranolazine test concentration. It was found that the correlation coefficient was higher than 0.99. All five impurities showed satisfactory recoveries, falling between 102.9% and 112.3%. A LOQ of 0.15 ppm was attained after the method was validated in accordance with ICH suggested parameters. All five contaminants could be quantified using the described approach at a concentration level of 1 ng/ml (0.5 ppm in relation to 2 mg/ml ranolazine).[12]

Rahman Zubaidur (2022) Chemically, ranolazine hydrochloride (RAN) is a derivative of piperizine that is prescribed as an anti-aginal medication. When myocardial ischemia occurs, ranolazine affects the sodium-dependent calcium channel, which is why it is used to treat it. Heart ischemia is caused by calcium excess, which is indirectly avoided by ranolazine. Chronic angina can be treated with ranolazine hydrochloride. Beta-blockers, nitrates, calcium channel blockers, ACE inhibitors, lipid-lowering therapies, antiplatelet treatment, and angiotensin receptor blockers can all be used with ranolazine. The several analytical techniques that have been published for the determination of ranolazine in synthetic mixtures are represented in this review article. There have been reports on chromatographic techniques such as GC, RP-HPLC, HPTLC, LC-MS, and LC-MS/MS. [13]

Chittireddy, Hari Naga Prasada Reddy (2022) The goal of this study was to create an analytical technique for measuring the potentially genotoxic impurity 7-nitroso-3-(trifluoromethyl)-5,6,7,8-tetrahydro-[1,2,4] triazolo [4,3-a] pyrazine (7-nitroso impurity). Given that sitagliptin is an anti-diabetic drug used to treat type 2 diabetes and that the treatment is meant to be long-term, it is necessary to manage the nitroso impurity content using appropriate methods. Ultraperformance liquid chromatography with triple quadrupole mass spectrometry (UHPLC-MS/MS) is a very sensitive and repeatable technology that was developed to quantify this contaminant. At about 40 °C in the oven, the analysis was carried out using a Kromasil-100 and a C18 column (100 mm × 4.6 mm, 3.5 µm particle size). With methanol serving as mobile phases A and B and 0.12% formic acid in water as the mobile phase, the flow rate was adjusted to 0.6 mL/min. Current International Council for Harmonization (ICH) requirements for permissible limits, specificity, repeatability, accuracy, linearity, precision, ruggedness, and robustness were followed in the validation process. The contaminant could be detected using this approach down to the lowest limit of quantification (LOQ) of 0.005 ppm and the lowest limit of detection (LOD) of 0.002 ppm. The linearity of this approach was observed in the 0.005 to 0.06 ppm range, and the correlation coefficient (R2) squared was found to be greater than 0.99. When sitagliptin drug ingredients and drug products are regularly analyzed, this method may be useful in identifying impurities.[14]

Pantula Srinivas Nagendra (2023) Using a gradient elution mode at 0.4 mL/min flow rate with an MS detector, an LC-MS/MS method was developed for the determination of MP-Epoxypropane. The method used a Hypersil BDS C18 column (125 x 4.6 mm, 3.5 µm) and a mobile phase consisting of 0.2% formic acid in water as mobile phase A, 0.02M ammonium formate in water as mobile phase B, and acetonitrile as mobile phase C. In ESI positive mode, the mass of MP-Epoxypropane was determined to be 180, and its retention time was measured to be 6.11 minutes. System compatibility, Specificity, Linearity, LOD and LOQ, Recovery, Precision, and Range were all verified for the suggested approach. Every parameter was discovered to be within allowable bounds. The range of MP-Epoxypropane's linearity was from LOQ to 150% of the specified amount. The LC-MS/MS approach was suitable for the analysis of MP-Epoxypropane in the drug compound ranolazine, as it was specific, accurate, and precise. [15]

Uppala Ravi (2023) 1-Methoxy-2-(oxiranyl methoxy) benzene (RC-A) and 2-Chloro-N-(2,6-dimethylphenyl) acetamide (RC-B) are produced as intermediates in the ranolazine (RLE) synthesis. RLE's efficacy may be impacted by the presence of RC-A and RC-B impurities. Establishing an LC-MS/MS approach to detect and assess RC-A and RC-B contaminants in RLE samples was the aim of this study. On an X-Select CSH C18 column with gradient elution, the RC-A and RC-B analytical method was established. The mobile phases used in the analysis were 0.1% ammonia (mobile phase A) and methanol (mobile phase B). RC-A (m/z181.1→151.1) and RC-B (m/z198.2→107.1) were analyzed using a mass spectrometer with electrospray ionization operating in the MRM mode. The LC-MS/MS methodology that was suggested demonstrated good linearity (0.251 to 1.128 ppm and 0.258 to 1.141 ppm), good quantitation limits (0.251 ppm and 0.258 ppm) and low detection limits (0.075 ppm and 0.077 ppm) for

RC-A and RC-B, respectively, good system precision (RSD = 0.8% and 2.9%), good method precision (RSD = 1.0% and 1.3%), and acceptable accuracy (94.1-106.0% and 96.2-99.2%). The quality of the RLE sample can be evaluated for the presence of RC-A and RC-B contaminants using the suggested LC-MS/MS approach.[16]

2. MATERIALS AND METHODS

Standards, Samples, and Reagents

Name: Ranolazine Prolonged Release (PR) tablets 750 mg
Batch No./Lot No.: AKA (7966) 001

Reagents & Materials:

Formic Acid: Make: Rankem, Grade: AR, Batch No./Lot No.: R133F23, Validity: 31/10/24
Methanol: Make: Duksan, Grade: LCMS, Batch No./Lot No.: M3E203, Validity: 13/12/24
Water: Make: In-house, Grade: HPLC, Batch No./Lot No.: NA, Validity: NA

Mobile Phase Preparation

Mobile Phase-A:

Prepare Mobile Phase-A by adding 2.0 mL of Formic acid to a 2000 mL reagent bottle containing 2000 mL of water. Mix the solution well by stirring or shaking.

Mobile Phase-B:

In another 2000 mL reagent bottle, mix 1000 mL of Methanol and 1000 mL of Acetonitrile. Then, add 2.0 mL of Formic acid to the mixture and shake it thoroughly until the components are fully combined.

Diluent Solution

For the Diluent Solution, simply use 100% Methanol.

Auto Sampler Rinsing Solution

To prepare the Auto Sampler Rinsing Solution, mix equal parts of Methanol and water (50:50 ratio) in an appropriate reagent bottle. Ensure the solution is well-mixed by shaking or stirring.

Preparation of Standard & Sample Solution

Standard Stock Solution (N-Nitroso Ranolazine):

Take 2.146 mg of the working standard and dissolve it in a 10 mL volumetric flask filled with Methanol. Then, dilute the solution to the mark with Methanol and mix it well.

Standard Solution-1:

Transfer 0.250 mL of the Standard Stock Solution into a 50 mL volumetric flask and fill it up to the mark with Diluent Solution. Ensure thorough mixing.

Standard Solution-2:

Take 0.270 mL of Standard Solution-1 and transfer it into a 100 mL volumetric flask. Fill the flask with Diluent Solution up to the mark and mix it properly.

Standard Solution:

Transfer 2 mL of Standard Solution-2 into a 10 mL volumetric flask and dilute it with Diluent Solution up to the mark. Mix the solution thoroughly.

LOD Solution:

Transfer 0.5 mL of Standard Solution into a 10 mL volumetric flask and fill it up to the mark with Diluent Solution. Make sure to mix the solution well.

Sample Solution:

Weigh 10 mg of Ranolazine powder and add it to a Tarson tube. Then, add 5 mL of Diluent Solution, vortex the mixture for 2 minutes at 3000 rpm, centrifuge it at 14500 RPM at 4°C for 5 minutes, and transfer the supernatant layer into an HPLC vial.

Acceptance Criteria

Ensure that the Relative Standard Deviation (RSD) of response area of N-Nitroso Ranolazine obtained from six replicate injections of Standard Solution and bracketing is not more than 15.0%. Additionally, the Signal to Noise ratio at the Limit of Detection (LOD) level must be greater than or equal to 3:1. There should not be any interference at the retention time of N-Nitroso Ranolazine peak due to Specificity Blank. If any interference is found, it should not be more than the LOD of the peak response of the impurity.

3.RESULTS

Table 1. Results of Prediction of LOD_LOQ for N-Nitroso Ranolazine

Sr. No.	Sample Details	Conc. (PPM)	Conc. (ng/mL)	Area
1.	Standard Solution – 2.5%	0.0000127	0.0127	7604
2.	Standard Solution – 5%	0.0000254	0.0254	12664

3.	Standard Solution – 10%	0.0000509	0.0509	24705
4.	Standard Solution – 20%	0.0001018	0.1018	49428

This table provides data on the concentration of N-Nitroso Ranolazine in standard solutions and the corresponding areas detected by the analytical method. It also includes the calculated limit of detection (LOD) and limit of quantification (LOQ) values, which indicate the lowest concentration of the analyte that can be reliably detected and quantified.

Table 2. Results of determination of LOD_LOQ for N-Nitroso Ranolazine

Sr. No.	**LOD**		**LOQ**	
	Area	**SN Ratio**	**Area**	**SN Ratio**
1	11311	113	22308	175
2	11843	89	23189	156
3	12598	84	23514	204
4	11961	95	23946	164
5	12730	101	23328	220
6	12463	97	23726	217
Average	**12151.0**	**96.5**	**23335.2**	**189.3**
%RSD	**4.46**	**10.44**	**2.45**	**14.71**
LOD & LOQ in PPM w.r.t. sample concentration	**0.012 PPM**		**0.024 PPM**	
LOD & LOQ (ng/mL)	**0.024 ng/mL**		**0.048 ng/mL**	

This table further elaborates on LOD and LOQ determination, providing additional parameters such as signal-to-noise (SN) ratio, average values, and percentage relative standard deviation (%RSD). These values assess the sensitivity and reliability of the analytical method for detecting and quantifying N-Nitroso Ranolazine.

Table 3. Results of Accuracy (Recovery) for N-Nitroso Ranolazine

Concentration Level	**Amount added (ppb)**	**Corrected Amount Recovered (ppb)**	**% Recovery**	**% Mean Recovery**	**%RSD**
LOQ	23.637	23.54	99.59	98.38	1.25
	23.457	22.785	97.14		
	23.459	23.087	98.41		
50%	118.456	104.211	87.97	86.76	1.28
	117.329	101.489	86.50		
	117.496	100.807	85.80		
100%	234.242	195.91	83.64	85.61	4.37
	235.999	196.491	83.26		
	236.269	212.444	89.92		
150%	351.114	294.522	83.88	84.30	1.17
	354.2	302.606	85.43		
	350.94	293.347	83.59		

Here, the accuracy of the analytical method is evaluated through recovery experiments conducted at different concentration levels of N-Nitroso Ranolazine. The table presents the amount added, corrected amount recovered, % recovery, % mean recovery, and %RSD, providing insights into the method's accuracy and precision.

Table 4. Results of Sample solution of method precision for N-Nitroso Ranolazine

Sr. No.	**Impurity (PPB)**	**%Recovery**
	100%	
1	195.91	83.64

2	196.491	83.26
3	212.444	89.92
4	192.935	81.96
5	196.062	83.13
6	203.735	86.48
Average	**199.596**	**84.732**
%RSD	**3.63**	**3.48**

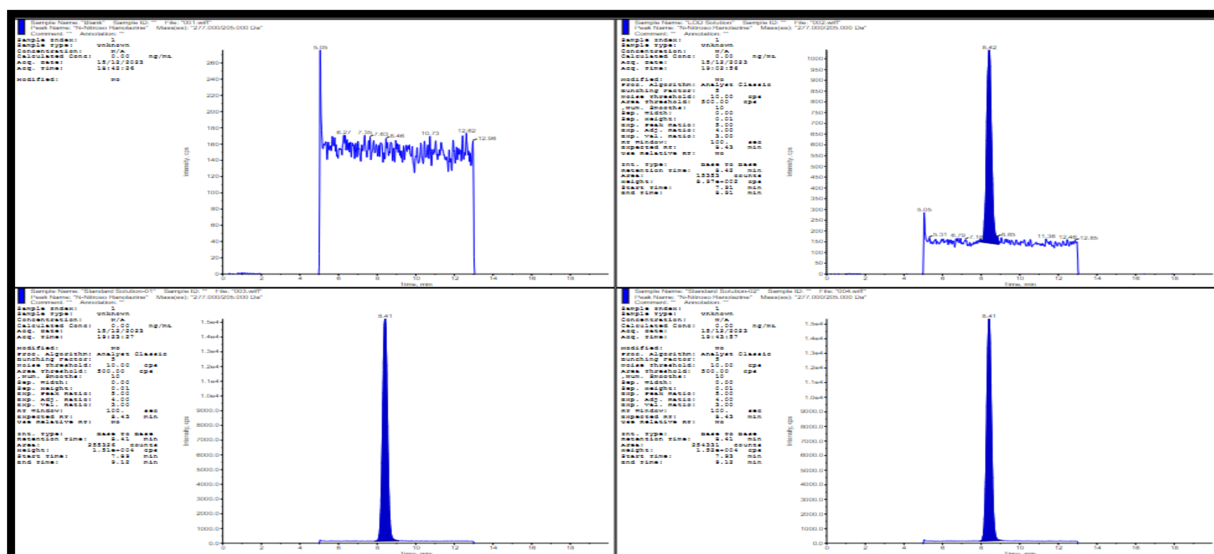
This table assesses the precision of the method by analysing sample solutions containing known impurity levels of N-Nitroso Ranolazine. It presents % recovery values at different impurity levels, along with the average and %RSD, indicating the method's precision and reproducibility.

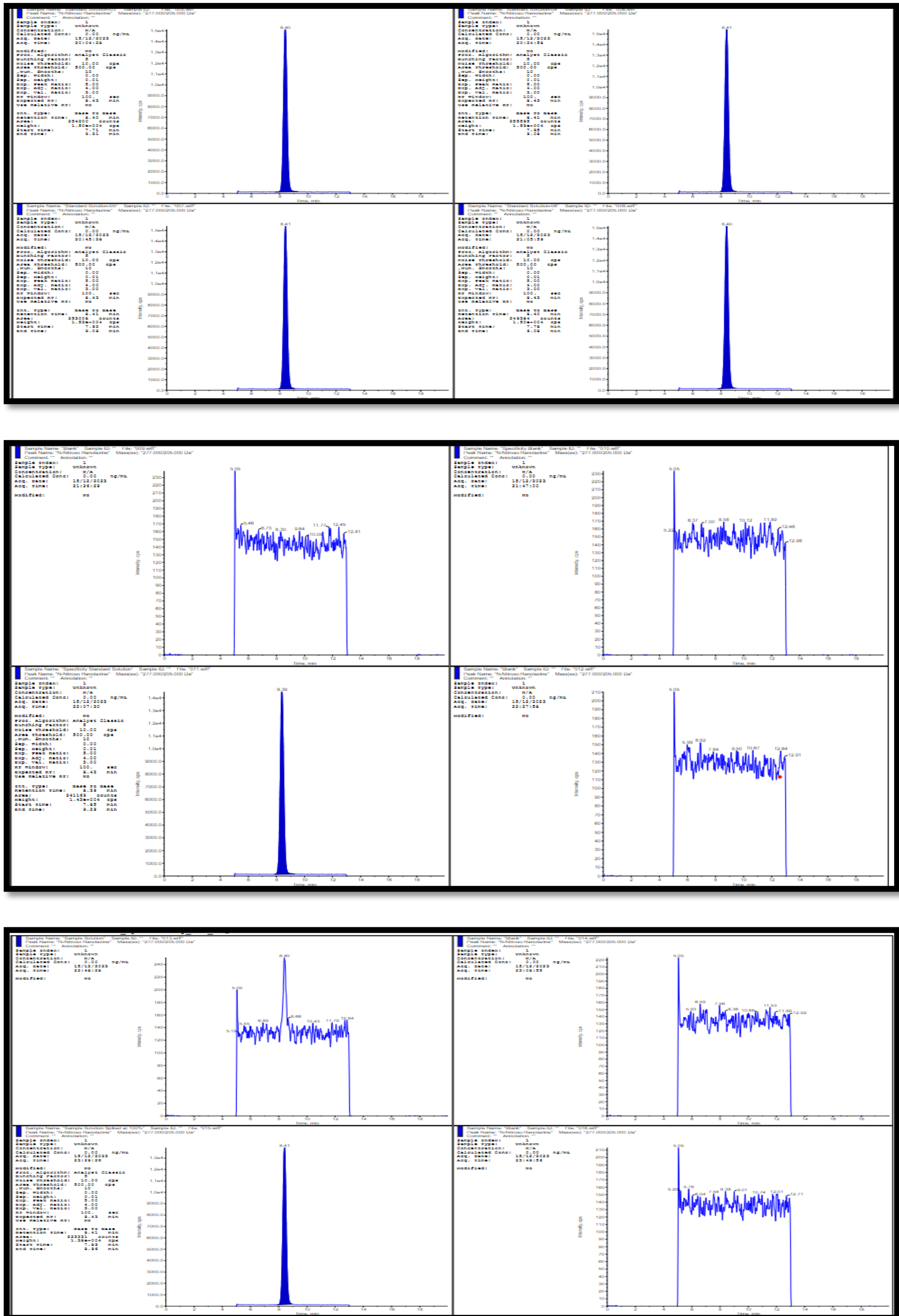
Table 5. Results of Standard solution for N-Nitroso Ranolazine

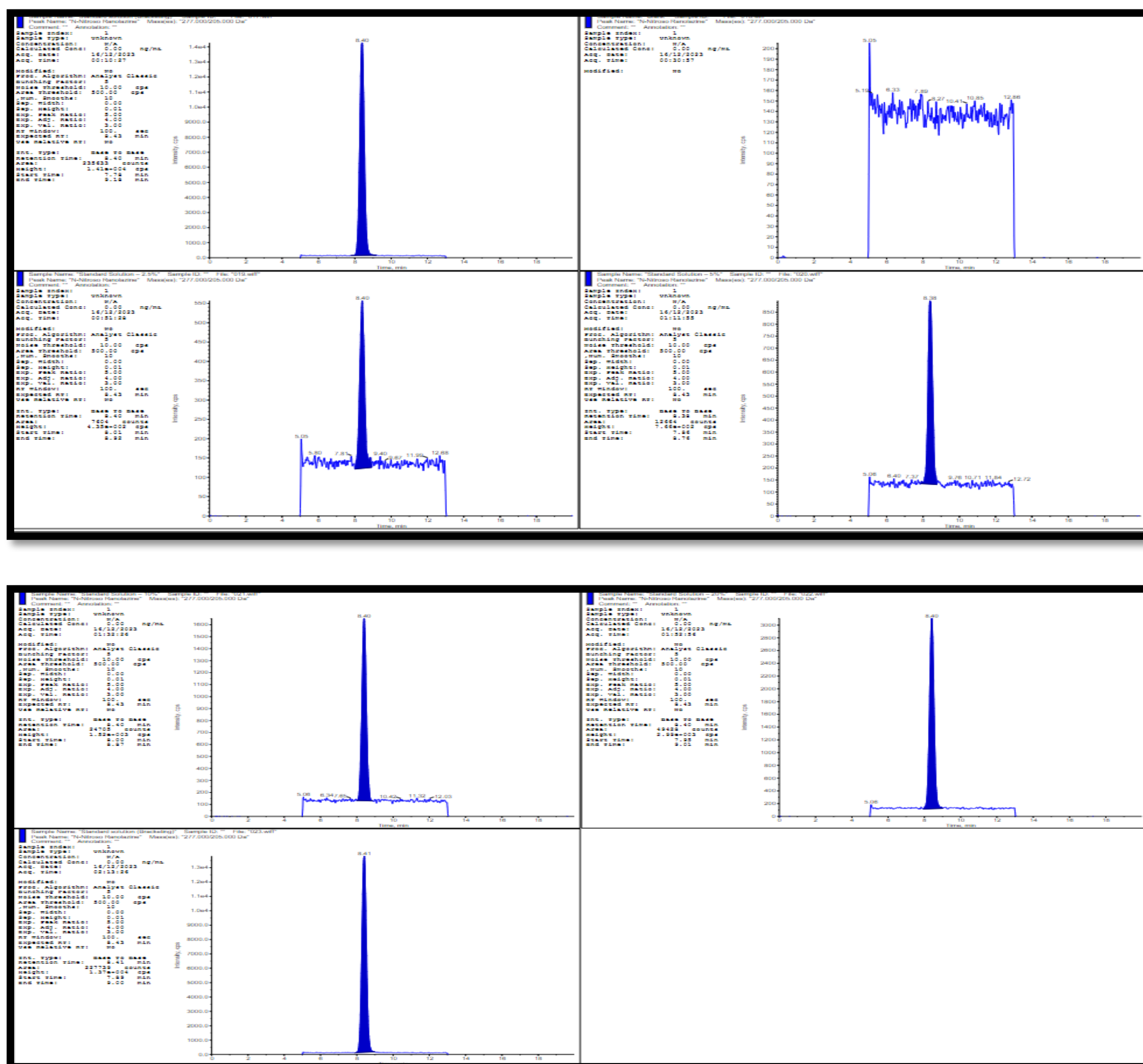
Sr. No.	**Flow Rate**		**Column Oven Temperature**		**Formic acid conc. in Mobile phase A**		**Gradient Composition (Mobile phase A)**	
	(-10%)	**(+10%)**	**(-5°C)**	**(+5°C)**	**(-10%)**	**(+10%)**	**(-2%)**	**(+2%)**
	Area							
1	226998	194506	237866	225481	212105	230160	199023	211534
2	216006	194553	238367	228466	208237	225574	198593	216177
3	215966	199218	230180	228471	211318	212096	194537	217303
4	223710	194519	229367	227714	210639	206702	192977	220266
5	223385	198813	229242	217996	209106	214136	186096	228967
6	223779	199286	225444	216636	212248	207094	183875	223812
Average	221640.7	196815.8	231744.3	224127.3	210608.8	215960.3	192516.8	219676.5
%RSD	2.06	1.28	2.25	2.41	0.77	4.52	3.28	2.79
Bracketing	224961	185668	224750	213676	193664	204897	163504	214070
Average	222115.0	230745.1	230745.1	222634.3	208188.1	214379.9	188372.1	218875.6
%RSD	1.96	2.46	2.36	2.84	3.16	4.59	6.58	2.73

This table presents data on the analysis of standard solutions of N-Nitroso Ranolazine under different experimental conditions, such as variations in flow rate, column oven temperature, and mobile phase composition. It includes the resulting areas detected by the analytical method, along with average values and %RSD, providing insights into the method's robustness and suitability for routine analysis.

Specificity_Lod_Loq Of Ranolazine







4.DISCUSSIONS

These tables collectively provide a comprehensive overview of the analytical methodology employed for the detection and quantification of N-Nitroso Ranolazine, a critical step in pharmaceutical analysis. [17]Table 1 outlines the prediction of the Limit of Detection (LOD) and Limit of Quantification (LOQ) for N-Nitroso Ranolazine. It presents data on standard solutions with varying concentrations and their corresponding peak areas detected by the analytical method. This table serves as the foundation for determining the sensitivity of the method in detecting trace amounts of the compound.[18] Building upon Table 1, Table 2 delves deeper into LOD and LOQ determination, offering additional parameters such as signal-to-noise (SN) ratio, average values, and percentage relative standard deviation (%RSD). These metrics are vital for assessing the robustness and reliability of the analytical method in quantifying N-Nitroso Ranolazine within samples.Table 3 shifts focus to the accuracy of the analytical method, presenting results from recovery experiments conducted at different concentration levels of N-Nitroso Ranolazine. This table evaluates the method's precision and accuracy by comparing the amount added with the corrected amount recovered, expressed as a percentage, alongside % mean recovery and %RSD values. [19]Furthermore, Table 4 assesses the method precision through the analysis of sample solutions containing known impurity levels of N-Nitroso Ranolazine. It presents % recovery values at various impurity levels, alongside average and %RSD values, demonstrating the method's reproducibility and reliability in quantifying impurities.Finally, Table 5 provides insights into the method's robustness by analysing the effects of variations in parameters such as flow rate, column oven temperature, and mobile phase composition on peak area measurements. This evaluation of robustness ensures the method's

consistency and suitability for routine analysis under different conditions.Together, these tables offer a comprehensive evaluation of the analytical method for N-Nitroso Ranolazine, covering aspects of sensitivity, accuracy, precision, and robustness crucial for its application in pharmaceutical analysis.[20]

5.CONCLUSIONS

The work presented in this study introduces a novel approach for the simultaneous determination of multiple genotoxic impurities in ranolazine active pharmaceutical ingredient using a single LC-MS/MS method. Unlike existing literature, which primarily focuses on individual impurity determination, our method offers a comprehensive solution for the concurrent analysis of multiple impurities. Through rigorous experimentation and adherence to ICH recommendations, we established critical parameters to assess the method's performance and conducted thorough validation.The determined Limit of Detection (LOD) and Limit of Quantification (LOQ) values for all five impurities signify the method's exceptional sensitivity, enabling the detection of trace amounts with high precision. Moreover, our method demonstrates excellent reproducibility, linearity, recovery, and robustness, as validated through extensive experiments.The implications of our findings are significant, particularly in the context of routine manufacturing processes. By streamlining impurity analysis into a single method, we can significantly enhance throughput and efficiency while ensuring the safety of the active pharmaceutical ingredient. Ultimately, our method holds promise for accelerating quality control procedures and contributing to the establishment of robust safety protocols in pharmaceutical manufacturing.

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