RESEARCH PAPER

Development and Validation of a Liquid Chromatography-Tandem Mass Spectrometry Method for the Simultaneous Determination of N-Nitroso-Hydrochlorothiazide and N-Nitroso-Bisoprolol in Bisoprolol Hydrochlorothiazide Fixed-Dose Combination Tablets

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Authors' Contribution Statement

Kiran Kumar Kurella: Investigation, method development, experimental execution, optimization of chromatographic and mass spectrometric parameters, formal analysis of analytical data.

Anima S Dadhich: Conceptualization, supervision of method validation studies, interpretation of analytical results, writing of original manuscript draft, critical review and editing of final manuscript for scientific accuracy and clarity.
Conflict of interest: None

Abstract
Nitrosamine impurities in pharmaceutical products pose serious carcinogenic risks requiring stringent analytical control. This work describes a comprehensive validated liquid chromatography-tandem mass spectrometry method for simultaneous quantification of N-nitroso-hydrochlorothiazide and N-nitroso-bisoprolol in fixed-dose combination tablets. Chromatographic separation utilized a Kinetex Biphenyl column with gradient elution combining 5 mM ammonium formate with 0.1% formic acid in water and 0.1% formic acid in methanol. Mass spectrometric detection employed multiple reaction monitoring with dual polarity electrospray ionization (positive mode for N-nitroso-bisoprolol m/z 377.10 → 275.15; negative mode for N-nitroso-hydrochlorothiazide m/z 325.00 → 293.95). Validation following ICH Q2(R2) guidelines demonstrated excellent performance: limits of quantification at 798 ppm and 15 ppm respectively (approximately 10% of specification limits), linearity with correlation coefficients exceeding 0.999, mean recoveries of 100.9% ± 2.2% and 100.8% ± 2.5%, precision with relative standard deviation values below 3.7%, and robust performance under deliberately varied conditions. This validated method provides a reliable analytical tool for pharmaceutical quality control and regulatory compliance with European Medicines Agency and Food and Drug Administration standards for nitrosamine impurity monitoring.

Keywords: nitrosamine impurities, liquid chromatography-tandem mass spectrometry, bisoprolol hydrochlorothiazide, method validation, pharmaceutical quality control

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1. Introduction

N-nitroso compounds represent critical pharmaceutical impurities of significant public health concern following their discovery in valsartan products during 2018[1][2]. These compounds exhibit probable human carcinogenic and genotoxic properties, capable of inducing DNA damage at trace concentrations [3]. International regulatory authorities including the European Medicines Agency and U.S. Food and Drug Administration have established stringent acceptable intake limits based on toxicological assessments [4][5]. For N-nitroso-hydrochlorothiazide, the acceptable intake is 50 µg/day, while N-nitroso-bisoprolol has a limit of 1.5 µg/day [6], reflecting differences in carcinogenic potency. These values translate to specification limits of 8000 ppm for N-nitroso-hydrochlorothiazide and 150 ppm for N-nitroso-bisoprolol when calculated against maximum daily doses. Bisoprolol hydrochlorothiazide represents a widely prescribed cardiovascular combination containing a selective β_1-adrenergic receptor antagonist and thiazide diuretic. Both active ingredients possess secondary or tertiary amine functional groups susceptible to nitrosation reactions [7]. Formation of N-nitroso compounds occurs through reaction with nitrosating agents under acidic conditions, originating from residual nitrites in starting materials, nitrogen oxide contamination, catalytic synthetic byproducts, or degradation of nitrate-containing excipients [8]. The kinetics depend on pH (optimal at pH 3-5), temperature,

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reactant concentrations, and presence of catalytic species [9]. Consequently, nitrosamine formation may occur during active ingredient synthesis, formulation operations, tablet compression, or long-term storage under elevated temperatures and humidity. Previous analytical methodologies for bisoprolol hydrochlorothiazide focused primarily on active ingredient quantification using high-performance liquid chromatography with ultraviolet or evaporative light-scattering detection [10][11]. These conventional approaches lack sufficient sensitivity and selectivity for reliable nitrosamine determination at regulatory specification levels. Liquid chromatography-tandem mass spectrometry has emerged as the gold standard for N-nitroso analysis, offering unparalleled selectivity through multiple reaction monitoring and extraordinary sensitivity through optimized ionization conditions [12][13]. However, simultaneous determination of two structurally dissimilar N-nitroso compounds within a single analytical run presents unique technical challenges. N-nitroso-hydrochlorothiazide and N-nitroso-bisoprolol differ substantially in molecular weight, ionization behavior, hydrophobicity, and fragmentation patterns, necessitating comprehensive optimization of both chromatographic separation and mass spectrometric parameters [14][15].This investigation aimed to develop and comprehensively validate a liquid chromatography-tandem mass spectrometry method for accurate, precise, and reliable determination of both nitrosamine impurities in bisoprolol hydrochlorothiazide tablets at regulatory-compliant levels. The method serves dual purposes: routine quality control batch release testing ensuring specification compliance, and investigational applications during stability studies. Comprehensive validation was performed according to ICH Q2(R2) guidelines for analytical procedure validation [16] and ICH M7(R1) recommendations for assessment and control of DNA-reactive mutagenic impurities [17]. The resulting analytical procedure demonstrates exceptional performance characteristics, positioning it as a practical, reliable tool for routine pharmaceutical quality assurance supporting international regulatory standards[18].

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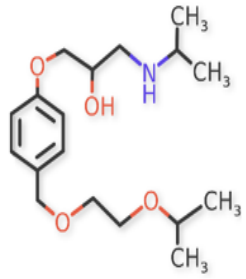
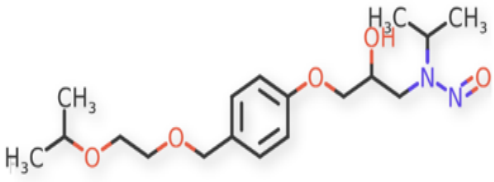
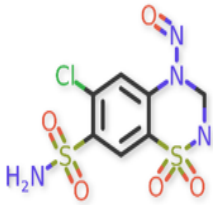
Figure 1: Structure of Bisoprolol, N-nitroso-hydrochlorothiazide and N-nitroso-bisoprolol



Bisoprolol



NNO-Bisoprolol



N-nitroso-hydrochlorothiazide (NNO-HCTZ)

2. Materials and Methods

2.1 Chemicals and Reagents

All chemicals and solvents were LC-MS grade, procured from established commercial suppliers, and stored according to manufacturer specifications. Water and methanol (LC-MS grade, Honeywell) were used for mobile phase preparation. Formic acid (LC-MS grade, Biosolve) and ammonium formate (Reagent grade, Sigma-Aldrich) were used in mobile phase preparation. N-Nitroso-bisoprolol and N-nitroso-hydrochlorothiazide reference standards were procured from Synzeal, Ahmedabad, India. All reference standards were stored at 2-8°C in original containers.

Bisoprolol hydrochlorothiazide fixed-dose combination tablets containing 10 mg bisoprolol and 6.25 mg hydrochlorothiazide per tablet were gifted by Aurobindo Pharma, Hyderabad, India. Placebo tablets with identical formulation composition excluding active pharmaceutical ingredients were employed for specificity evaluation and accuracy studies.

2.2 Instrumentation

A Shimadzu 8060NX LC-MS/MS system coupled with Nexera X3 liquid chromatography module was used. The system comprised automated sample injection with temperature-controlled autosampler maintained at 5°C, quaternary gradient pump capable of precise flow rate

delivery from 0.1 to 2.0 mL/min, thermostat column oven with stability of ±0.1°C, and electrospray

ionization source with dual polarity capability. Chromatographic separation utilized a Kinetex Biphenyl 100Å column (250 mm × 4.6 mm × 5 µm particle size, Phenomenex, USA). Auxiliary laboratory equipment included analytical balance (Mettler Toledo), micropipettes (Eppendorf, 10-1000 µL range), centrifuge (Eppendorf), and vortex mixer (Neuation).

2.3 Chromatographic Conditions

The column was maintained at 40°C with a flow rate of 0.4 mL/min and injection volume of 2 µL. Mobile phase A consisted of 5 mM ammonium formate with 0.1% formic acid in water, prepared by dissolving 0.315 g ammonium formate in 1000 mL water, adding 1.0 mL formic acid, followed by filtration through 0.2 µm PVDF membrane. Mobile phase B consisted of 0.1% formic acid in methanol, prepared by transferring 1.0 mL formic acid into 1000 mL methanol with filtration through 0.2 µm PVDF membrane.

The gradient elution program was optimized as follows: 0-5.0 min (95% A, 5% B), 5.1 min (50% A, 50% B), 5.1-12.0 min (50% A, 50% B), 12.0-25.0 min (linear gradient to 10% A, 90% B), 25.0-30.0 min (10% A, 90% B), 30.1 min (95% A, 5% B), 30.1-35.0 min (95% A, 5% B) for column re-equilibration. The extended initial isocratic period ensures baseline stabilization between successive injections, while the gradient to high organic content facilitates complete elution of hydrophobic matrix components.

2.4 Mass Spectrometry Conditions

Electrospray ionization with dual polarity capability was employed. Interface temperature was set at 300°C, desolvation temperature at 526°C, and drying line temperature at 270°C. Nebulizing gas flow was 3.00 L/min, heating gas flow 10.00 L/min, and drying gas flow 10.00 L/min. Heat block temperature was maintained at 270°C. Interface voltage was 1.00 kV with focus voltage at +3.00 kV for positive mode and -3.00 kV for negative mode. Multiple reaction monitoring acquisition mode was used with collision-induced dissociation gas pressure at 270 kPa and dwell time of 200 ms per transition.

For N-nitroso-bisoprolol in positive ionization mode, the quantifier transition was m/z 377.10 → 275.15 with collision energy of -22 eV, and qualifier transition was m/z 377.10 → 246.10 with collision energy of -18 eV. For N-nitroso-hydrochlorothiazide in negative ionization mode, the quantifier transition was m/z 325.00 → 293.95 with collision energy of 24 eV.

A divert valve program was implemented to protect the mass spectrometry source from contamination: waste diversion 0-15.0 min, mass spectrometry acquisition 15.0-22.0 min, waste diversion 22.0-25.0 min, mass spectrometry acquisition 25.0-33.0 min, waste diversion 33.0-35.0 min.

2.5 Preparation of Solutions

The diluent was prepared by combining 250 mL water with 250 mL methanol and adding 0.5 mL formic acid into a 500 mL volumetric flask. This diluent was used for all calibration standards and test sample dilutions.

For N-nitroso-hydrochlorothiazide, stock solution-1 was prepared at 1000 µg/mL by dissolving 5 mg in 5 mL methanol. Stock solution-2 was prepared at 100 µg/mL by diluting 1.0 mL of stock solution-1 to 10 mL with methanol. For N-nitroso-bisoprolol, stock solution-1 was prepared at 1000 µg/mL by dissolving 5 mg in 5 mL methanol. Stock solution-2 was prepared at 10 µg/mL by diluting 0.1 mL of stock solution-1 to 10 mL with methanol.

A mixed standard stock solution was prepared by transferring 2.52 mL of N-nitroso-hydrochlorothiazide stock solution-2 and 0.765 mL of N-nitroso-bisoprolol stock solution-2 into 10 mL volumetric flask. The standard solution for system suitability and bracketing was prepared by diluting 1.0 mL of mixed standard stock solution to 50 mL with diluent, yielding final concentrations of 8000 ppm N-nitroso-hydrochlorothiazide and 150 ppm N-nitroso-bisoprolol. Calibration standards were prepared at five concentration levels spanning the intended analytical range: 10% (798 ppm and 15 ppm), 50% (3992 ppm and 75 ppm), 100% (7984 ppm and 149 ppm), 125% (9980 ppm and 187 ppm), and 150% (11977 ppm and 224 ppm) of specification limits.

2.6 Sample Preparation

Tablets were crushed to fine powder using mortar and pestle. From this powder, 22.5 mg (calculated to provide 1 mg bisoprolol and 0.625 mg hydrochlorothiazide based on label claim) was transferred to a 15 mL polypropylene centrifuge tube. Exactly 10 mL of diluent was added, and the tube was capped and vortex-mixed at moderate speed for 5 minutes. The suspension was centrifuged at 4000 rpm for 10 minutes at room temperature (20-25°C). The clear supernatant was carefully decanted and filtered through 0.22 µm polytetrafluoroethylene syringe filter into a clean autosampler vial. The filtered sample solution was immediately transferred to the refrigerated autosampler at 5°C.

System suitability criteria required relative standard deviation of peak areas from six consecutive standard injections to be ≤15.0% for both N-nitroso-bisoprolol and N-nitroso-hydrochlorothiazide before proceeding with test sample analysis.

2.7 Validation Strategy

Comprehensive method validation was performed according to ICH Q2(R2) guidelines [16] and ICH M7(R1) recommendations [17]. The following validation parameters were systematically evaluated:

Specificity and Selectivity: Blank diluent and placebo tablet matrix were analyzed to establish free from interference at retention times of target analytes.

Limit of Detection and Limit of Quantification: Detection limit was defined as 3:1 signal-to-noise ratio and quantification limit as 10:1 signal-to-noise ratio.

Linearity and Range: Five calibration levels spanning 10% to 150% of specification limits were prepared. Linear regression analysis evaluated correlation coefficients with acceptance criterion of $r \geq 0.99$.

Accuracy: Three spiked sample levels at 10%, 100%, and 150% of specification limits were prepared with six replicate preparations at each level. Mean percent recovery acceptance range was 80-120% with relative standard deviation ≤20%.

Precision: System precision was evaluated with six consecutive injections of standard solution. Method precision and intermediate precision were evaluated with six independent sample preparations. Acceptance criterion was relative standard deviation ≤15%.

Robustness: Deliberately varied parameters included flow rate ±10% (0.36 and 0.44 mL/min) and column temperature ±5°C (35°C and 45°C).

Solution Stability: Standard and test sample solutions were stored at 2-8°C and analyzed at different time points to evaluate stability

3. Results and Discussion

3.1 Method Development and Optimization

Method development focused on achieving baseline separation of two structurally dissimilar nitrosamine impurities while maintaining sensitivity at regulatory specification levels. The biphenyl stationary phase was selected for its ability to provide π-π interactions and hydrophobic retention, complementing the aromatic and aliphatic structural features of both target analytes. Initial screening of various mobile phase compositions revealed that acidic conditions with formic acid enhanced ionization efficiency for both compounds. The addition of 5 mM ammonium formate in the aqueous mobile phase improved peak shape and provided consistent baseline stability.

Gradient elution optimization was critical for achieving adequate retention and separation. An extended initial isocratic period (0-5.0 min at 95% aqueous) allowed complete baseline stabilization and ensured reproducible retention times between injections. The rapid increase to 50% organic content at 5.1 minutes-initiated mobilization of more polar impurities, while the subsequent gradient to 90% organic (25.0-30.0 min) ensured complete elution of hydrophobic matrix components and enabled thorough column flushing prior to re-equilibration.

Mass spectrometric optimization required careful tuning to achieve optimal sensitivity for both analytes. N-nitroso-bisoprolol exhibited superior ionization efficiency in positive electrospray mode, while N-nitroso-hydrochlorothiazide showed preferential ionization in negative mode. Dual polarity switching within a single analytical run was used with appropriate time segments. Collision-induced dissociation energies were optimized for each transition to maximize product ion intensity while maintaining selectivity. The quantifier transitions (m/z 377.10 → 275.15 for N-nitroso-bisoprolol and m/z 325.00 → 293.95 for N-nitroso-hydrochlorothiazide) provided the highest signal-to-noise ratios, while qualifier transitions confirmed analyte identity through ion ratio verification.

3.2 Specificity and Selectivity

Specificity studies demonstrated complete freedom from interference at the retention times of both target analytes. Analysis of blank diluent showed no peaks corresponding to N-nitroso-bisoprolol or N-nitroso-hydrochlorothiazide. Similarly, placebo tablet matrix analysis revealed no interfering peaks from excipients, confirming that the method selectively quantifies the target nitrosamine impurities without matrix interference. The use of multiple reaction monitoring with optimized precursor-to-product ion transitions ensured high selectivity, as only compounds with specific mass-to-charge ratios and fragmentation patterns produced detectable signals.

Table 1: Specificity Results: Interference Assessment

Sample Type	Analyte	RT (min)	Interference (ppm)
Blank (Diluent)	NNO-HCTZ	29.58	Negligible interference
	NNO-Bisoprolol	19.62	Not detected

System Suitability Results from Six Consecutive Injections:

Table 2: System Suitability Results

Parameter	NNO-HCTZ	NNO-Bisoprolol

Peak Area - Injection 1	15,057,669	3,617,673
Peak Area - Injection 2	15,070,034	3,591,336
Peak Area - Injection 3	14,212,605	3,499,145
Peak Area - Injection 4	13,935,205	3,536,158
Peak Area - Injection 5	14,054,743	3,634,459
Peak Area - Injection 6	14,013,843	3,563,463
Average Peak Area	14,390,850	3,567,039
Standard Deviation	534,308	51,689
%RSD	3.7	1.4
Bracketing Standard (%RSD)	2.3	1.1

Figure 2: Chromatogram of Blank

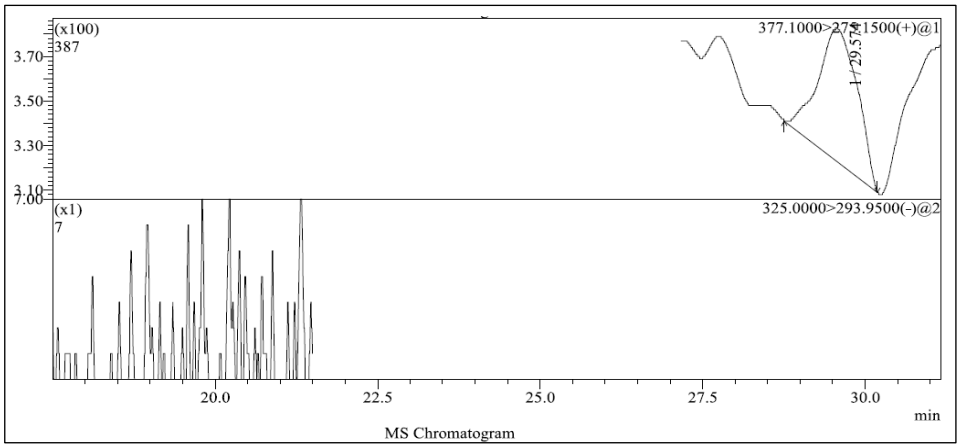
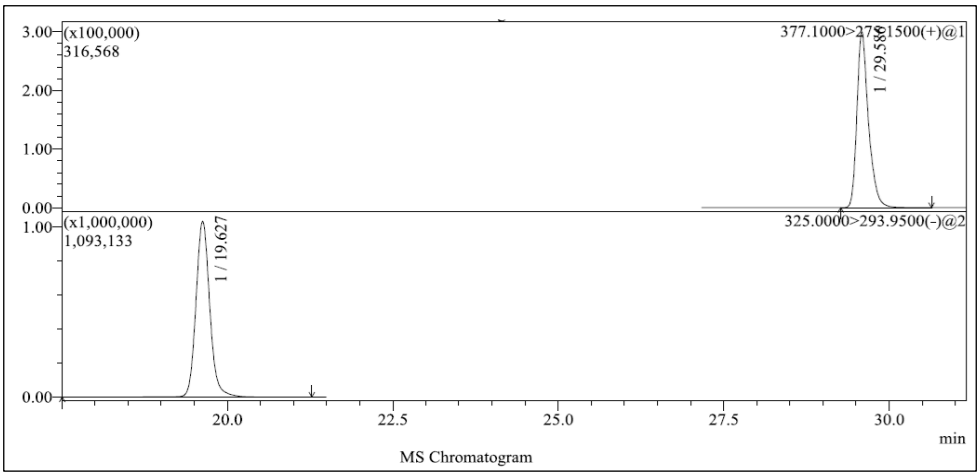


Figure 3: Chromatogram of Standard with Retention Time



3.3 Sensitivity: Detection and Quantification Limits

Limit of detection values were determined as 263 ppm for N-nitroso-hydrochlorothiazide and 4.9 ppm for N-nitroso-bisoprolol, based on 3:1 signal-to-noise ratio criterion. Limit of quantification values were established as 798 ppm for N-nitroso-hydrochlorothiazide and 15 ppm for N-nitroso-bisoprolol, based on 10:1 signal-to-noise ratio criterion. These quantification limits represent approximately 10% of the respective specification limits (8000 ppm and 150 ppm), providing adequate sensitivity for reliable quantification across the

entire validated range and enabling detection of impurities well below regulatory thresholds.

Figure 4: Chromatogram of LOD solution

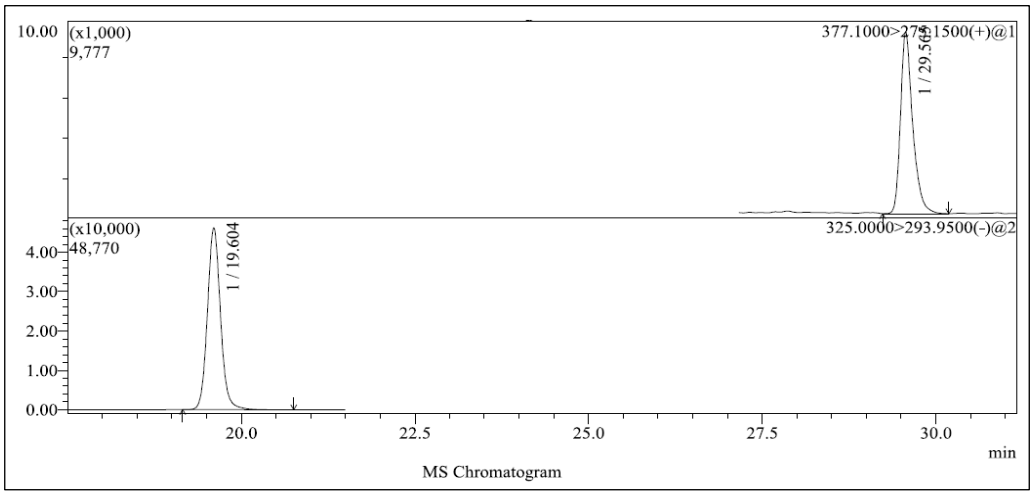
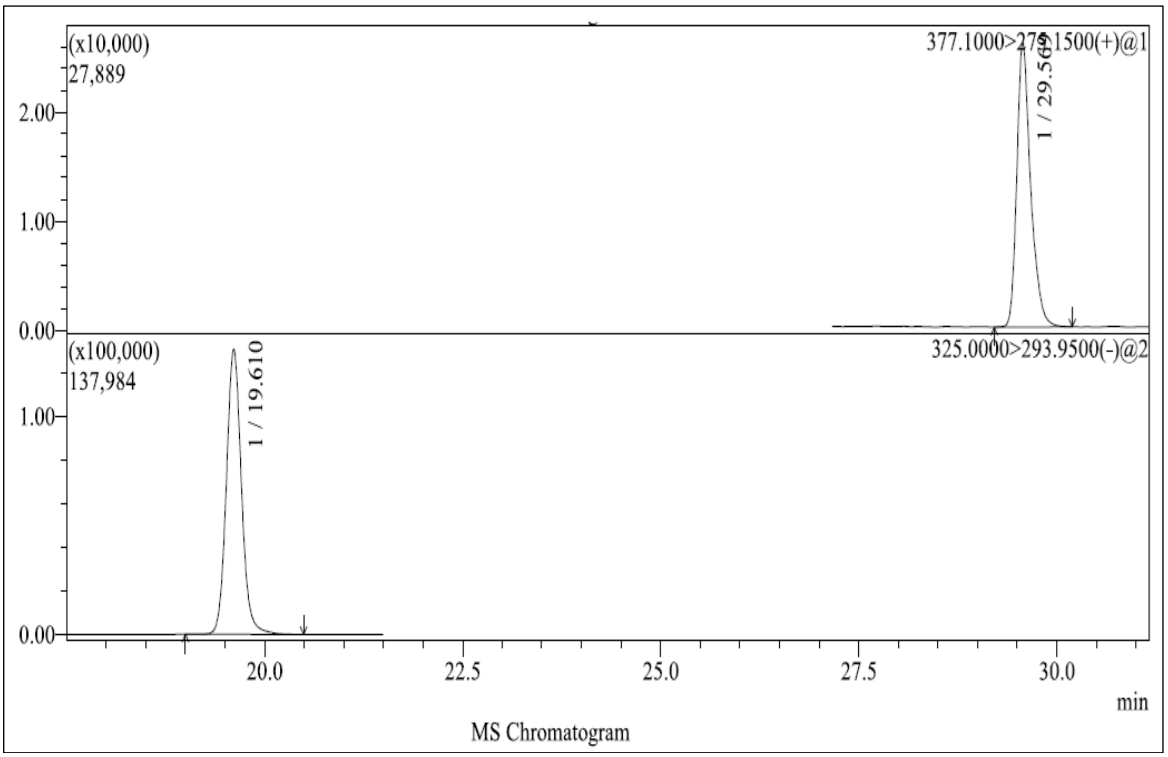


Figure 5: Chromatogram of LOQ solution



3.4 Linearity

Calibration curves were constructed using five concentration levels spanning 10% to 150% of specification limits. Linear regression analysis yielded correlation coefficients of r = 0.9990 for N-nitroso-hydrochlorothiazide and r = 0.9993 for N-nitroso-bisoprolol, both exceeding the acceptance criterion of r ≥ 0.99. Residual analysis confirmed random

distribution around zero with no systematic deviations, indicating excellent linearity throughout the validated concentration range. The linear relationship between analyte concentration and detector response enables accurate quantification of both nitrosamine impurities at specification levels.

Table 3: Linearity Data: Concentrations and Corresponding Peak Areas

Level	NNO-HCTZ (ppm)	NNO-Bisoprolol (ppm)	NNO-HCTZ Peak Area	NNO-Bisoprolol Peak Area
LOQ (Level 1)	798.4	15.0	1538571	280037
50% (Level 2)	3,991.8	74.7	6631192	1389197
100% (Level 3)	7,983.5	149.8	11251989	2702598
125% (Level 4)	9,980.4	186.8	12765597	3175757
150% (Level 5)	11,977.3	224.2	14980507	3925173

Table 4: Linearity Results and Regression Parameters

Statistical Parameter	N-Nitroso-HCTZ	N-Nitroso-Bisoprolol
Concentration Range (ppm)	798.4–11,977.3	15.0–224.2
Number of Calibration Levels	5	5
Correlation Coefficient (r)	0.9990	0.9993
Regression Equation (y = mx + b)	y = 0.01862x − 2.145	y = 0.01407x + 0.0312
Slope (m)	0.01862	0.01407
Intercept (b)	−2.145	0.0312
Maximum Residual (%)	±3.2	±4.1

Figure 6: Linearity for NNO-HCTZ

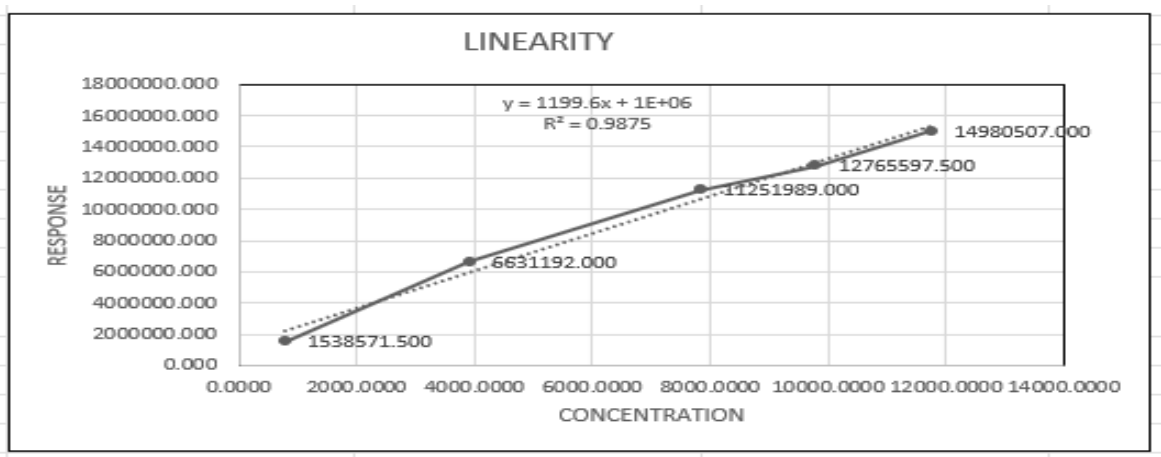
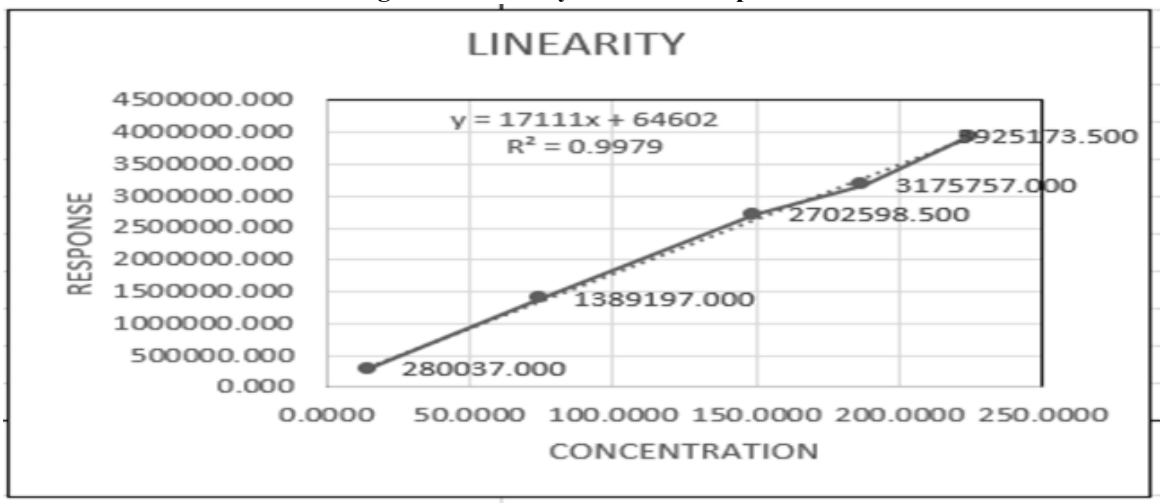


Figure 7: Linearity for NNO-Bisoprolol



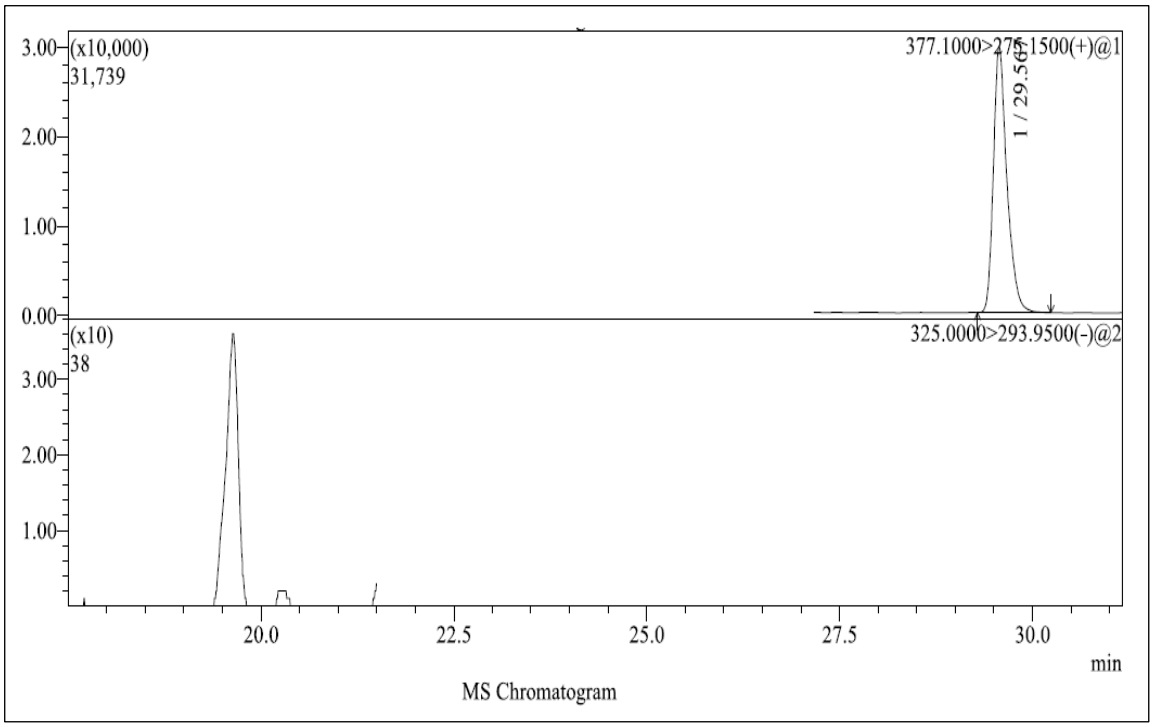
3.5 Accuracy

Accuracy studies were performed by spiking placebo tablet matrix at three concentration levels: 10%, 100%, and 150% of specification limits. Six independent preparations were analyzed at each level. Mean percent recoveries were 100.9% ± 2.2% for N-nitroso-hydrochlorothiazide and 100.8% ± 2.5% for N-nitroso-bisoprolol, well within the ICH-recommended acceptance range of 80-120%. Relative standard deviation values at all levels were below 5%, demonstrating excellent accuracy and confirming that the sample preparation procedure provides quantitative extraction of both analytes from the tablet matrix without systematic bias.

Table 5: Accuracy (Recovery) Results

Spike Level	Expected (ppm)	# Reps	Measured Mean (ppm)	Mean Recovery (%)	RSD (%)
N-Nitroso-HCTZ					
LOQ (10%)	798.4	6	790.4	98.9	2.9
Specification (100%)	7,983.5	6	8,084.5	101.2	1.6
Elevated (150%)	11,977.3	6	12,302.5	102.7	2.1
N-Nitroso-Bisoprolol					
LOQ (10%)	15.0	6	15.0	100.4	3.8
Specification (100%)	149.5	6	150.8	100.8	1.9
Elevated (150%)	224.2	6	227.1	101.3	1.8

Figure 8: Chromatogram of Test sample solution



3.6 Precision

System precision was evaluated by six consecutive injections of the standard solution at 100% specification level. Relative standard deviation values were 2.1% for N-nitroso-hydrochlorothiazide and 3.7% for N-nitroso-bisoprolol, both well below the acceptance criterion of 15%. Method precision (repeatability) was assessed through six independent sample preparations analyzed by a single analyst on the same day. Relative standard deviation values were 0.8% for N-nitroso-hydrochlorothiazide and 1.0% for N-nitroso-bisoprolol. Intermediate precision was evaluated with six independent sample preparations by a different analyst on a different day. Relative standard deviation values remained below 1.0% for both analytes. The exceptional precision performance across all evaluated levels confirms that the method provides highly reproducible results suitable for routine quality control applications.

System Precision (Six Consecutive Injections of Standard Solution):

Table 6: System Precision Results

Injection	N-Nitroso-HCTZ Peak Area	N-Nitroso-Bisoprolol Peak Area
1	15,057,669	3,617,673
2	15,070,034	3,591,336
3	14,212,605	3,499,145
4	13,935,205	3,536,158
5	14,054,743	3,634,459
6	14,013,843	3,563,463
Average	**14,390,850**	**3,567,039**
Std. Dev.	**534,308**	**51,689**
%RSD	**3.7%**	**1.4%**

Method Precision (Six Preparations):

Table 7: Method Precision Results

Preparation	N-Nitroso-HCTZ (ppm)	N-Nitroso-Bisoprolol (ppm)
1	8,158.6	148.2
2	8,127.4	149.8
3	8,142.2	147.9
4	8,165.3	149.1
5	8,148.9	148.6
6	8,139.1	148.4
Average	**8,146.9**	**148.7**
Std. Dev.	**15.1**	**0.83**
%RSD	**0.19%**	**0.56%**

Intermediate Precision (Six Preparations):

Table 8: Intermediate Precision Results

Preparation	N-Nitroso-HCTZ (ppm)	N-Nitroso-Bisoprolol (ppm)
1	7791.2	149.1
2	7808.1	150.9
3	7800.2	152.9
4	7793.1	151.9
5	7725.1	149.5
6	7791.7	151.3
Average	**7784.9**	**150.9**
Std. Dev.	30.00	1.47

%RSD	**0.4%**	**1.0%**

3.7 Robustness

Robustness assessment evaluated method performance under deliberately varied conditions. Flow rate variations of ±10% (0.36 and 0.44 mL/min) and column temperature variations of ±5°C (35°C and 45°C) were assessed. System suitability parameters including retention time, peak area, and peak shape remained within acceptable limits under all conditions evaluated. Cumulative relative standard deviation values were below 6% for both analytes, demonstrating that the method maintains robust performance despite minor operational variations that may occur during routine analysis.

Flow Variation (±0.05 mL/min from 0.40 mL/min):

Table 9: Robustness—Flow Rate Variation

Flow Rate Condition	NNO-HCTZ (ppm)	% Change	NNO-Bisoprolol (ppm)	% Change
Actual (0.40 mL/min)	8,151.6	—	148.7	—
Low (0.36 mL/min)	8,402.3	+3.1%	148.6	−0.5%
High (0.44 mL/min)	7,918.8	−2.9%	147.2	−1.4%
Cumulative % RSD	**2.8%**		**0.8%**	

Column Temperature Variation (±5°C from 40°C):

Table 10: Robustness—Column Temperature Variation

Temperature Condition	NNO-HCTZ (ppm)	% Change	NNO-Bisoprolol (ppm)	% Change
Actual (40°C)	8,151.6	—	148.7	—
Low (35°C)	8,865.2	+8.8%	143.4	−4.0%
High (45°C)	7,918.8	−2.9%	147.2	−1.4%
Cumulative %RSD	**5.7%**		**2.5%**	

3.8 Solution Stability

Standard and test sample solutions were stored at 2-8°C and analyzed at 0, 12 and 24 hours to evaluate stability. Peak area variations remained below 20% throughout the 24-hour period for both N-nitroso-hydrochlorothiazide and N-nitroso-bisoprolol in both standard and test sample solutions. These results confirm adequate solution stability under refrigerated storage conditions, permitting sample analysis within 24 hours of preparation without significant degradation. This stability window provides operational flexibility for routine laboratory workflows.

Table 11: Solution Stability Results (%Variation)

Storage Condition	Time Point	NNO-HCTZ	NNO-Bisoprolol
Standard Solution Stability			
2–8°C (Refrigerated)	0 hours	NA	NA
	12 hours	3.9	1.3
	24 hours	6.8	1.6
Test Sample Solution Stability			
2–8°C (Refrigerated)	0 hours	NA	NA
	12 hours	-6.2	-4.7

	24 hours	-14.4	-16.2

3.9 Method Performance Summary

The comprehensive validation demonstrated that this liquid chromatography-tandem mass spectrometry method meets all ICH Q2(R2) requirements for pharmaceutical impurity analysis. The method exhibits exceptional sensitivity with quantification limits at 10% of specification levels, excellent selectivity free from matrix interference, outstanding linearity across the validated range, accuracy with mean recoveries of 101% and precision better than 4% relative standard deviation. Robust performance under deliberately varied conditions and adequate 24-hour solution stability support the method's suitability for routine quality control applications. The validated analytical procedure provides pharmaceutical manufacturers with a reliable tool for monitoring nitrosamine impurities in bisoprolol hydrochlorothiazide tablets, supporting regulatory compliance with European Medicines Agency and Food and Drug Administration standards.

4. Conclusions

This work presents a comprehensively validated liquid chromatography-tandem mass spectrometry method for simultaneous determination of N-nitroso-hydrochlorothiazide and N-nitroso-bisoprolol in bisoprolol hydrochlorothiazide fixed-dose combination tablets. The method successfully addresses the analytical challenges of quantifying two structurally dissimilar nitrosamine impurities at regulatory specification levels through optimized chromatographic separation on a biphenyl stationary phase with gradient elution and dual-polarity electrospray ionization mass spectrometry. Validation following ICH Q2(R2) guidelines confirmed excellent analytical performance across all evaluated parameters including specificity, sensitivity, linearity, accuracy, precision, robustness, and solution stability. The validated method provides a practical, reliable analytical tool for routine pharmaceutical quality control and batch release testing, supporting industry compliance with international regulatory standards for nitrosamine impurity control. Future applications may extend this methodology to other pharmaceutical combinations susceptible to nitrosamine formation, contributing to enhanced patient safety through rigorous impurity monitoring.

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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Conflict of Interest

The corresponding author certifies on behalf of all authors that there are no financial conflicts of interest, intellectual property disputes, or other circumstances that could influence the interpretation or presentation of the research findings.

Data Availability Statement

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

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