

Article

Development and Validation of a Sensitive LC-MS/MS Method for the Determination of N-Nitroso-Atenolol in Atenolol-Based Pharmaceuticals

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Abstract: The recent detection of N-nitroso-atenolol, a mutagenic and potentially carcinogenic impurity in atenolol-based pharmaceuticals, has raised serious safety concerns and emphasized the need for stringent analytical control. This study developed and validated a highly sensitive LC-MS/MS method for quantifying N-nitroso-atenolol in both active pharmaceutical ingredients (APIs) and finished products. Quantification was carried out using multiple reaction monitoring (MRM) under positive-mode electrospray ionization (ESI). Separation was performed on a C18 reversed-phase column with a gradient of water and methanol containing 0.1% formic acid. The method was validated to meet a specification limit of 15 ng/mg, with a linear range of 0.5–80 ng/mL, effectively covering 10–400% of the regulatory threshold. The method exhibited an excellent performance in terms of specificity, accuracy, precision, linearity, and robustness. It achieved a limit of detection (LOD) of 0.2 ng/mL (0.30 ng/mg) and a limit of quantification (LOQ) of 0.5 ng/mL (0.75 ng/mg), alongside a comprehensive uncertainty analysis with an expanded uncertainty of ± 3.86 mg/kg. Application to commercial atenolol products confirmed the reliability and practical utility of the method. This validated approach offers a critical tool for pharmaceutical manufacturers and regulatory agencies to monitor and control N-nitroso-atenolol, ensuring compliance and enhancing patient safety.

Keywords: N-nitroso-atenolol; NDSRIs; LC-MS/MS



Academic Editor: Paraskevas D. Tzanavaras

Received: 7 April 2025

Revised: 7 May 2025

Accepted: 9 May 2025

Published: 12 May 2025

Citation: Kwon, S.; Ahn, S.-H.; Chang, Y.; Park, J.-S.; Cho, H.; Kim, J.-B. Development and Validation of a Sensitive LC-MS/MS Method for the Determination of N-Nitroso-Atenolol in Atenolol-Based Pharmaceuticals. *Separations* **2025**, *12*, 122. <https://doi.org/10.3390/separations12050122>

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1. Introduction

Nitrosamine impurities remain a persistent challenge for the pharmaceutical industry. The 2018 discovery of nitrosodimethylamine (NDMA) in valsartan escalated this issue beyond safety concerns, directly impacting public trust [1,2]. Furthermore, the consistent detection of nitrosamine drug substance-related impurities (NDSRIs) in multiple drugs reinforces the ongoing and evolving nature of this problem [3–6].

Nitrosamine drug substance-related impurities (NDSRIs) are mutagenic and potentially carcinogenic contaminants that resemble the structural characteristics of active pharmaceutical ingredients (APIs) [7,8]. These impurities may form during manufacturing when nitrosating agents interact with APIs containing amine groups or their byproducts under specific conditions [9–11].

The detection of N-nitroso-atenolol in an atenolol drug substance from an Indian manufacturer in April 2023 intensified existing concerns (Figure 1). Atenolol, a commonly prescribed beta-blocker, is widely used to manage hypertension, angina, and arrhythmias [12,13]. Therefore, the detection of N-nitroso-atenolol poses a severe threat to the safety of numerous patients worldwide.

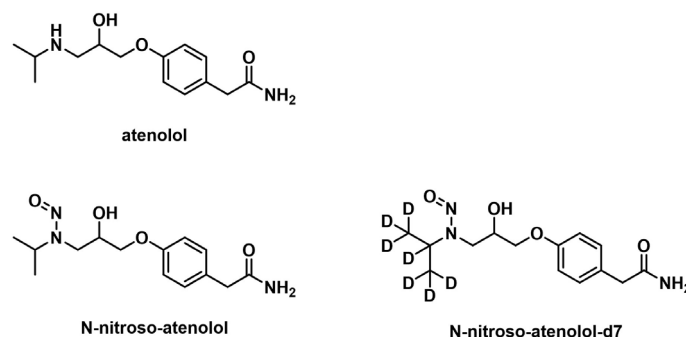


Figure 1. Structure of N-nitroso-atenolol, N-nitroso-atenolol-d7, and atenolol.

Regulatory bodies, including the Ministry of Food and Drug Safety (MFDS) in South Korea, responded promptly to the N-nitroso-atenolol detection. Prompted by international reports of contamination, the MFDS initiated a comprehensive investigation, requesting impurity assessments from local pharmaceutical manufacturers and evaluating potential safety measures. This proactive approach aligns with the global regulatory momentum led by agencies such as the Food and Drug Administration (FDA) and the European Medicines Agency (EMA), which implements strict limits on nitrosamine levels in drugs including sartans, histamine antagonists, and metformin [14,15].

Accurate detection and control of nitrosamines require stringent analytical methods and adherence to defined acceptable intake (AI) limits. The Carcinogenic Potency Categorization Approach (CPCA), revised by the EMA and FDA, classifies nitrosamines based on their structural relationship to carcinogenic potency, assigning AI limits from 18 ng/day to 1500 ng/day [16–18]. Consequently, the pharmaceutical industry must emphasize sensitive, reliable, and practical analytical methods for detecting and quantifying nitrosamines and NDSRIs at trace levels [15,19].

Quantifying N-nitroso-atenolol involves several analytical challenges. Trace levels (parts per million or billion) require highly sensitive and specific techniques, often exceeding the capabilities of conventional methods. Additionally, the structural similarity between atenolol and N-nitroso-atenolol complicates separation and quantification, especially when high concentrations of the parent API are present. To address these challenges, advanced technologies like liquid chromatography coupled with mass spectrometry (LC-MS/MS) and gas chromatography–mass spectrometry (GC-MS) have been developed [20–24]. Notably, tandem mass spectrometry (MS/MS) and high-resolution mass spectrometry (HRMS) have become the preferred methods due to their exceptional sensitivity and selectivity, ensuring robust and precise quantification of this impurity [25–27]. In line with these technological advancements, the lower limit of quantification (LLOQ) for nitrosamine impurities using LC-MS/MS has generally been reported in the range of approximately 0.1 ng/mL to 10 ng/mL. Regulatory agencies such as the EMA and FDA often mandate detection limits of 0.03 ppm (30 ng/g) or lower for nitrosamine compounds, underscoring the need for highly sensitive analytical methods in pharmaceutical quality control.

This research aims to develop and validate a sensitive, reproducible LC-MS/MS method for quantifying N-nitroso-atenolol in atenolol-based products, with an emphasis on practical implementation in routine quality control. The optimized method for trace-level analysis will help enhance the safety and quality of pharmaceutical prod-

ucts and provide essential tools for regulatory agencies and manufacturers in addressing nitrosamine impurities.

2. Materials and Methods

2.1. Chemicals and Reagent

Reference standards, N-nitroso-atenolol (99.5%) and N-nitroso-atenolol-d7 (98.4%), were obtained from TLC Pharmaceutical Standards (Newmarket, ON, Canada). Solvents used for sample preparation and LC-MS analysis, including ultra-pure water, methanol, and formic acid, were of LC-MS grade and purchased from Fisher Chemical (Fair Lawn, NJ, USA). PVDF syringe filters (VIVAGEN Co., Ltd., Seongnam, Republic of Korea) facilitated sample cleanup. All other reagents were of analytical or HPLC grade. Youngil Pharmaceutical Co., Ltd., Korea, supplied the atenolol active pharmaceutical ingredient (IPCA, Mumbai, India) and the tablets of their drug product.

2.2. HPLC Conditions

Chromatographic separation was performed using a Waters Acquity UPLC system (Milford, MA, USA), comprising a pump, column oven, autosampler, and photodiode array (PDA) detector. Separation was achieved using a Waters BEH C18 column (2.1 × 50 mm, 1.7 µm) maintained at 40 °C. The autosampler was cooled to 10 °C, and a 1 µL injection volume was used for the LC-MS/MS analysis. The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B), delivered at a flow rate of 0.33 mL/min using the following gradient: 20% B (0–1.5 min); 20–23.5% B (1.5–2.5 min); 23.5% B (2.5–3 min); 23.5–26% B (3–9.5 min); 26–99% B (9.5–9.9 min); 99% B (9.9–12.4 min); followed by a return to 20% B (12.4–12.5 min) and equilibration at 20% B (12.5–15 min). Identification of high atenolol API concentrations in the matrix was achieved using a PDA detector operating at 225 nm.

2.3. Mass Spectrometer Conditions

The UPLC system was coupled to a Waters Xevo TQ-Absolute mass spectrometer (Milford, MA, USA) optimized for LC-MS/MS analysis. Electrospray ionization (ESI) was performed in positive ion mode. Multiple reaction monitoring (MRM) transitions were monitored at m/z 296 → 222 and 145 for N-nitroso-atenolol, and m/z 303 → 229 and 152 for N-nitroso-atenolol-d7. Cone voltages were set to 2 and 34 V for N-nitroso-atenolol and N-nitroso-atenolol-d7, respectively. The capillary voltage was 0.71 kV. The source and desolvation temperatures were 150 °C and 400 °C, respectively. Desolvation and cone gas flows were 800 L/h and 150 L/h, respectively. To minimize ion source contamination, the initial eluent was diverted to waste using a switching valve. Data acquisition and processing were performed using Waters MassLynx v.4.2 software.

2.4. Preparation of Standard Solution

Individual stock solutions of N-nitroso-atenolol and its deuterated analog, N-nitroso-atenolol-d7, were prepared at 1 mg/mL in methanol. These were then diluted with 75% methanol to a working concentration of 600 ng/mL. A series of calibration standards were prepared with N-nitroso-atenolol concentrations of 0.5, 2, 10, 20, 40, and 80 ng/mL. The 100% standard, defined as the specification limit, was 10 ng/mL. Recovery experiments involved preparing N-nitroso-atenolol solutions at 0, 0.5, 10, and 40 ng/mL in the drug substance and drug product samples. The internal standard was added to all solutions to achieve a final concentration of 20 ng/mL.

2.5. Sample Preparation

2.5.1. Drug Substance Preparation

A 20 mg sample of atenolol API was precisely weighed into a 50 mL centrifuge tube. One milliliter of the internal standard solution was added, followed by 29 mL of 75% methanol. The mixture was vortexed for 1 min and centrifuged at 4000 rpm for 10 min. The supernatant was filtered through a 0.22 μ m PVDF syringe filter (DAIHAN, Seoul, Republic of Korea), discarding the initial 1 mL. A 1 mL aliquot of the filtered solution was then transferred to an autosampler vial for LC-MS/MS analysis.

2.5.2. Drug Product Preparation

Ten atenolol tablets were crushed, and a portion of the powder equivalent to 20 mg of atenolol API was accurately weighed and transferred to a 50 mL centrifuge tube. One milliliter of the internal standard solution was added, followed by 29 mL of 75% methanol. The subsequent sample preparation steps were identical to those described for the atenolol API.

2.6. Method Validation

The analytical method for quantifying N-nitroso-atenolol in atenolol was rigorously validated according to ICH Q2 (R2) guidelines [28], encompassing assessments of system suitability, specificity, linearity, limits of detection (LOD) and quantitation (LOQ), precision, and accuracy and robustness.

To assess specificity, blank (75% methanol), standard solution, drug substance, and drug product were analyzed to verify the absence of interference at retention time for the analyte. Linearity was evaluated by performing triplicate analyses at each concentration level across the range of 0.5 to 80 ng/mL (corresponding to 5–800% of the limit). Residual plot analysis and R^2 values exceeding 0.998 confirmed the linearity of the method. LOD and LOQ were determined based on signal-to-noise ratios (S/N) of ≥ 3 and ≥ 10 , respectively. Accuracy and precision were evaluated by analyzing the concentrations detected after extracting samples spiked into both the drug substance and drug product. The assessment was conducted using triplicate analyses at three concentrations (0.5, 10, and 40 ng/mL) within the calibration range. For each concentration, the mean assay values, standard deviations, relative standard deviations (%RSD), and percent recoveries were calculated. To evaluate method robustness, the %RSD of peak response and retention time for N-nitroso-atenolol was measured after adjusting the eluent flow rate and column temperature by $\pm 10\%$. System suitability was confirmed by verifying that the %RSD of the peak areas for six injections of the standard solution did not exceed 10%, and the variation in retention time was within 2%. Additional parameters such as plate count (N), symmetry factor, and capacity factor (k') were also calculated to confirm the chromatographic performance.

2.7. Measurement Uncertainty

To improve the reliability of the quantitative analysis, measurement uncertainty was estimated with reference to the Guide to the Expression of Uncertainty in Measurement (GUM) and the draft EURACHEM/CITAC guide [29,30]. Key sources of uncertainty, such as the reference standard, preparation of standard solutions, calibration curve construction, and sample analysis, were identified, and the standard uncertainty associated with each component was individually evaluated. Additionally, regression analysis of the calibration curve was performed to support the quantification process. Based on the individual standard uncertainties, the combined standard uncertainty was calculated. Subsequently, the expanded uncertainty was estimated and applied to the analytical results, enabling the final measurement uncertainty to be determined and reported.

2.8. Specification Limit

In 2023, regulatory authorities such as the FDA and EMA established an acceptable intake (AI) limit of 1500 ng per day for N-nitroso-atenolol, based on the Carcinogenic Potency Categorization Approach (CPCA). Applying the formula of Limit (ng/mg) = acceptable intake of impurity (ng/day)/maximum dose of atenolol (mg/day) to a 100 mg maximum daily atenolol dose, the resulting 15 ng/mg specification limit highlights the critical regulatory focus on minimizing NDSRIs risks.

3. Results and Discussion

3.1. Method Development

For mass spectrometric analysis, a 100 ng/mL solution of N-nitroso-atenolol was introduced directly into the instrument. Utilizing positive mode electrospray ionization (ESI), a strong signal was observed for the protonated molecular ion $[M + H]^+$ at m/z 296. Subsequent fragmentation via collision-induced dissociation (CID) revealed major product ions at m/z 222 and 145 (Figure 2). The consistent ion ratios of the quantifier and qualifier ions in both the spiked samples and standard solution confirmed the identity of the analyte and demonstrated that the optimized LC-MS/MS method effectively minimizes interference at the retention time of N-nitroso-atenolol. Likewise, the deuterated form, N-nitroso-atenolol-d7, showed a protonated molecular ion at m/z 303, with corresponding fragment ions at m/z 229.

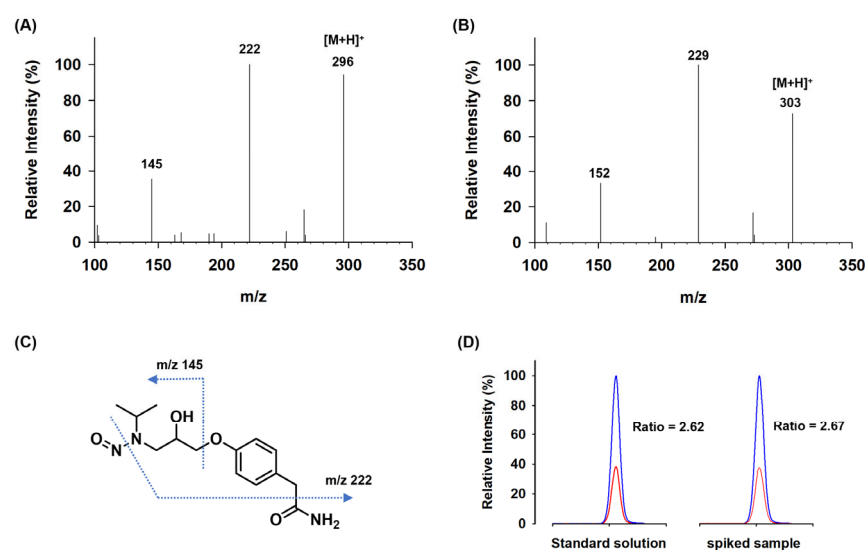


Figure 2. Result of fragmentation of N-nitroso-atenolol and N-nitroso-atenolol-d7 with LC-MS/MS. (A) Product ion spectra of N-nitroso-atenolol, (B) product ion spectra of N-nitroso-atenolol-d7, (C) fragmentation of N-nitroso-atenolol, and (D) quantifier (blue) to qualifier (red) ion ratios of N-nitroso-atenolol.

A robust chromatographic method was established to quantify N-nitroso-atenolol in the presence of high concentrations of atenolol API. The method employed a C18 stationary phase with an initial mobile phase consisting of 0.1% formic acid in 20% methanol. Under these conditions, atenolol eluted rapidly, followed by trace-level N-nitroso-atenolol, with a sufficient retention time gap that enabled accurate and distinct quantification (Figure 3). N-nitroso-atenolol was detected at 4.5 min under optimized LC-MS/MS conditions. To ensure complete removal of late-eluting matrix components or residual high-concentration substances, a wash step using a high-percentage methanol mobile phase was incorporated. Consequently, the total run time was extended and optimized to 15 min. To prevent mass

spectrometer overload due to the high API concentration, the flow was diverted to waste during the first four minutes and after six minutes of the chromatographic run.

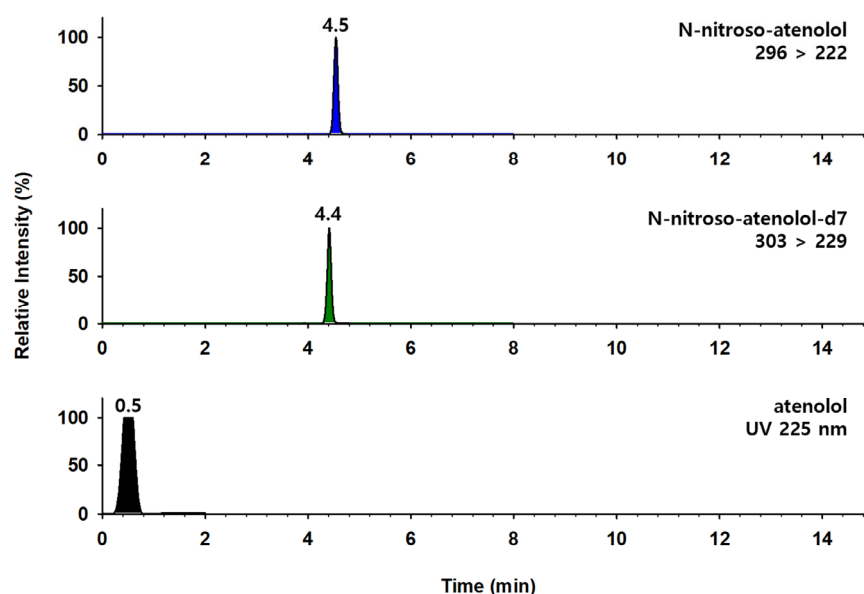


Figure 3. Separation of N-nitroso-atenolol, N-nitroso-atenolol-d7, and atenolol.

3.2. Validation Studies

3.2.1. Linearity, Limit of Detection (LOD) and Limit of Quantification (LOQ)

For the determination of N-nitroso-atenolol, a calibration strategy was employed using standard solutions ranging from 0.5 to 80 ng/mL, corresponding to 0.75 to 120 µg/g within atenolol (Table 1). The resulting calibration curve, generated by plotting analyte-to-internal standard peak area ratios against concentration, exhibited excellent linearity with an R^2 value of 0.9996 and a regression equation of $y = 0.0515x + 0.0026$. The accuracy of this calibration was verified, with all points falling within 15% of their predicted values. Furthermore, a residual analysis confirmed the robustness of the linear model, showing no systematic errors.

Table 1. Linearity, LOD, and LOQ.

Linearity Range				LOQ			LOD		
ng/mL	ng/mg	Equation	R^2	ng/mL	ng/mg	S/N	ng/mL	ng/mg	S/N
0.5–80	0.75–120	$Y = 0.0515x + 0.0026$	0.9993	0.5	0.75	30.8	0.2	0.30	13.3

To ensure reliable detection and quantitation, the method was designed to achieve signal-to-noise ratios of at least 3 and 10. These criteria were met with limits of detection and quantitation established at 0.2 ng/mL (0.30 ng/mg) and 0.5 ng/mL (0.75 ng/mg), respectively.

3.2.2. Selectivity and Specificity

Comparison of the diluent solution (75% methanol), API, finished drug product, and standard solution revealed no interfering substances at the retention time of the analyte peak (Figure 4). However, a distinct peak corresponding to N-nitroso-atenolol exceeding the detection limit of 0.2 ng/mL was observed. Following measurements of the pharmaceutical matrices and subsequent interpolation using the calibration curve, it was determined that the API matrix contained an average of 0.3 ng/mL, and the drug product matrix an average of 1.1 ng/mL of N-nitroso-atenolol.

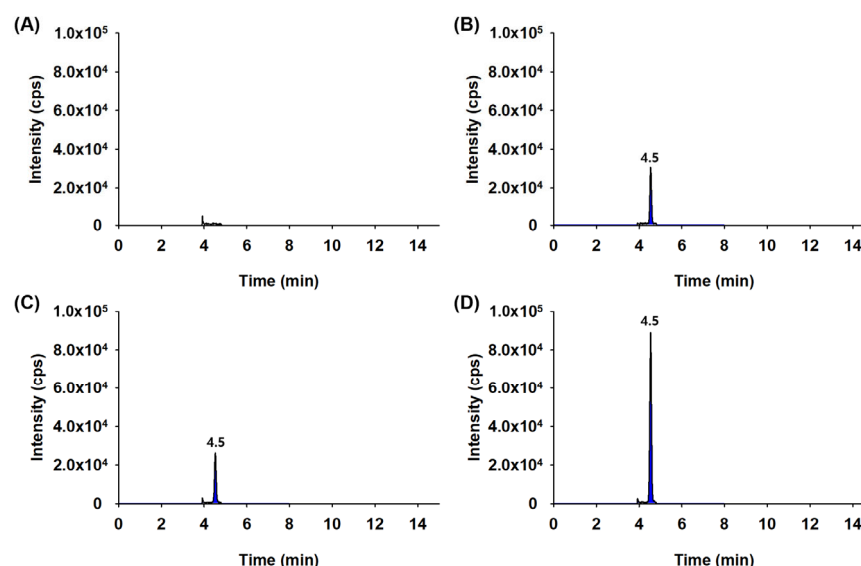


Figure 4. LC-MS/MS chromatogram of (A) diluent, (B) LOQ solution, (C) API solution, and (D) drug product solution.

3.2.3. Accuracy and Precision

The accuracy (as percentage recovery) and precision (as %RSD) of the N-nitroso-atenolol assay were evaluated in triplicate at 5% (LOQ), 100%, and 400% of the specification limit, using both the API and tablet formulation. As endogenous levels of N-nitroso-atenolol were detected in both the API and drug product, recovery calculations were performed using background-corrected concentrations. Corrections were applied based on the mean concentrations observed in the API blank ($n = 3$) and drug product blank ($n = 3$). As shown in Table 2, the API assay yielded overall intra-day and inter-day accuracy values of 102.94% and 101.27%, respectively, while the tablet formulation showed 102.85% and 102.51% for intra-day and inter-day accuracy, respectively. For both the API and the drug product, the %RSD values at the LLOQ, 100%, and 400% of the specification limit were below 5.19%, 1.72%, and 2.15%, respectively. These results demonstrate that the method provides reliable and consistent quantification of N-nitroso-atenolol.

Table 2. Recovery results from spiked samples.

Sample Type	Nominal Concentration (ng/mL)	Intra-Day			Inter-Day		
		Measured Concentration (ng/mL)	RSD (%)	Recovery (%)	Measured Concentration (ng/mL)	RSD (%)	Recovery (%)
API	0	0.30 ± 0.01	-	-	0.31 ± 0.01	-	-
	0.5	0.84 ± 0.02	4.60	107.33	0.83 ± 0.02	3.11	104.13
	10	10.45 ± 0.17	1.72	101.47	10.31 ± 0.09	0.86	99.98
	40	40.31 ± 0.64	1.59	100.01	40.19 ± 0.86	2.15	99.69
Drug product	0	1.12 ± 0.01	-	-	1.14 ± 0.00	-	-
	0.5	1.67 ± 0.03	4.82	109.13	1.69 ± 0.03	5.19	109.80
	10	11.02 ± 0.07	0.68	99.21	10.97 ± 0.07	0.68	106.61
	40	41.79 ± 0.23	0.68	100.22	40.90 ± 0.82	2.06	99.41

3.2.4. Robustness

To evaluate the robustness of method, intentional variations were introduced to key chromatographic parameters. These included adjusting the mobile phase flow rate by ± 0.03 mL/min and modifying the column temperature by ± 4 °C. N-nitroso-atenolol demonstrated a consistent peak area and retention time, with %RSD values remaining within acceptable limits. These results confirm that the method maintains a reliable performance despite minor fluctuations in operating conditions (Table 3).

Table 3. Robustness results.

Robustness (RSD%, $n = 9$)			
Column Temperature (40 ± 4 °C)		Flow Rate (0.33 ± 0.03 mL/min)	
Area	RT	Area	RT
3.14	0.88	5.28	5.01

3.2.5. System Suitability

System suitability was assessed using six consecutive injections of a 10 ng/mL standard (Table 4). The %RSD of the peak area ratio remained within the 10% acceptance limit, and the retention time variation for the N-nitroso-atenolol peak did not exceed 2%. The symmetry factor of 1.07 indicated a good peak shape.

Table 4. Results of system suitability parameters.

Parameter	Value
Retention time (RT, min)	4.55
Plate number (N)	17,095
HETP (mm)	0.0029
Retention factor (k')	9.1
Symmetry factor (S)	1.28
System precision (%RSD, $N = 6$)	Peak area ratio Retention time
	0.09

3.3. Uncertainty Evaluation

To ensure the precision and reliability of the quantitative analysis of N-nitroso-atenolol, key sources of uncertainty were identified. These included the reference standard, preparation of standard solutions, sample pretreatment, calibration curve, and sample analysis results. The standard uncertainty associated with each factor was calculated individually (Figure 5). The combined standard uncertainty was then obtained by summing the individual standard uncertainties. For the calibration curve, regression analysis was used to estimate its corresponding standard uncertainty. To further enhance the reliability of the combined standard uncertainty, the expanded uncertainty was calculated by applying a coverage factor (k) with a confidence level of 95%. This expanded uncertainty, accounting for potential matrix effects, was applied to the sample analysis results to determine the final measurement uncertainty. As a result, the expanded uncertainty was calculated to be ± 3.86 mg/kg at a 95% confidence level ($k = 2$), leading to a final reported value of 63.11 ± 3.86 mg/kg (Table 5). These results demonstrate that potential sources of error in the analysis of N-nitroso-atenolol were rigorously and quantitatively assessed, thereby improving the overall reliability of the analytical outcome.

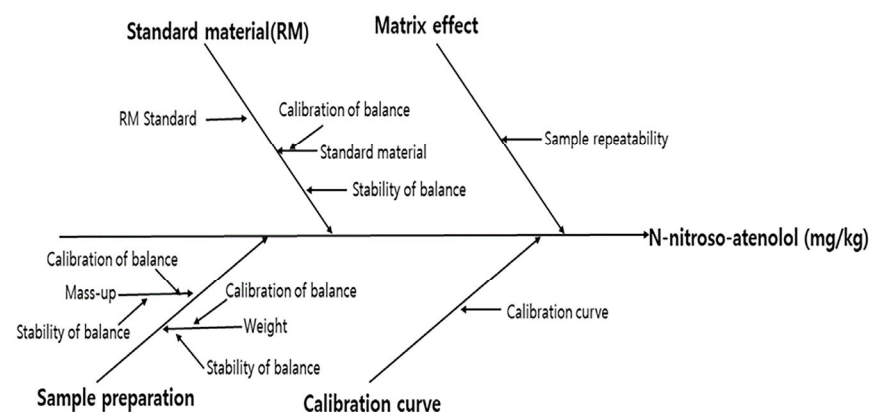


Figure 5. Fish bone diagram of measurement uncertainty in the N-nitroso-atenolol assay.

Table 5. Result of uncertainty in the N-nitroso-atenolol assay.

Parameters of Uncertainty	Result	Unit
Certified Concentration	63.11	mg/kg
The result of uncertainty	63.11 ± 3.86	mg/kg
Expanded uncertainty (U)	(k = 2, 95% confidence level)	
Coverage factor (k)	3.86	mg/kg
degrees of freedom, (veff)	2	dimensionless unit
Combined Standard	4281	dimensionless unit
Uncertainty (u_c)	1.9	mg/kg
Combined relative Standard		
Uncertainty (u_r/r)	0.031	dimensionless unit

3.4. Application of the Method for Real Sample Analysis

Using the validated analytical method, 12 atenolol-containing pharmaceutical products were analyzed to quantify N-nitroso-atenolol and assess compliance with the acceptable limit of 1500 ng/day, as specified in the MFDS guideline. Peaks exceeding the detection limit (0.2 ng/mL) were observed in all samples; however, all 12 products met the impurity control criteria (Table 6). Furthermore, the fact that all active pharmaceutical ingredients (APIs) were sourced from a single manufacturer highlights the importance of supply chain management. Even in cases where N-nitroso-atenolol is not detected, continuous monitoring and stringent oversight of suppliers are essential. Additionally, improvements to the manufacturing process are necessary to minimize the formation of this impurity. Accordingly, the establishment of a robust monitoring system for both APIs and finished drug products is critical to proactively manage potential risks. These findings confirm that the validated method is a reliable and widely applicable analytical tool for the detection and quantification of N-nitroso-atenolol across various pharmaceutical formulations.

Table 6. Results of the drug product analysis.

Drug Product No. *	Manufacturer	Strength	N-Nitroso-Atenolol Contents	
			ng/mL	ng/mg
1	Imported API	-	0.65	0.98
2	Manufacturer A	50 mg	0.51	0.77
3	Manufacturer A	50 mg	0.40	0.60
4	Manufacturer A	50 mg	0.84	1.26
5	Manufacturer A	50 mg	1.64	2.47
6	Manufacturer B	50 mg	1.40	2.09
7	Manufacturer B	50 mg	0.56	0.84
8	Manufacturer B	50 mg	0.94	1.41
9	Manufacturer B	50 mg	1.49	2.23
10	Manufacturer C	50 mg	0.69	1.03
11	Manufacturer C	50 mg	0.75	1.13
12	Manufacturer C	50 mg	0.78	1.16
Specification limit			N-nitroso-atenolol ≤ 15 ppm (ng/mg)	

* No. 1 is API, while No. 2–12 are tablet.

4. Conclusions

As N-nitroso-atenolol is classified as a genotoxic carcinogen that can cause DNA damage and increase the risk of cancer according to the ICH M7 guideline, it is essential to establish a high-sensitivity analytical method that enables preemptive responses to minimize human exposure. In this study, LC-MS/MS, which has a high sensitivity and specificity, was utilized to quantify N-nitroso-atenolol, a residual substance in atenolol finished drug products, and to validate the analytical method. The validation items included specificity, accuracy, precision, linearity and range, limit of quantification (LOQ), limit of

detection (LOD), system suitability, and measurement uncertainty, which were confirmed through LC-MS/MS according to strict standards, ensuring the reliability of the analytical results. The analytical method established in this study was applied to quantify N-nitroso-atenolol in various finished drug products, demonstrating its immediate applicability in actual drug production and quality control settings with a high sensitivity and accuracy. Based on the results of this study, it has become possible to significantly enhance the safety of commercially available antihypertensive finished drug products and establish preemptive safety management systems with residual impurity standards that prioritize patient health. This contributes to raising the level of domestic drug quality control and regulation and provides important scientific evidence for future related research and regulatory direction setting. In conclusion, this study clearly confirms that the results can be utilized as an essential reference not only for establishing strict standards and preemptive safety management for trace amounts of N-nitroso-atenolol in raw materials and finished drug products, but also for innovating drug management systems that prioritize patient safety.

Author Contributions: Conceptualization, J.-S.P.; methodology, J.-S.P. and S.K.; writing—original draft, S.K. and J.-B.K.; writing—review and editing, J.-B.K. and H.C.; data curation, Y.C. and S.-H.A.; formal analysis: S.-H.A.; investigation: S.-H.A. and Y.C.; visualization, S.-H.A. and Y.C.; validation: J.-S.P.; supervision, J.-S.P.; resources: J.-S.P.; project administration, S.K. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: All data are included in the article.

Conflicts of Interest: Authors Soonho Kwon, Sang-Hyun Ahn, Yongha Chang, and Joon-Sang Park were employed by the company Corporation VIVAGEN. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

References

1. Sörgel, F.; Kinzig, M.; Abdel-Tawab, M.; Bidmon, C.; Schreiber, A.; Ermel, S.; Wohlfart, J.; Besa, A.; Scherf-Clavel, O.; Holzgrabe, U. The contamination of valsartan and other sartans, part 1: New findings. *J. Pharm. Biomed. Anal.* **2019**, *172*, 395–405. [CrossRef] [PubMed]
2. Tuesuwan, B.; Vongsutilers, V. Nitrosamine Contamination in Pharmaceuticals: Threat, Impact, and Control. *J. Pharm. Sci.* **2021**, *110*, 3118–3128. [CrossRef] [PubMed]
3. EMA 2021. CHAMPIX (Varenicline)-Lots to Be Recalled Due to Presence of Impurity N-Nitroso-Varenicline. Available online: https://www.ema.europa.eu/en/documents/dhpc/direct-healthcare-professional-communication-dhpc-champix-varenicline-lots-be-recalled-due-presence_en.pdf (accessed on 23 November 2022).
4. Bharate, S.S. Critical Analysis of Drug Product Recalls due to Nitrosamine Impurities. *J. Med. Chem.* **2021**, *64*, 2923–2936. [CrossRef] [PubMed]
5. FDA. Pfizer Expands Voluntary Nationwide Recall to include All Lots of CHANTIX® (Varenicline) Tablets Due to N-Nitroso Varenicline Content. U.S. Food and Drug Administration. 16 September 2021. Available online: <https://www.fda.gov/safety/recalls-market-withdrawals-safety-alerts/pfizer-expands-voluntary-nationwide-recall-include-all-lots-chantix-varenicline-tablets-due-n> (accessed on 9 May 2025).
6. FDA. Pfizer Voluntary Nationwide Recall of Lots of ACCUPRIL® (Quinapril HCl) Due to N-Nitroso-Quinapril Content, U.S. Food and Drug Administration. 22 April 2022. Available online: <https://www.fda.gov/safety/recalls-market-withdrawals-safety-alerts/pfizer-voluntary-nationwide-recall-lots-accupril-quinapril-hcl-due-n-nitroso-quinapril-content> (accessed on 9 May 2025).
7. Snodin, D.J. Mutagenic impurities in pharmaceuticals: A critical assessment of the cohort of concern with a focus on N-nitrosamines. *Regul. Toxicol. Pharmacol.* **2023**, *141*, 105403. [CrossRef]
8. Schlingemann, J.; Burns, M.J.; Ponting, D.J.; Martins Avila, C.; Romero, N.E.; Jaywant, M.A.; Smith, G.F.; Ashworth, I.W.; Simon, S.; Saal, C.; et al. The Landscape of Potential Small and Drug Substance Related Nitrosamines in Pharmaceuticals. *J. Pharm. Sci.* **2023**, *112*, 1287–1304. [CrossRef]

9. Boetzel, R.; Schlingemann, J.; Hickert, S.; Korn, C.; Kocks, G.; Luck, B.; Blom, G.; Harrison, M.; François, M.; Allain, L.; et al. A Nitrite Excipient Database: A Useful Tool to Support N-Nitrosamine Risk Assessments for Drug Products. *J. Pharm. Sci.* **2023**, *112*, 1615–1624. [\[CrossRef\]](#)
10. Holzgrabe, U. Nitrosated Active Pharmaceutical Ingredients—Lessons Learned? *J. Pharm. Sci.* **2023**, *112*, 1210–1215. [\[CrossRef\]](#)
11. Sharma, N.; Patel, R.; Bothara, T.; Jain, S.; Shah, R.P. Modified NAP test: A simple and Responsive Nitrosating Methodology for Risk Evaluation of NDSRIs. *J. Pharm. Sci.* **2023**, *112*, 1333–1340. [\[CrossRef\]](#)
12. Wadworth, A.N.; Murdoch, D.; Brogden, R.N. Atenolol. *Drugs* **1991**, *42*, 468–510. [\[CrossRef\]](#)
13. Cruickshank, J.M. The Role of Beta-Blockers in the Treatment of Hypertension. In *Hypertension: From Basic Research to Clinical Practice*; Islam, M.S., Ed.; Springer International Publishing: Cham, Switzerland, 2017; pp. 149–166.
14. EMA. EMA recommendations on nitrosamine impurities in sartans. *React. Wkly.* **2020**, *1832*, 4. [\[CrossRef\]](#)
15. Shaik, K.M.; Bhaskar, S.; Suresh, W.G.; Kumar, P. Regulatory Updates and Analytical Methodologies for Nitrosamine Impurities Detection in Sartans, Ranitidine, Nizatidine, and Metformin along with Sample Preparation Techniques. *Crit. Rev. Anal. Chem.* **2022**, *52*, 53–71. [\[CrossRef\]](#)
16. EMA. Questions and Answers for Marketing Authorisation Holders/Applicants on the CHMP Opinion for the Article 5 (3) of Regulation (EC) No 726/2004 Referral on Nitrosamine Impurities in Human Medicinal Products. European Medicines Agency. 2020. Available online: https://www.ema.europa.eu/en/documents/opinion-any-scientific-matter/nitrosamines-emea-h-a53-1490-questions-answers-marketing-authorisation-holders-applicants-chmp-opinion-article-53-regulation-ec-no-726-2004-referral-nitrosamine-impurities-human-medicinal-products_en.pdf (accessed on 9 May 2025).
17. U.S. Food and Drug Administration. *Recommended Acceptable Intake Limits for Nitrosamine Drug Substance-Related Impurities (NDSRIs)—Guidance for Industry*; U.S. Food and Drug Administration: Silver Spring, MD, USA, 2023.
18. Kruhlak, N.L.; Schmidt, M.; Froetschl, R.; Graber, S.; Haas, B.; Horne, I.; Horne, S.; King, S.T.; Koval, I.A.; Kumaran, G.; et al. Determining recommended acceptable intake limits for N-nitrosamine impurities in pharmaceuticals: Development and application of the Carcinogenic Potency Categorization Approach (CPCA). *Regul. Toxicol. Pharmacol.* **2024**, *150*, 105640. [\[CrossRef\]](#)
19. Liu, Y.; Guo, X.; Wang, Z.; Qing, Z.; Qiang, M.; Lv, Q. Research progress of N-nitrosamine detection methods: A review. *Bioanalysis* **2022**, *14*, 1123–1135. [\[CrossRef\]](#) [\[PubMed\]](#)
20. Kalauz, A.; Tiringier, K.V.; Horváth, V.; Kapui, I. Simultaneous determination of low molecular weight nitrosamines in pharmaceutical products by fast gas chromatography mass spectrometry. *J. Chromatogr. A* **2023**, *1708*, 464323. [\[CrossRef\]](#) [\[PubMed\]](#)
21. Sieira, B.J.; Carpinteiro, I.; Rodil, R.; Quintana, J.B.; Cela, R. Determination of N-Nitrosamines by Gas Chromatography Coupled to Quadrupole–Time-of-Flight Mass Spectrometry in Water Samples. *Separations* **2020**, *7*, 3. [\[CrossRef\]](#)
22. Vogel, M.; Norwig, J. Analysis of genotoxic N-nitrosamines in active pharmaceutical ingredients and market authorized products in low abundance by means of liquid chromatography–tandem mass spectrometry. *J. Pharm. Biomed. Anal.* **2022**, *219*, 114910. [\[CrossRef\]](#)
23. Lim, Y.; Kim, A.; Lee, Y.-M.; Cho, H. Development of a sensitive LC-MS/MS method for determination of N-nitrosopiperazine in levocetirizine. *Anal. Methods* **2024**, *16*, 6494–6500. [\[CrossRef\]](#)
24. Vogel, M.; Escher, S.E.; Weiler, E.; Londenberger, A.; Deppenmeier, U.; Whomsley, R. Quantitative Investigation of Nitrosamine Drug Substance-Related Impurities (NDSRIs) Under Artificial Gastric Conditions by Liquid Chromatography–Tandem Mass Spectrometry and Structure–Activity Relationship Analysis. *Drug Test. Anal.* **2025**, ahead of print. [\[CrossRef\]](#)
25. Parfant, T.P.; Roškar, R. A comprehensive approach for N-nitrosamine determination in pharmaceuticals using a novel HILIC-based solid phase extraction and LC-HRMS. *Talanta* **2025**, *282*, 126752. [\[CrossRef\]](#)
26. Ngongang, A.D.; Duy, S.V.; Sauvé, S. Analysis of nine N-nitrosamines using liquid chromatography-accurate mass high resolution-mass spectrometry on a Q-Exactive instrument. *Anal. Methods* **2015**, *7*, 5748–5759. [\[CrossRef\]](#)
27. Zhang, J.; Raghavachari, R.; Kirkpatrick, D.C.; Keire, D.A.; Xu, X.; Faustino, P.J. Analytical Procedure Development and Proposed Established Conditions: A Case Study of a Mass Spectrometry Based NDSRI Analytical Procedure. *J. Pharm. Sci.* **2024**, *113*, 3028–3033. [\[CrossRef\]](#)
28. International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH). *Validation of Analytical Procedures Q2 (R2)*; ICH: Geneva, Switzerland, 2023.
29. Joint Committee for Guides in Metrology. *Evaluation of Measurement Data—Guide to the Expression of Uncertainty in Measurement*; Joint Committee for Guides in Metrology: Geneva, Switzerland, 2008.
30. Eurachem. *Eurachem/CITAC Guide: Quantifying Uncertainty in Analytical Measurement*, 3rd ed.; Eurachem: Teddington, UK, 2012.

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