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Development and application of an LC-MS/MS method for the detection of N-nitrosochlordiazepoxide as a potential genotoxic impurity

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ABSTRACT

In this study, we synthesized and characterized N-nitrosochlordiazepoxide, a potential genotoxic impurity originating from chlordiazepoxide hydrochloride, assessing its toxicity via quantitative structure-activity relationship (QSAR) analysis. Characterization techniques including UV spectroscopy and HPLC demonstrated purity at 97.0%, complemented by Fourier transform infrared spectroscopy analysis indicating characteristic peaks at 1,500 cm^{-1} (nitroso group) and 945 cm^{-1} (N-O bond), as well as mass spectrometry (m/z 329.2 [M+H]+) and NMR. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis, utilizing an HPLC octyldecylsilane column, revealed ion transitions from (Q1) m/z 329.20 to (Q3) m/z 281.90, 299.25, and 240.80. Method validation confirmed linearity (0.18–3 ppm) following The International Conference of Harmonization Q2 (R1) guidelines, with LOD and LOQ determined at 0.18 ppm and 0.375 ppm, respectively. Robustness studies showed <3% RSD with minor adjustments, and method precision exhibited <2.3% RSD for the main fragment (Q3) m/z 281.90, with intermediate precision below 1.8% RSD. Method accuracy was verified by recovery rates between 92.01% and 104.55%, and solution stability was confirmed for 24 hours at room temperature. Notably, analysis of three batches of chlordiazepoxide hydrochloride samples detected no traces of N-nitrosochlordiazepoxide, underscoring the enhanced sensitivity and accuracy of this LC-MS/MS method in pharmaceutical analytical procedures, crucial for ensuring product safety and compliance.

INTRODUCTION

Chlordiazepoxide hydrochloride is a benzodiazepine class molecule. Moreover, it is well known for its safety and effectiveness in the treatment of various psychological conditions, such as anxiety in patients, pre-surgical anxiety, and alcohol withdrawal symptoms [1–15]. As an old and effective medicine, it has become important to carry out regular control of medicinal quality and safety in terms of pharmacopeia quality systems. For instance, the manufacture of active pharmaceutical ingredients for medicine may lead to the appearance of potential genotoxic impurities (PGIs), which may be hazardous for public health even at trace levels [16–19]. N-nitroso compounds are known for their genotoxicity [20–32]. Nitrosamines are known mutagens and can form DNA adducts during metabolism. These adducts have the potential to cause miscoding errors in DNA replication, which can result in mutations and increase the risk of cancer [33]. Notably, our research identified a reaction pathway in which chlordiazepoxide and sodium nitrite under acidic conditions (pH 0.5–5) react and yield N-nitrosochlordiazepoxide [34–37]. Based on this reaction pathway, we successfully synthesized N-nitrosochlordiazepoxide from its active pharmaceutical ingredient (API) and characterized this PGI using different analytical tools, including UV spectroscopy, which provides invaluable insights into electronic transitions; infrared spectroscopy (IR), which elucidates the molecular

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structure; and high-pressure liquid chromatography (HPLC), which facilitates meticulous separation. Mass spectrometry augments our understanding by revealing molecular weight and structural information, while nuclear magnetic resonance (NMR) spectroscopy delves into the intricate molecular framework. We validated the Liquid chromatography-tandem mass spectrometry (LC-MS/MS) method following the guidelines outlined in The International Conference of Harmonization (ICH) Q2 (R1). For the assurance of drug safety, the International Council of Harmonization, the European Medicines Agency, and the United States Food and Drug Administration, on regulating PGIs in drug substances have released relevant guidelines [38–40]. Our study offers insights into the formation of N-nitrosochlordiazepoxide from chlordiazepoxide, leading to a pharmaceutical analysis by providing precise quantification of PGI. Through this ground-breaking research, we aim to accelerate progress in the analytical sciences, thereby contributing to improving pharmaceutical product quality and safety.

The evaluation of N-nitrosochlordiazepoxide (Structure as shown in Fig. 2) was conducted following regulatory guidelines issued by the US FDA CDER on August 2023, Recommended Acceptable Intake (AI) [41] as shown in Figure 1.

A method for analyzing chlordiazepoxide's nitrosation products using "high-pressure liquid chromatography" was reported [42]. However, to date, there are no LC-MS/MS methods tailored for specifically detecting and quantifying N-nitrosochlordiazepoxide in chlordiazepoxide hydrochloride.

EXPERIMENTAL STUDY

Materials and methods

Chemicals and reagents

Sodium nitrite (99% AR grade, Loba Chemie), Glacial acetic acid (99.8% AR grade, Rankem), sodium sulfate anhydrous (purity 99.0% LR grade, Thomas Bakers), dichloromethane (99.7% AR grade, standard reagents), potassium bromide (≥ 99.5% IR Spectroscopic grade), in-house HPLC grade water, HPLC grade methanol (≥99%, standard reagents), LCMS grade methanol (purity 99.9%, J.T.Baker), LCMS grade water (Honeywell), formic acid (98.3%, TCI), chlordiazepoxide samples (purity by HPLC >99.5%, free sample from Flowchem Pharma Pvt Ltd.), Whatman filter paper (grade 41), and membrane filters (0.45 µm, Axiva) were used.

Instrumentation

An analytical weighing balance (Shimadzu, model AP225WD, 0.1 mg accuracy), UV–visible spectrometer (Shimadzu, model UV1800), HPLC (Shimadzu, Model LC2030C Prominence-I), IR spectra were recorded using Fourier transform infrared spectroscopy (FTIR) (Shimadzu, model IRAffinity-1S), and LCMS chromatograms were recorded using a Shimadzu 8045 LC-MS/MS system with a high-pressure switch valve model FCV20AH2 equipped with Lab solution software. An NMR spectrometer (Bruker, model Ascend Evo 400MHz) was used to record the 1H and 13C-NMR spectra. The quantitative structure-activity relationship (QSAR) software tool used for toxicity estimation is TOXTREE. Class A standard volumetric flasks and pipettes were used for standard and sample preparations.

QSAR of N-Nitrosochlordiazepoxide

The QSAR of N-nitrosochlordiazepoxide in Toxtree software, according to the Cramer rule, classified it as Class III, indicating high toxicity and a structural alert for *S. typhimurium* mutagenicity and genotoxic carcinogenicity due to its functional groups linked to increased toxicity. N-nitrosochlordiazepoxide contains a nitroso group and alpha carbons (C2 and C3) likely devoid of hydrogen atoms, involved in forming the diazepine ring and potentially binding with other functional groups.

Method

N-Nitrosochlordiazepoxide synthesis

N-nitrosochlordiazepoxide was synthesized from chlordiazepoxide based on the observation of N-nitroso impurities manifested upon exposing the drug substance to an acidic

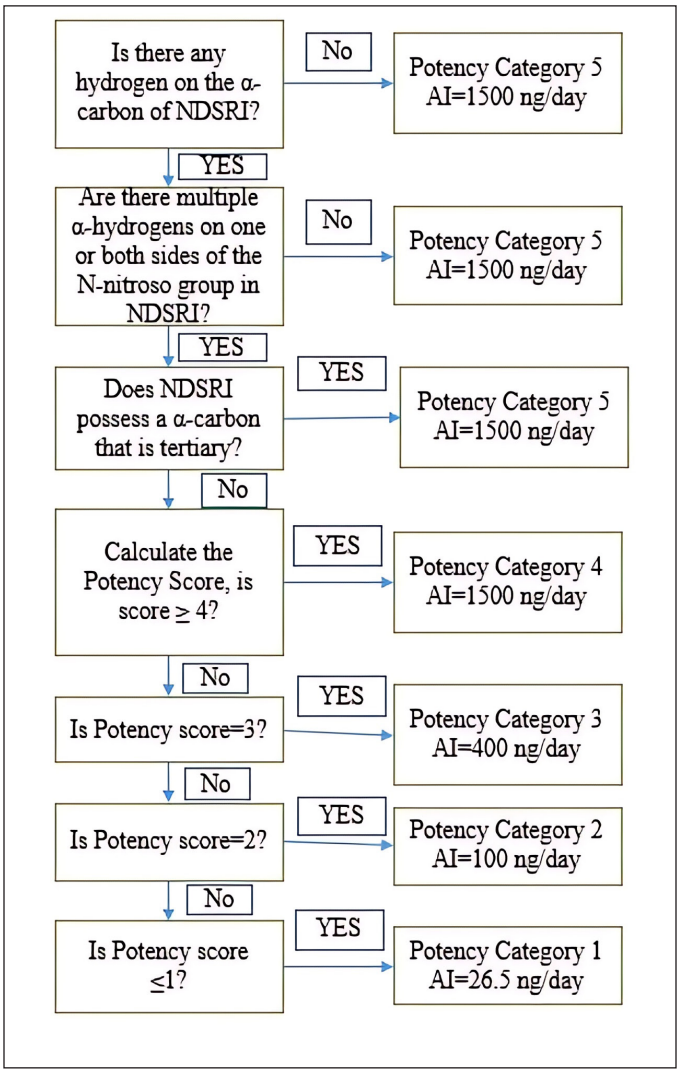

Figure 1. Flowchart of the USFDA CDER Guidelines of August 2023 for predicting the carcinogenic potency category of an NDSRI and identifying an associated recommended acceptable intake (AI).

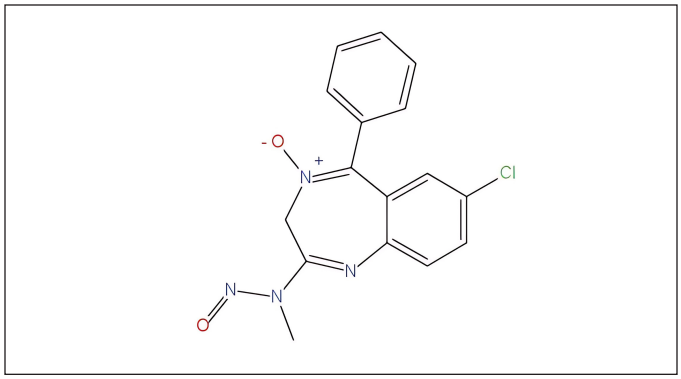

Figure 2. Structure of N-nitrosochlordiazepoxide.

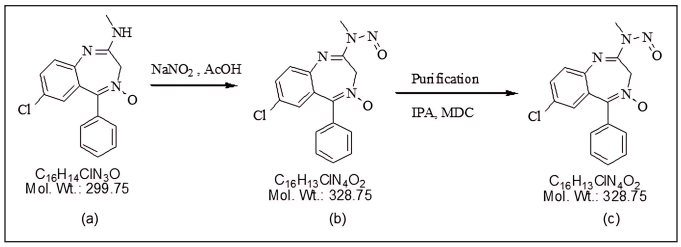

Figure 3. Synthetic route of N-nitrosochlordiazepoxide, (a) chlordiazepoxide, (b) N-nitrosochlordiazepoxide crude, and (c) pure N-nitroso chlordiazepoxide.

environment in the presence of sodium nitrite. The synthesis procedure, as depicted in Figure 3, commenced by dissolving 3.0 g (0.01 moles) of chlordiazepoxide hydrochloride in 20 ml of HPLC-grade water at room temperature (RT). Subsequently, 0.90 grams (0.013 moles) of sodium nitrite was slowly added at RT. The pH of the reaction mass was adjusted to 4.5–4.6 using 20 ml of acetic acid at RT, resulting in the formation of a light yellowish precipitate at pH 3.2. Following this, 300 ml of HPLC-grade water was added to the reaction mixture, which was then stirred for an additional 2 hours at RT. The solid product was filtered using Whatman filter paper, and the resulting filtrate was dissolved in 20 ml of dichloromethane with the aid of ultrasonication. The solution was then transferred to a separating funnel and basified with 5% sodium bicarbonate. The dichloromethane layer was separated, filtered, and dried using sodium sulphate to remove water. Subsequently, the residue was further concentrated via vacuum distillation, resulting in a final weight of 0.987 grams

Recrystallization

The residue was dissolved in 2 ml of dichloromethane and filtered. The resulting filtrate was then concentrated to 50% of its volume, after which 5 ml of isopropyl alcohol was added. The solid crystals were allowed to settle at 10°C–15°C for 30 minutes before filtration. Subsequently, the wet material underwent vacuum drying at 60–°C65°C for 8 hours. The resulting dry material weighed 0.940 g, yielding 29%.

spectroscopy

The spectroscopic analyses provided crucial structural insights into the synthesized compounds. UV-visible spectroscopy showed distinct absorption patterns, with chlordiazepoxide hydrochloride peaking at 263.30 nm and N-nitrosochlordiazepoxide at 291.0 nm. FTIR analysis revealed characteristic stretching vibrations for the *N* = O nitroso group (1501 cm^{-1}) and N–O bonds (945 cm^{-1}). Mass spectrometry confirmed molecular identities, with mass–charge ratios of 300.05 [M+H]+ for chlordiazepoxide and 329.05 [M+H]+ for N-nitrosochlordiazepoxide. NMR spectroscopy elucidated proton and carbon distributions, showcasing 1H NMR signals ranging from δ 7.034 to 7.628 (m, 8H, Aromatic-H), δ 5.358-(m, 2H, CH2), and δ 3.340–3.419 (s, 3H, CH3), and 13C NMR chemical shifts at various positions.

Physical characterization

We characterized the synthesized material, which appeared as a light-yellowish solid with a melting point between 155°C and 158°C.

Chromatography purity by HPLC

The purity of the synthesized compound was evaluated by HPLC under the following chromatographic conditions. Detection carried at 254 nm wavelength. A C18 HPLC column measuring 30 cm in length, 0.4 cm inner diameter, and particle size of 5 µm was used, and the oven temperature of the column was adjusted to 40°C. The mobile phase was prepared by adding 600 ml of methanol and 400 ml of water in a 1,000ml flask, the flow rate was adjusted to 1 ml/minute, and the mobile phase was used as the diluent to prepare 1 mg/ml samples. We injected 10 ml of each sample into the HPLC system, the purity of the synthesized N-nitrosochlordiazepoxide was 97.0%, and the purity of the key starting material chlordiazepoxide hydrochloride was 99.90%, as shown in Figure 4.

Peak Purity Analysis using Photodiode array detector (PDA)-HPLC

The observed peak purity index of N-nitrosochlordiazepoxide was 0.999993, there was no impurity detected in the main peak, the minimum peak purity index was 147, and the purity curve was above the zero line, which indicates that the peak was spectrally pure, as shown in Figure 5.

Method development (LC-MS/MS)

Preparation of the impurity stock solution

After precise weight measurements, N-nitrosochlordiazepoxide (10 mg) was dissolved and subsequently diluted in 100 ml of methanol, to attain a concentration of 100 ppm. Following this, a 3 ppm impurity stock solution was prepared by transferring 3 ml of the solution to a 100 ml volumetric flask and diluting it with methanol.

Preparation of the impurity standard solution

From the impurity stock solution (3 ppm), a series of linear dilutions were prepared to generate impurity standard solutions with concentrations of 0.18 ppm, 0.375 ppm, 0.75 ppm, 1.20 ppm, 1.50 ppm, and 2.25 ppm.

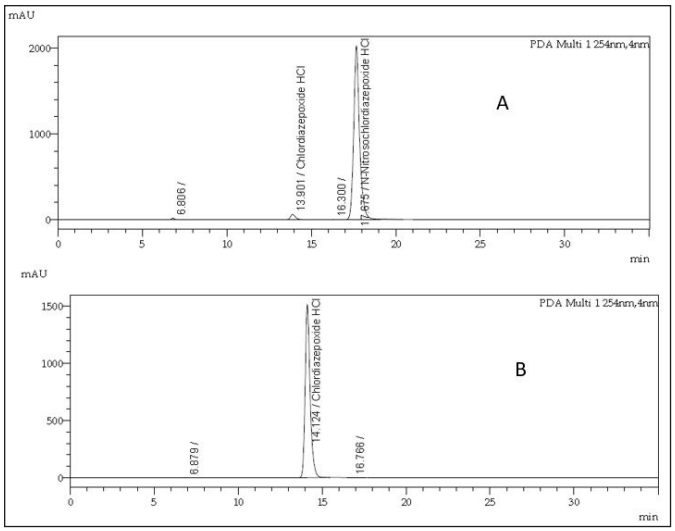

Figure 4. HPLC chromatograms of (A) N-nitrosochlordiazepoxide hydrochloride (97.0% purity) and (B) key starting material chlordiazepoxide hydrochloride (99.90% purity).

Preparation of spiked sample solution

We prepared spiked samples by adding 10 mg of chlordiazepoxide hydrochloride API to 10–ml standard flasks. The API was dissolved and diluted to volume using impurity standard solutions at 50%, 80%, 100%, and 150% concentrations, corresponding to the quantification limit.

Method optimization and chromatographic column selection

To refine analytical methods, various HPLC columns featuring distinct stationary phases were scrutinized for their efficacy in achieving optimal separation. Specifically, a C18 HPLC column, length of 15 cm, inner diameter of 0.46 cm, and 5µm particle size, C18 column length of 100 cm, 0.4 cm inner diameter, and particle size of 5µm, and a C8 column length of 15 cm, inner diameter 0.46 cm with a 5µm particle size were evaluated. The evaluation encompassed assessments of separation efficiency and sensitivity.

After extensive experimentation, the Shimadzu Zest Column was identified as the preferred option, with dimensions matching those of the previously mentioned C18 HPLC column (15 cm in length, inner diameter 0.46 cm, and particle size 5 µm). Multiple iterations were conducted to refine the methodology, particularly optimizing chromatographic parameters. In this endeavor, diverse LCMS/MS suitable mobile phases were explored, including Ammonium acetate buffers, Formic acid buffers, Ammonium formate, Trifluoroacetic acid buffer, as well as organic mobile phases such as Acetonitrile and methanol. Each mobile phase was scrutinized for its ability to facilitate efficient separation while ensuring compatibility with the LCMS/MS system. Through rigorous experimentation and evaluation, the optimal combination of the mobile phase and chromatographic column has been determined, laying the foundation for robust and reliable LCMS/MS methods in subsequent studies.

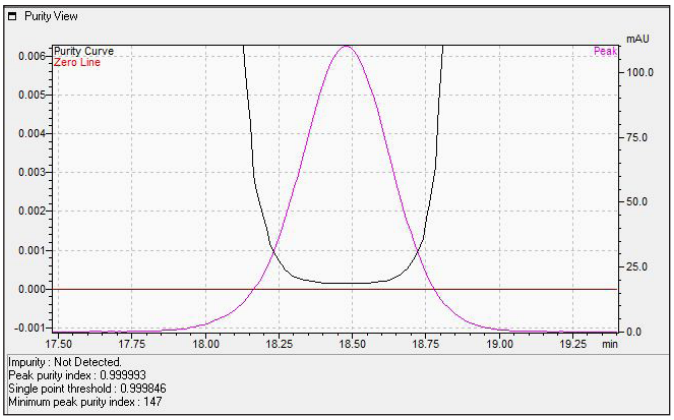

Figure 5. HPLC Photodiode array detector (PDA) peak purity view of N-nitrosochlordiazepoxide.

The final optimized LCMS/MS chromatographic conditions

To prepare mobile phase-A, a mixture of 40% HPLC-grade water and 60% HPLC-grade methanol was combined with 0.2% formic acid, while mobile phase-B consisted of 100% acetonitrile. Chromatographic separation was accomplished employing a Shimadzu zest HPLC C18 Column of length 15 cm, inner diameter 0.46 cm, and a particle size of 5 µm, 40°C temperature was set to column oven. The wavelength of UV Detection was selected to 254 nm, while the flow rate of the mobile phase was set to 0.6 ml/minute, and 10 µl of the sample was injected into the system. Cooling was performed at 15°C to maintain stability. A linear gradient program was used, with mobile phase A starting at 60% and mobile phase B at 40%. The acquisition was performed in MRM mode under positive polarity, with a run time of 10 minutes. At 4.5 kV, the interface voltage was set, utilizing an electron spray ionization (ESI) source interface at a temperature of 300°C. Further desolvation was achieved at a temperature of 526°C, with the desolvation line temperature set to 250°C. The nebulizer gas flow was adjusted to 3.00 l per minute, the heating gas flow was adjusted to 10.00 l per minute, and the 400°C temperature was set to the heating block. The drying gas (nitrogen) flow was maintained at 10.00 l per minute. Impurity is introduced into the mass detector using a flow line selection-switching valve. These meticulously optimized conditions ensure precise separation and efficient detection in LC-MS/MS analysis, facilitating accurate results in analytical studies.

RESULTS

Spectroscopic characterization of synthesized N-Nitrosochlordiazepoxide

Ultraviolet-visible spectroscopy (UV-VIS)

In UV-VIS the chlordiazepoxide hydrochloride and N-nitrosochlordiazepoxide samples were prepared in methanol at a concentration of 0.005 mg/ml and scanned in the range of 200 nm to 400 nm, the maximum absorbance was recorded at 263.30 nm and 243.80 nm, respectively, for the chlordiazepoxide hydrochloride sample, as shown in Figure 6. UV–visible (A) and maximum absorbance were recorded at 291.0 nm and 238.3 nm, respectively, for the N-nitrosochlordiazepoxide sample, as depicted in Figure 6 B.

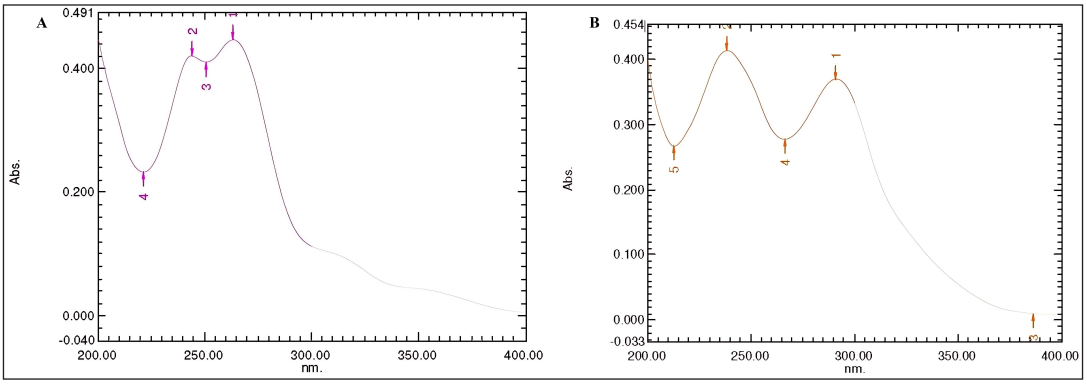

Figure 6. Ultraviolet-visible spectra of chlordiazepoxide hydrochloride (A) and (B) N-nitrosochlordiazepoxide.

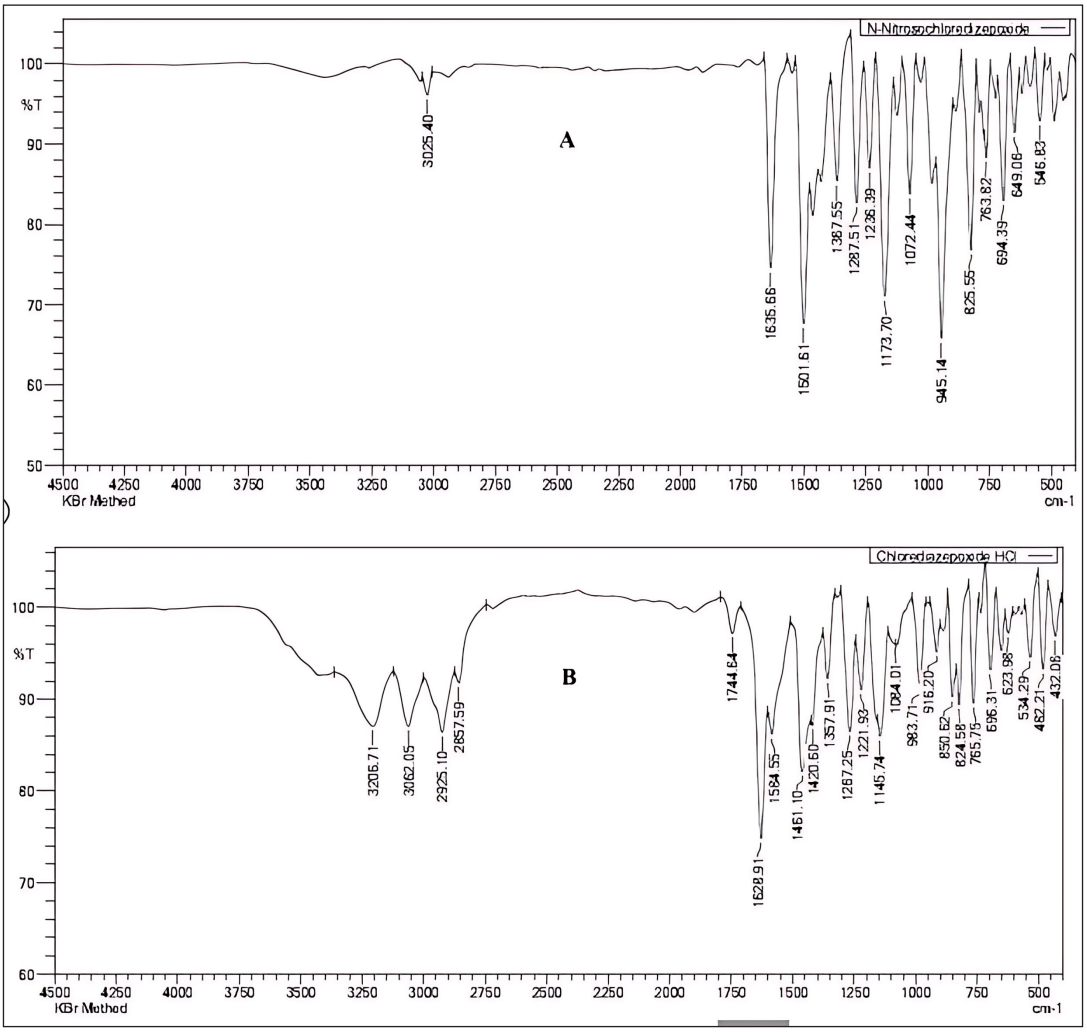

Figure 7. Comparison of Fourier transform infrared spectra: (A) N-nitrosochlordiazepoxide and (B) Chlordiazepoxide Hydrochloride.

FTIR spectroscopy

FTIR analysis was conducted by combining 2 mg of the sample with 200 mg of potassium bromide, followed by thoroughly grinding it with an agate mortar and pestle, and then compressing it with a hydraulic press to form a thin and transparent pellet. The pellet was placed in the FTIR and scanned in the infrared region 400 cm^{-1}–4,500 cm^{-1}, and the spectrum of the synthesized materials revealed the distinct stretching vibrations associated with the *N* = O nitroso group at a wave number of 1,501 cm^{-1} and N–O bonds at 945 cm^{-1} as shown in Figure 7.

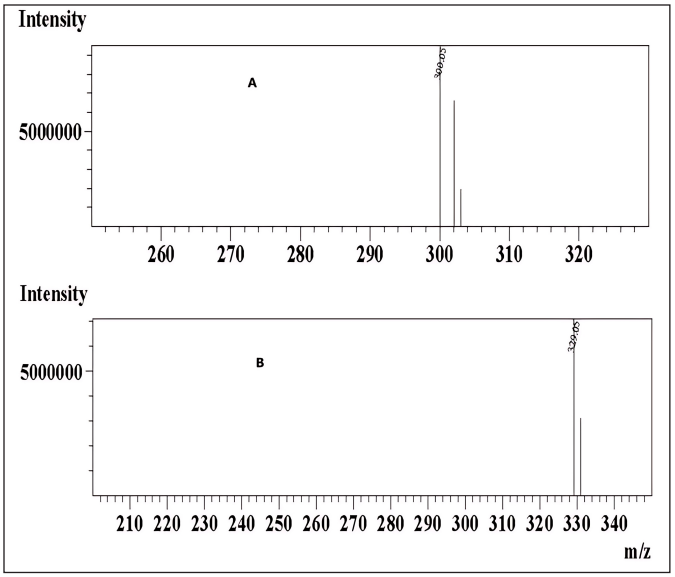

Figure 8. Mass spectrum (A) chlordiazepoxide and (B) N-nitrosochlordiazepoxide.

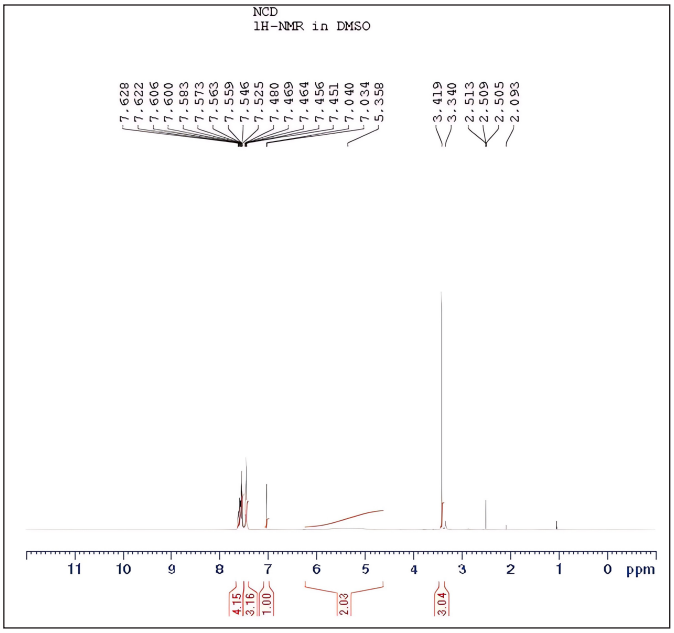

Figure 9. Proton NMR signals of N-nitrosochlordiazepoxide ranged from 0 to 12 ppm.

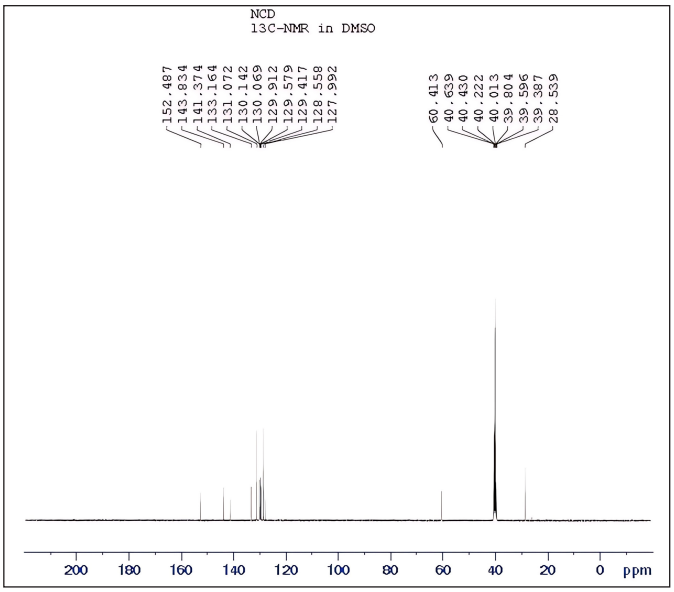

Figure 10. ^{13}C NMR spectra of N-nitrosochlordiazepoxide at 0–200 ppm.

Mass spectroscopy

In mass spectrometry, the resulting mass–charge ratios of the synthesized N-nitrosochlordiazepoxide and key starting material chlordiazepoxide were 329.05 $[M+H]^+$ and 300.05 $[M+H]^+$, as shown in Figure 8.

NMR spectroscopy

In NMR Spectrometry, the ^1H NMR δ values in DMSO-d6, as shown in Figure 10 are 7.034 to 7.628 (m, 8H, Aromatic-H), δ 5.358-(m, 2H, CH2), and δ 3.340–3.419 (s, 3H, CH_3). These results show the proton distribution within N-nitrosochlordiazepoxide as shown in figure 9.

Method validation

Specificity

From the resulting chromatograms of the spiked sample solution, impurity standard solution, sample solution, and blank solution, no interference was detected during the impurity retention period (5.5–6.5 minute). A comparison of these chromatograms confirmed that the method was specific, as demonstrated in Figures 11. and 12.

Precision and intermediate precision

The reproducibility of findings from several measurements made under the same circumstances is called precision. In the context of this method, the precision and intermediate precision were assessed through the analysis of 6 preparations of N-nitrosochlordiazepoxide at a concentration of 1.5 ppm using the developed method. The process utilized MRM transitions, yielding the precursor at m/z 329.20 and the product ions at m/z 281.90, 299.25, and 240.80. The percentage RSD of the mass areas was calculated, and for the main fragment (Q3) m/z 281.90, the method precision was less than 2.3% RSD, and for intermediate precision, less than 1.8% RSD, as shown in Table 1.

Additionally, intermediate precision was demonstrated by calculating the mean recovery results (25% LOQ, 50%, 100%, and 150%) for both analytes. The percentage RSD for both analysts was less than 2%, as depicted in Table 2. These findings provide evidence of the method's repeatability and intermediate precision.

Linearity and range

In LC-MS/MS method development, linearity and range are critical factors. Linearity ensures a direct correlation

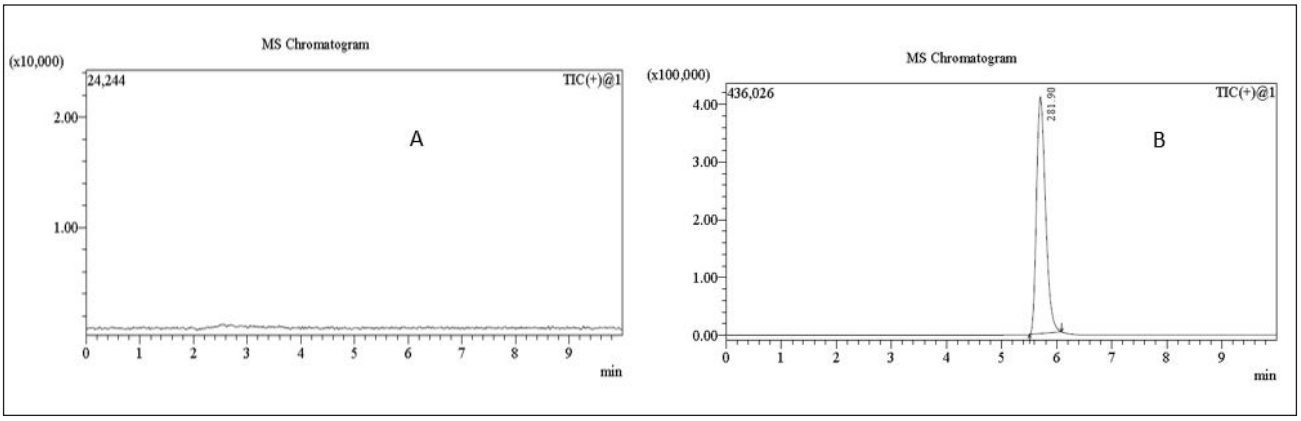

Figure 11. MRM chromatograms of (A) blank solution and (B) 0.18 ppm N-nitrosochlordiazepoxide hydrochloride (LOD) solution.

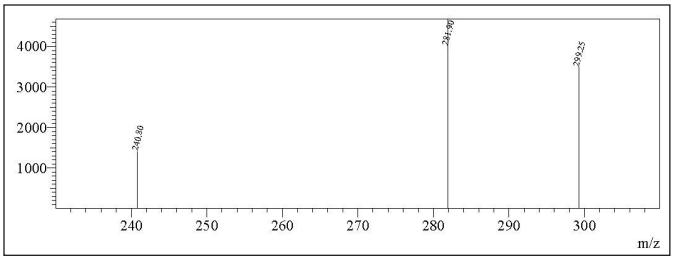

Figure 12. MRM spectrum of 1.5 ppm N-nitrosochlordiazepoxide.

between the analyte concentration and the mass detector response. Calibration curves were constructed for three product ions with m/z values of 281.90, 299.25, and 240.80, showing correlation coefficients falling within the range from 0.9980 to 0.9988.

Furthermore, the range determines the concentration range within which the method delivers accurate and precise results, encompassing the lower and upper limits of quantification. The method exhibits linearity between 0.18 ppm and 3.0 ppm in concentration, with the coefficient of determination (R^2) ranging

Table 1. The precision and intermediate precision table contains results from six replicates of the N-nitrosochlordiazepoxide impurity solution (1.5 ppm).

Analysts	Injections	Retention time (min)	Mass Area 329.20>281.90	Mass Area 329.20>299.25	Mass Area 329.20>240.80
	1	5.729	6854441	4505313	1909071
	2	5.731	6679046	4506860	1898166
Analyst-1	3	5.731	6739340	4421200	1855310
(Precision)	4	5.732	6616576	4335636	1818737
	5	5.733	6471038	4242051	1781434
	6	5.733	6471727	4282530	1749782
Average		5.732	6638694.7	4382265.0	1835416.7
SD		0.002	151512.6	113103.5	63692.7
%RSD		0.026	2.28	2.58	3.47
	1	5.713	6742641	4739666	1911275
	2	5.714	6670024	4630190	1913108
Analyst-2	3	5.714	6693034	4776485	1914799
(Intermediate Precision)	4	5.714	6844705	4544182	1835068
	5	5.715	6557226	4379443	1789159
	6	5.715	6578032	4420899	1799834
Average		5.738	5843314.3	3851453.5	1614635.0
SD		0.002	102567.3	68769.2	28166.1
%RSD		0.031	1.76	1.79	1.74

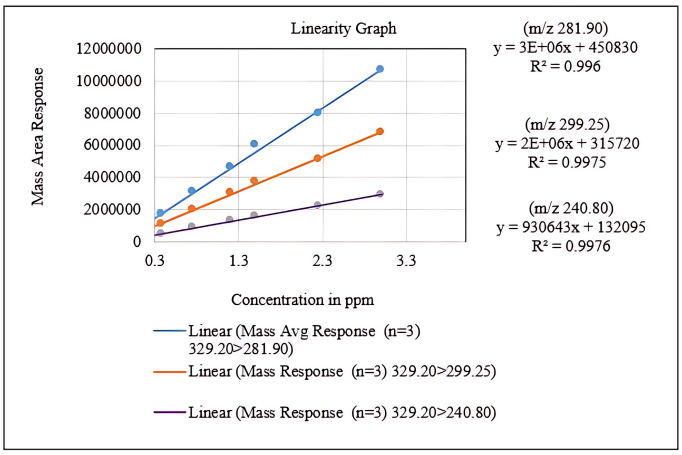

Figure 13. Linearity graph of the Q3 mass area versus concentration in ppm.

from 0.9960 to 0.9976 for all product ions. These results are illustrated in Figure 13, which shows the linearity graph.

Accuracy

Based on the recovery of the spiked samples, the method accuracy was assessed with concentrations ranging from the LOQ (0.375 ppm) to 150% (2.3 ppm), yielding a mean recovery percentage between 92.01% and 104.55%. A correlation coefficient of 0.9970 to 0.9985 was obtained for all the product-ion and the TIC. These results, as indicated in Table 3, were used to assess the method's accuracy, and regression statistics are tabulated in Table 4.

Detection and quantification limits

Based on the signal-to-noise (S/N) ratio, methods with a limit of detection ≥ 3 and quantification limit ≥ 10 were validated, as detailed in Table 5.

Table 2. Intermediate Precision 25% LOQ, 50%, 100% and 150% TIC Mass area (n = 3).

	SL.No	25% (LOQ)	50%	100%	150%
Analyst-1	1	5218741	8109094	12770947	18220163
	2	5199456	8119177	12568101	18008616
	3	5096989	8119621	12406688	17714515
	Average mass Area (TIC)	5171729	8115964	12581912	17981098
	SD	65440.75	5953.73	182521.81	253944.69
	%RSD	1.27	0.07	1.45	1.41
	Mean recovery (ppm)	0.35	0.78	1.45	2.26
	Mean recovery%	92.01	104.55	96.68	100.24
	SL.No	25% (LOQ)	50%	100%	150%
Analyst-2	1	5311953	8102768	12773677	18223017
	2	5281958	8101140	12569411	18014862
	3	5082993	8102882	12407130	17715201
	Average mass Area (TIC)	5225635	8102263	12583406	17984360
	SD	124438.37	974.50	183673.82	255278.38
	%RSD	2.38	0.01	1.46	1.42
	Mean recovery (ppm)	0.35	0.78	1.45	2.26
	Mean recovery%	93.12	103.91	96.62	100.30
% RSD (Analyst-1 and Analyst- 2)		1.91	0.06	1.31	1.27

Table 3. The accuracy ranged from 0.375 ppm to 2.3 ppm (n = 3).

Percentage spiked (%) Level	Spiked impurity Concentration (ppm)	Mass Average Area (n = 3) 329.20>281.90	Mass Average Area (n = 3) 329.20>299.25	Mass Average Area (n = 3) 329.20>240.80	TIC Mass Average Area (n = 3)	Mean Recovery (TIC) ppm	Percentage (%) Recovery
25	0.375	2640949	1761531	703108	5171729	0.35	92.01
50	0.75	4397131	2887517	1158670	8115964	0.78	104.55
80	1.2	5828364	3816330	1535882	11174908	1.24	103.36
100	1.5	6569327	4285352	1728604	12581912	1.45	96.68
150	2.25	9400333	6113738	2464823	17981098	2.26	100.24
Correlation		0.9970	0.9970	0.9971	0.9985	0.9985	
Slope		3495355	2250864	911696	670546		
Intercept		1520364	1038093	410506	2857984		

Robustness

To evaluate the robustness of the method, slight modifications were implemented to the mobile phase composition (±5%), column oven temperature (±5°C), and mobile phase flow rate (±0.05 ml/minute), and the TIC mass area was recorded for the 1.5 ppm impurity spike solution, as displayed in Table 6.

Solution stability

A spiked impurity solution concentration of 1.5 ppm was injected (n = 3), the solution stability was monitored for 48 hours, and no notable alterations were detected in the mass areas of any of the (Q3) product-ion of the impurity standard solutions. Based on these data, the solution was stable for a period of 24 hours. With a percentage RSD less than 4. As shown in Table 7.

At 48 hour, a 20%–24% reduction in the average mass area of all the product ions (m/z) was observed, as shown in Table 8.

Batch analysis

Three batches of chlordiazepoxide hydrochloride were analyzed, and no traces of N-nitrosochlordiazepoxide were detected in any of the samples as shown in Figure 14.

Table 4. Regression Statistics from 0.375 ppm to 2.3 ppm (n = 3) versus the TIC Mass Average Area.

Multiple correlation coefficient	**0.99848096**
Coefficient of determination (R^2)	0.99696422
Adjusted R^2	0.99595230
The standard deviation of the sample mean	0.04581745
Observations	5

Table 5. The LOD and LOQ mean values (n = 3).

Test	**Concentration (ppm)**	**Mass average area (TIC)**	**Signal-to-noise ratio**
Limit of Detection 12.5%	0.18	1651741	753
Limit of Quantitaion 25%	0.375	5194124	2135

Table 7. Solution stability for up to 24 hours.

SL. No	**Time interval**	**Mass area 329.20>281.90**	**Mass area 329.20>299.25**	**Mass area 329.20>240.80**
1	Initial	6854441	4505313	1909071
2		6679046	4506860	1898166
3		6739340	4421200	1855310
4	After 24 hours	6736535	4470628	1885531
5		6765657	4433265	1813458
6		6706786	4414396	1766613
Average		6746967.5	4458610.3	1854691.5
Standard deviation		60489.25	41594.28	55238.83
% RSD		0.89654	0.93290	2.97833

Table 8. Solution stability for up to 48 hours (n = 3).

Time interval	**Average mass area 329.20>281.90**	**Average mass area 329.20>299.25**	**Average mass area 329.20>240.80**
Initial	6757609	4477791	1887516
24 Hours	6736326	4439430	1821867
48 Hours	5326031	3571675	1429709

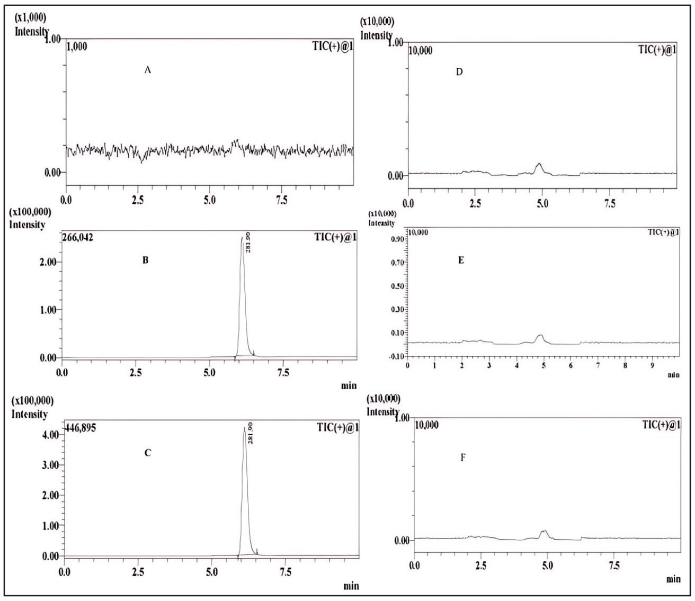

Figure 14. MRM chromatograms. A is blank, B is the LOD, C is the LOQ, and D, E, and F are sample batches 1, 2, and 3, respectively.

Table 6. Robustness of the method.

SL.No	**Actual condition**	**Column oven Temperature (35°C)**	**Column oven Temperature (45°C)**	**Mobile Phase Flow Rate 0.60 (ml/minute)**	**Mobile Phase Flow Rate 0.70 (ml/minute)**	**Methanol (%) in Mobile phase (65%)**	**Methanol (%) in Mobile phase (55%)**
1	13292922	13062348	13269374	13125663	13109950	12944181	12935782
2	13280389	13041500	13367677	12915309	12901409	12915487	12915398
3	13015848	13015595	13495726	12550834	12542688	12550834	12550834
Avg	13196386	13039814	13377592	12863935	12851349	1280350	12800671
SD	156476.3	23422.0	113501.3	290837.6	286925.2	219285.6	216605.4
%RSD	1.186	0.180	0.848	2.261	2.233	1.713	1.692

DISCUSSION

The formation of PGI in the pharmaceutical drug manufacturing process should be controlled. To understand its structure and formation, we referred to studies conducted by Robbiano *et al.* [35] and Mereto *et al.* [36], which involved *in vivo* experiments with living organisms. However, in our study, we synthesized N-nitrosochlordiazepoxide under controlled laboratory conditions, this approach enabled us to produce N-nitrosochlordiazepoxide from chlordiazepoxide in an acidic environment in the presence of sodium nitrite. We extensively characterized its physical and chemical properties, including solubility, melting point, and various spectroscopic techniques such as UV-VIS, FTIR, mass spectrometry, NMR spectroscopy, and HPLC. Additionally, we employed the Toxtree QSAR tool to explore potential structural activity relationships, determining that the compound falls within potency category 5, with an acceptable daily intake limit of 1,500 ng/day or 1.5 µg/day (1.5 ppm).

Mazzei *et al.* [42] investigated the products resulting from the interaction between sodium nitrite and chlordiazepoxide hydrochloride using HPLC. The quantification of trace-level impurities is challenging due to the lower sensitivity of the HPLC method compared to LC-MS/MS. However, no method was reported to quantify N-nitrosochlordiazepoxide in chlordiazepoxide using LC-MS/MS. Therefore, we developed a novel, precise, and accurate LC-MS/MS method, validated in compliance with ICH Q2 (R1) guidelines. Our experiment demonstrates the method's reliability under various conditions, yielding consistent results over time. This research significantly enhances our ability to accurately analyze N-nitrosochlordiazepoxide, ensuring the quality and safety of pharmaceutical products. By addressing analytical limitations and meeting regulatory requirements, our study contributes to improving drug safety and efficacy, ultimately benefiting patient health.

CONCLUSION

In conclusion, after reviewing the study references [34–37], we have selected a synthetic route for the production of N-Nitrosochlordiazepoxide from chlordiazepoxide. This synthetic route capitalizes on the observed formation of N-nitroso impurities under acidic conditions, in the presence of sodium nitrite. The comprehensive characterization of the synthesized compound included analysis of its physical and chemical properties, solubility, melting range, UV spectrum, purity assessment, FTIR analysis, mass spectrometry, and interpretation of ^{13}C and ^{1}H data, furthermore, structural activity relationship assessed using Toxtree QSAR tool.

Additionally, a novel method employing LC-MS/MS was developed for the detection and quantification of N-nitrosochlordiazepoxide, addressing a notable analytical gap in the field. This method underwent rigorous validation following ICH Q2 (R1) guidelines, demonstrating the specificity, detection, and quantification limits, linearity, range, accuracy (recovery), precision, intermediate precision, robustness, and solution stability for a period of 48 hours. This underscores the method's reliability and suitability for use in the pharmaceutical industry, furthermore, the established method was effectively utilized to analyze chlordiazepoxide hydrochloride samples across three separate batches, all of which did not exhibit any trace of N-nitrosochlordiazepoxide. This method applicability extends beyond API analysis, providing a valuable tool across the pharmaceutical sectors, including process development, quality control, and research aimed at controlling the formation of nitrosochlordiazepoxide impurity in chlordiazepoxide hydrochloride upholding its commitment to ensuring patient safety, the pharmaceutical sector can confidently rely on this robust analytical approach to maintain the quality and integrity of the active drug substance.

LIST OF ABBREVIATIONS

FTIR: Fourier transform infrared spectroscopy; ICH: The International Conference of Harmonization; LC-MS/MS: Liquid chromatography-tandem mass spectrometry;

PDA: Photodiode array detector; PGI: Potential genotoxic impurity; QSAR: Quantitative structural activity relationship; TIC: Total ion chromatogram; UV-VIS: Ultraviolet-visible spectroscopy

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All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

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This study does not involve experiments on animals or human subjects.

DATA AVAILABILITY

All data generated and analyzed are included in this research article.

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USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declares that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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