DEVELOPMENT AND VALIDATION OF A STABILITY-INDICATING HPLC METHOD FOR ANALYSIS OF 5-(4-NITROPHENYL)-1,2,4-TRIAZOLIDINE-3-ONE, USING QUALITY BY DESIGN APPROACH

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ABSTRACT:

The compound 5-(4-nitrophenyl)-1,2,4-triazolidine-3-one (4-NPTZ) was synthesized and assessed for its antimicrobial potential. Structural identity was confirmed through spectral analysis, and biological activity was compared with standard drugs. A novel reversed-phase high-performance liquid chromatography (RP-HPLC) method was developed for purity confirmation and validated for precise analysis of 4-NPTZ, emphasizing sensitivity, accuracy, and robustness. Chromatographic separation was achieved on a Cosmosil C18 column (250 mm × 4.6 mm, 5 µm) using methanol–water as the mobile phase at a 0.8 ml/min flow rate, with detection at 331 nm. The optimized method yielded a retention time of 4.374 minutes, established using Design Expert-12 software. Linearity was demonstrated over a 10–50 ppm range with an excellent correlation coefficient (r^2 = 0.9995). Precision studies indicated %RSD below 2.0%, confirming reliability, while recovery studies showed 99.79% accuracy. The limits of detection and quantitation were 0.8247 ppm and 2.4992 ppm, respectively. Stability testing under acidic, basic, oxidative, thermal, and photolytic conditions using a validated RP-HPLC method. Overall, the developed RP-HPLC method is efficient, reliable, and suitable for routine quality control and analysis of 4-NPTZ in industrial applications.

Keywords: Reversed-Phase HPLC, QbD, method development, validation, stability, Triazoles.

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INTRODUCTION

Triazole, an aromatic heterocyclic compound with a nitrogen bridge, has recently gained significant attention due to its biological activities [1]. The term "triazole" was introduced by Bladin in 1855 to refer to the carbon-nitrogen ring structure $C_2H_3N_3$ [2]. This compound is a crystalline solid with a melting point of 120°C and a boiling point of 260°C, ranging in color from white to light yellow. It dissolves in alcohol and water and has a subtle, recognizable odor. The four triazole isomers (1H-1,2,3-, 2H-1,2,3-, 1H-1,2,4-, and 2H-1,2,4-) are distinguished by the positions of three hydrogen atoms[3–4] shown in fig. 1.

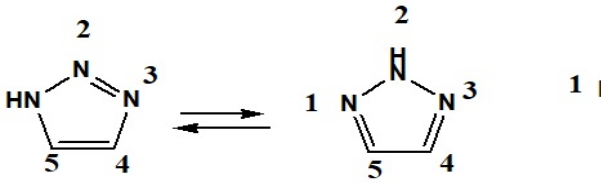


Fig. 1: Tautomeric and structural isomers of 1,2,4-triazole and 1,2,3-triazole.

Due to their wide range of biological activities, triazole derivatives have gained attention in modern medicinal chemistry, particularly with the careful application of bioisosteric replacement techniques [5–6]. Prominent medications having a triazole moiety include antifungal medications like paclobutrazole, myclobutanil, tebuconazole, posaconazole, itraconazole, and fluconazole, as well as anti-cancer medications like anastrozole, letrozole, and vorozole. Furthermore, Ribastriptan, an anti-migraine medication, has gained significant attention [7–10]. The growing apprehension over antimicrobial resistance necessitates the investigation and creation of novel compounds that can battle a wide range of bacterial and fungal strains, especially those that exhibit resistance [11–12]. The complex interactions of 1,2,4-triazole compounds, which are highlighted by their anti-urease characteristics, are a focus, revealing possible directions for treatment interventions [13]. The scientific community has paid significant attention to 1,2,4-triazole due to its wide range of biological activities, which include antimigraine, antioxidant, anti-urease, antimicrobial, anti-inflammatory, anticonvulsant, anticancer, antiviral, and antiparasitic properties [9,12,14,18-21]. The synthesis of 5-(4-Nitrophenyl)-1,2,4-triazolidine-3-one (hereafter abbreviated as 4-NPTZ), as described by Vikas D. Jadhav et al., is an example of the continuous search for novel triazole derivatives [18–20]. Shown in fig.2.

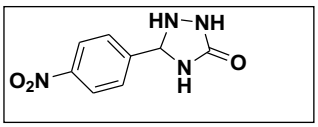
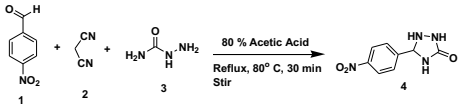


Fig. 2. Chemical structure of 4-NPTZ

The strategic creation of a high-performance liquid chromatography (HPLC) method, painstakingly crafted to enable the quantitative measurement of 4-NPTZ and purity confirmation, is essential to this research study. The proposed approach is expected to be simple, economical, accurate, reproducible, selective, and, most importantly, stability-indicating. Since HPLC is widely used in the pharmaceutical, biotechnology, environmental monitoring, and food and beverage industries, according to the International Council for Harmonisation (ICH) guidelines Q1A(R2) and Q1B, forced degradation studies are essential to evaluate the stability of pharmaceutical compounds and to establish a stability-indicating analytical method. The suggested analytical technique complies with ICH standards for drug substance stability testing, guaranteeing its ability to identify contaminants and degradation products under stressful circumstances. The Validation of the developed method follows the ICH guideline [Q2(R1)]. The ability of a stability indicator test to distinguish degradation byproducts from parent molecules adds even more evidence to support the analytical strength of the suggested approach [21–30]. Validated RP-HPLC techniques for the assay of 4-NPTZ should be developed using the Quality by Design (QbD) methodology following ICH Q8 (R2) principles to preserve process consistency throughout the product lifetime [31–35].

MATERIALS AND METHODS

Synthesis of biologically active compound 4-NPTZ: 4-NPTZ was synthesised using a controlled process involving 4-Nitrobenzaldehyde (2 mmol), malononitrile (2 mmol), and semicarbazide (2 mmol) were mixed in a solvent matrix of 80% acetic acid (4:1 Mixture of glacial acetic acid and water, glacial acetic acid (16 ml) and water (4 ml). Then the mixture was stirred and heated under reflux conditions at 80 °C for about 30 min. The yellow solid product formed was filtered, washed with water, and recrystallized from ethanol. Temperature was found to be highly effective for this chemistry, and the corresponding target 4-NPTZ was obtained in 90 %. The spectral data confirmed the formation of the unexpected compound 4-NPTZ and are in fine agreement with the literature data. Synthetic process shown in scheme 1



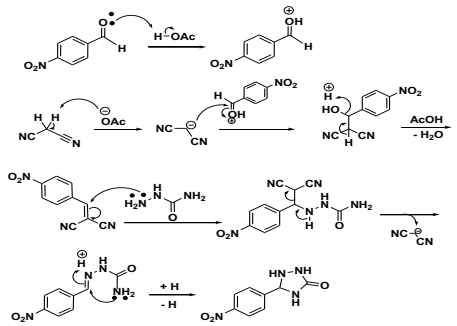
Scheme 1: Cyclocondensation reaction for the synthesis of 4-NPTZ

Spectral Data of 4-NPTZ

^{1}H NMR spectrum of the compound 4 exhibited three notable singlets at δ 10.63,6.70 ppm, confirming the presence of 3–NH proton, and the singlet at δ 7.93 ppm highlights the presence of benzylic methane proton.IR spectrum shows dissimilar absorption bands at 3441,3178, and 3070 cm^{-1}, respectively, which indicate the existence of 3-NH stretching and conjointly a band at 1666 cm^{-1} representing the presence of –C=O stretching. In the ^{13}C NMR spectrum, a peak at δ 156.9 ppm denotes the presence of the oxy-carbonyl moiety.

In addition, the LC-MS showed a molecular ion peak m/z at 207.01 M^-.

Plausible mechanism:



Scheme 2: Plausible mechanism for synthesis of 4-NPTZ

Reagents:

All reagents used in the present study were of analytical grade unless otherwise noted. The compound 4-NPTZ was synthesized in-house and characterized, with its purity confirmed at 99%. It was employed as both the test analyte and reference standard throughout the analysis. Deionized water was produced using a Milli-Q purification system (Millipore) equipped with ion-exchange and organic-removal cartridges. HPLC-grade methanol (Fisher Scientific) and orthophosphoric acid (Merck) were used without any additional purification.

Instrumentation:

Chromatographic analysis was carried out using a binary gradient HPLC system (Analytical Technologies Ltd., India) equipped with an HPLC 3000 series reciprocating pump (Model P-3000-M, 40 MPa), a UV detector (Model UV-3000-M), an inbuilt degasser, and ambient column temperature control**.** Data acquisition and processing were performed using HPLC Workstation software**.** The determination of the maximum absorbance wavelength (λ_{max}) for 4-NPTZ was achieved using a double-beam UV-Visible spectrophotometer (Model 2012, Analytical Technologies Ltd.) operating in the range of 190–1100 nm with a wavelength resolution of 0.1 nm**.** Additional instruments employed during method development and validation included a digital pH meter (Metrohm, Switzerland), an acyclomixer (Remi, India), an ultrasonic bath sonicator (Wenser Ultra Sonicator WUC-4L), and a high-precision analytical balance (Wenser PGB100). Buffer solutions were filtered through 0.45 µm nylon membrane filters (Millipore) before use.

Chromatographic Conditions:

The RP-HPLC method was optimized using a reversed-phase octadecylsilane (C18) column, specifically a Cosmosil C18 column (250 mm × 4.6 mm i.d., 5 µm particle size), selected due to the moderate polarity of the analyte. A matching C18 guard column was employed to protect the analytical column and enhance column longevity. The chromatographic separation was carried out under isocratic elution using a mobile phase composed of methanol and water (80:20, v/v)**,** with the pH adjusted to 3.0 using orthophosphoric acid. Before use, the mobile phase was filtered through a 0.45 µm nylon membrane and degassed by ultrasonication to eliminate particulate matter and dissolved gases. The flow rate was maintained at 0.8 mL/min**,** and the UV detection was performed at a wavelength of 331 nm**,** corresponding to the λ_{max} of the analyte. A 20 µL injection volume was used for all analyses, and the column was operated at ambient temperature**.** Before sample injection, the system was equilibrated with the mobile phase at 1.0 mL/min for one hour to ensure baseline stability and reproducibility. The total run time for the chromatographic analysis was 7.76 minutes**.** System suitability parameters, including retention time**,** peak symmetry (tailing factor)**,** and number of theoretical plates, were assessed before sample analysis to ensure reliable system performance and method reproducibility.

Software:

To facilitate experimental design and statistical optimization, Design-Expert software (version 12.0.10, Stat-Ease Inc., Minneapolis, MN, USA) was employed under a trial license. This software was utilized to generate the experimental matrix, assess the influence of critical method variables, and statistically evaluate the response outcomes based on a Quality by Design (QbD) approach. Analysis of variance **(**ANOVA**)** was conducted within the software environment to determine the significance of model terms and to validate the adequacy of the fitted model. In addition, Microsoft Excel was used for basic data organization, preliminary calculations, and tabulation of system suitability results. HPLC Workstation Software (Analytical Technologies Ltd., India) was used for data acquisition, chromatographic integration, and evaluation of peak parameters during the RP-HPLC analysis.

EXPERIMENTAL

Solution Preparations:

Standard Stock Solution (1000 ppm):

A standard stock solution of 4-NPTZ was prepared to support method calibration and validation. An

accurately weighed 10 mg quantity of the high-purity, well-characterized, structurally confirmed synthesized compound was transferred into a 10.00 mL volumetric flask and dissolved in methanol (used as the mobile phase). The solution was sonicated for 15 minutes to ensure complete dissolution, followed by filtration through a 0.45 µm Millipore membrane filter to eliminate particulate matter. This solution corresponds to a concentration of 1000 µg/mL (ppm) and was used as the reference standard throughout the study.

Test Solution Preparation (100 ppm):

For assay evaluation, a test solution was freshly prepared from the synthesized batch of 4-NPTZ. Initially, 10 mg of the compound was precisely weighed and dissolved in methanol in a 10 mL volumetric flask, sonicated for 15 minutes, and filtered through a 0.45 µm nylon filter to obtain a 1000 ppm stock solution. From this, 1.00 mL of the stock was transferred into another 10 mL volumetric flask, diluted to volume with methanol, sonicated again for 15 minutes, and filtered to obtain a 100-ppm intermediate solution.

Working Solution Preparation (30 ppm):

To prepare the working solution, 3.00 mL of the 100 ppm test solution was pipetted into a 10 mL volumetric flask, made up to volume with methanol, and sonicated for 15 minutes. The final solution was filtered through a 0.45 µm Millipore membrane filter, resulting in a working concentration of 30 ppm, used for analytical runs.

Mobile Phase Preparation:

The composition of the mobile phase was selected based on the pKa and physicochemical behavior of 4-NPTZ. A series of mobile phase compositions was evaluated using various combinations of methanol, water, and acetonitrile at different pH values. The optimized mobile phase consisted of methanol: water (80:20, v/v), with the pH adjusted to 3.0 ± 0.05 using orthophosphoric acid. For preparation, 800 mL of methanol was combined with 200 mL of distilled water in a 1000 mL HPLC-grade bottle. The mixture was sonicated, the pH adjusted accordingly, and the solution was filtered through a 0.45 µm nylon membrane filter and degassed by ultrasonication before use.

Preparation of 10.00 mM Phosphate Buffer (pH 3.0):

A 10.00 mM phosphate buffer was prepared by dissolving 0.136 g of potassium dihydrogen orthophosphate (KH_2PO_4) in 100.0 mL of distilled water. The pH of the solution was carefully adjusted to 3.0 ± 0.05 using a diluted orthophosphoric acid (OPA) solution. This buffer was evaluated during method development but not selected in the final optimized mobile phase.

Detection Wavelength Determination:

The UV-Vis absorption spectrum of the standard 4-NPTZ solution (100 ppm) was recorded using a double-beam UV-visible spectrophotometer in the wavelength range of 200–400 nm. The maximum absorbance (λ_{max}) was observed at 331 nm, which was selected as the detection wavelength for all RP-HPLC analyses as shown in fig. 3.

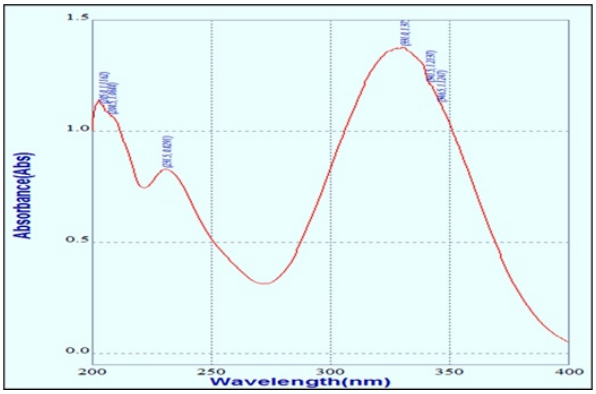


Fig. 3: UV spectrum of 4-NPTZ.

Design of Experiments (DoE) for Method Optimization:

To optimize the chromatographic conditions, a Box-Behnken design (BBD) was applied as part of a response surface methodology (RSM) strategy. A total of 17 experimental runs were generated from a 3^3 factorial design, which systematically evaluated the influence of three independent variables on the analytical performance of the method. The selected independent factors were: mobile phase composition (Factor A), flow rate (Factor B), and detection wavelength (Factor C). These variables were studied at three different levels.

The method performance was assessed based on three dependent (response) variables: retention time (RT), peak asymmetry, and number of theoretical plates. Statistical evaluation of the experimental data was performed using analysis of variance (ANOVA), and all model fitting and diagnostics were carried out using Design-Expert software (version 12.0.10.0; Stat-Ease Inc., Minneapolis, MN, USA). Three-dimensional response surface plots and corresponding contour plots were generated to visualize the effect of the input variables on each response. The results of the optimization study, including the selected method conditions, are presented in Table 1, while the graphical outputs of the model are illustrated in fig. 4.

Table 1: Box -Behnken design of DOE

Run	Factor A Composition (MeOH)	Factor B Flow rate	Factor C Wavelength	Response 1 R.T. (min)	Response 2 Asymmetry	Response 3 TP
1	70	1	329	4.148	1.32	7312
2	60	0.9	329	6.693	1.32	6983
3	60	0.9	333	6.386	1.31	7056
4	80	1	331	3.689	1.29	7456
5	70	0.9	331	4.768	1.3	7470
6	70	0.9	331	4.768	1.3	7470
7	70	0.9	331	4.768	1.3	7470
8	70	0.8	329	6.243	1.32	6912
9	60	0.8	331	7.078	1.34	6054
10	80	0.9	333	4.033	1.28	7415
11	70	0.9	331	4.768	1.3	7470
13	70	1	333	4.112	1.34	7319
14	70	0.9	331	4.768	1.3	7470
15	80	0.8	331	4.489	1.24	8437
16	70	0.8	333	5.886	1.33	7896
17	60	1	331	5.639	1.29	7232

The response surface and contour plots as shown in fig. 4. reveal the combined influence of composition (Factor A) and flow rate (Factor B) on three critical responses: retention time, asymmetry factor and theoretical plates, while holding wavelengh (Factor C) constant at 331.

Retention Time:

A clear inverse trend was observed for retention time as shown in fig. 4. (a). Increasing composition and flow rate both led to a reduction in retention time, with the lowest values achieved at higher levels of both factors. This reflects improved elution efficiency under optimized chromatographic conditions.

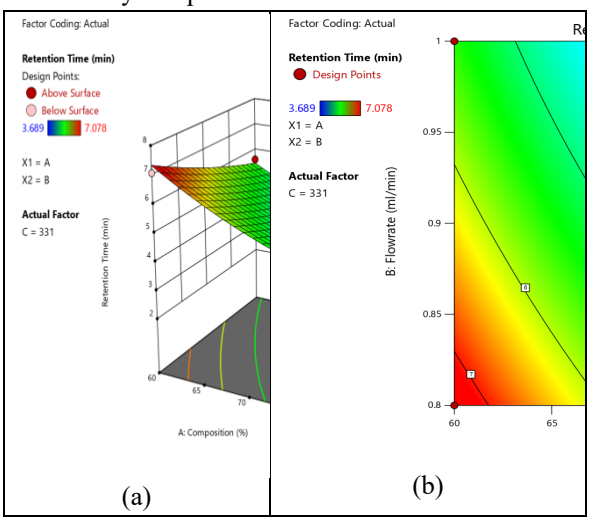
Asymmetry Factor:

The asymmetry factor remained relatively stable across the studied range, with only minor fluctuations as shown in fig. 4. (c). The values stayed within an acceptable range (1.24–1.34), indicating that neither factor significantly distorted peak symmetry. This highlights the robustness of the method with respect to peak shape.

Theoretical Plates

The number of theoretical plates increased moderately with higher composition and lower flow rate, signifying improved column efficiency under these conditions as shown in fig. 4. (e). The response surface suggests that composition has a greater influence compared to flow rate on plate count. Maximum efficiency was obtained at high composition and moderate flow rates.

Overall, the RSM analysis indicates that optimal chromatographic performance characterized by higher reduced retention time, acceptable asymmetry factor, and higher theoretical plates is achieved at higher composition and flow rate levels. These findings provide a reliable basis for method optimization and robust analytical performance.



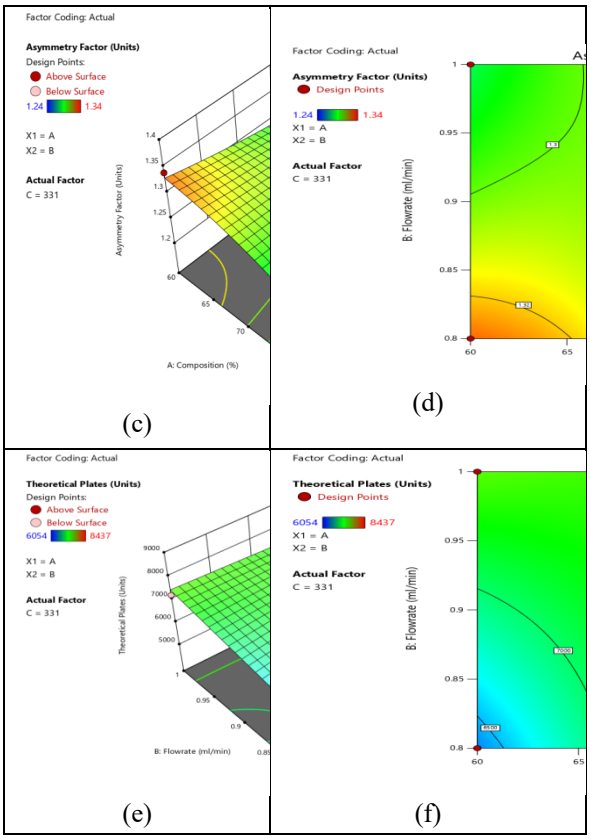


Fig. 4: Response surface and contour plots for method optimization.

Methanol and water (80:20) at pH 3 (pH is modified with o-phosphoric acid) made up the mobile phase. A Millipore 0.45µm filtration membrane was used to filter the mobile phase. Before the actual analysis, the HPLC system was run through baseline monitoring for a minimum of one hour at a flow rate of one milliliter per minute. 4-NPTZ was detected at 331 nm with a mobile phase flow rate of 0.8 mL/min. A 20 µL injection volume was used. Optimizing a range of factors, including detection wavelength, temperature of column, flow rate, and mobile phase composition, among others, in order to accomplish the intended results.

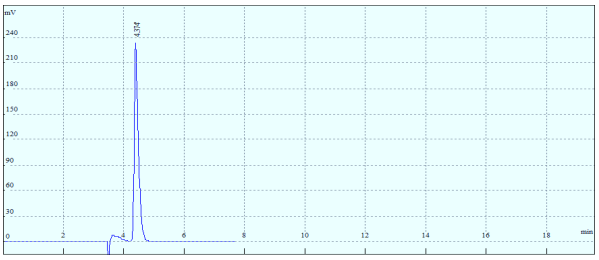


Fig.5: Typical chromatogram of optimized run

Conducted the HPLC run using 4-NPTZ at a concentration of 30 ppm. A strong peak with a retention time of 4.374 minutes is detected under these optimised chromatographic conditions, as illustrated in Figure 04, with a sample volume of 20µL injected at a flow rate of 0.8 mL/min and pressure of 9–10 MPa. The run time was 7.75 min. Chromatographic circumstances in the trial were therefore employed to validate the technique. The analytical data of optimized run as summarized in table 2,

Table 2: Analytical data of the optimized run

Parameter	R.T. (min)	Area	Asymmetry	Theoretical plate (USP)
Observation	4.374	3532729	1.18	8007

Forced Degradation Study of 4-NPTZ:

Forced degradation studies are critical in the development of stability-indicating methods [26-27], allowing for the identification of degradation pathways and the chemical behavior of pharmaceutical substances under various stress conditions. According to ICH guideline Q1A(R2), such studies are essential to demonstrate the specificity of an analytical method and to ensure the separation of the active pharmaceutical ingredient (API) from its degradation products. In the present study, forced degradation of 4-NPTZ was carried out under acidic, alkaline, oxidative, thermal, and photolytic conditions using a validated RP-HPLC method with UV detection to evaluate its chemical stability and degradation profile. The data of forced degradation study presented in table 3.

Acid Hydrolysis:

A quantity of 10.70 mg of 4-NPTZ was accurately weighed and transferred into a 10 mL volumetric flask. To this, 2 mL of mobile phase and 3 mL of 1 N hydrochloric acid were added, and the mixture was kept at room temperature for 6 hours to induce acid hydrolysis. The reaction mixture was then allowed to cool and neutralized with 1 N sodium hydroxide, followed by dilution to volume with the mobile phase. An aliquot of 0.1 mL was further diluted to 10 mL using the mobile phase before analysis. The resulting chromatogram is presented in fig.6 (b).

Alkali Hydrolysis:

For alkaline degradation, 10.38 mg of the compound was placed in a 50 mL volumetric flask, followed by the addition of 2 mL of mobile phase and 0.5 mL of 0.1 N sodium hydroxide. The solution was stored at room temperature for 75 minutes, after which it was neutralized using 0.1 N hydrochloric acid and diluted appropriately with mobile phase. A 0.1 mL aliquot was then transferred to a 10 mL volumetric flask, diluted to volume with mobile phase, and analyzed. The chromatogram is shown in fig. 6 (c).

Oxidative (Peroxide) Degradation:

For oxidative stress, 10.69 mg of 4-NPTZ was dissolved in 2 mL of mobile phase, and 8 mL of 30% hydrogen peroxide was added. The solution was maintained at room temperature for 1 hour, cooled, and transferred into a 25 mL volumetric flask, then diluted to volume with mobile phase. Subsequently, 0.1 mL was pipetted into a 10 mL flask, diluted with mobile phase, and analyzed. The peroxide degradation chromatogram is provided in fig. 6 (d).

Photolytic Degradation:

To study photolytic stability, 10.13 mg of the sample was weighed and dissolved in mobile phase in a 10 mL volumetric flask. The solution was exposed to light and diluted to the mark. A 0.1 mL aliquot was further diluted to 10 mL with mobile phase before chromatographic analysis. The corresponding chromatogram is presented in fig.6 (e).

Thermal Degradation:

For thermal degradation, the solid drug sample was subjected to 105 °C in a hot air oven for 10 days. After exposure, 10.32 mg of the thermally stressed sample was weighed, transferred to a 10 mL volumetric flask, dissolved in mobile phase, and diluted to volume. A 0.1 mL aliquot was then taken, diluted to 10 mL, and analyzed. The thermal degradation profile is depicted in fig.6 (f).

Among all applied stress conditions**,** significant degradation was observed under acidic and alkaline hydrolysis, with distinct separation between the parent compound and its degradation products. In contrast, the compound exhibited minimal or no degradation under oxidative, thermal, and photolytic stress**,** as indicated by the stable chromatographic profiles and unchanged physical appearance in solid and solution states during photolytic exposure.

Furthermore, the degree of degradation under each condition remained within the target range of 10% to 30%**,** validating the specificity and reliability of the method for detecting degradants. These results confirm that the developed RP-HPLC method is specific, robust, and suitable for the detection of degradation products of 4-NPTZ, making it an effective tool for the stability assessment of the compound under ICH-recommended stress conditions.

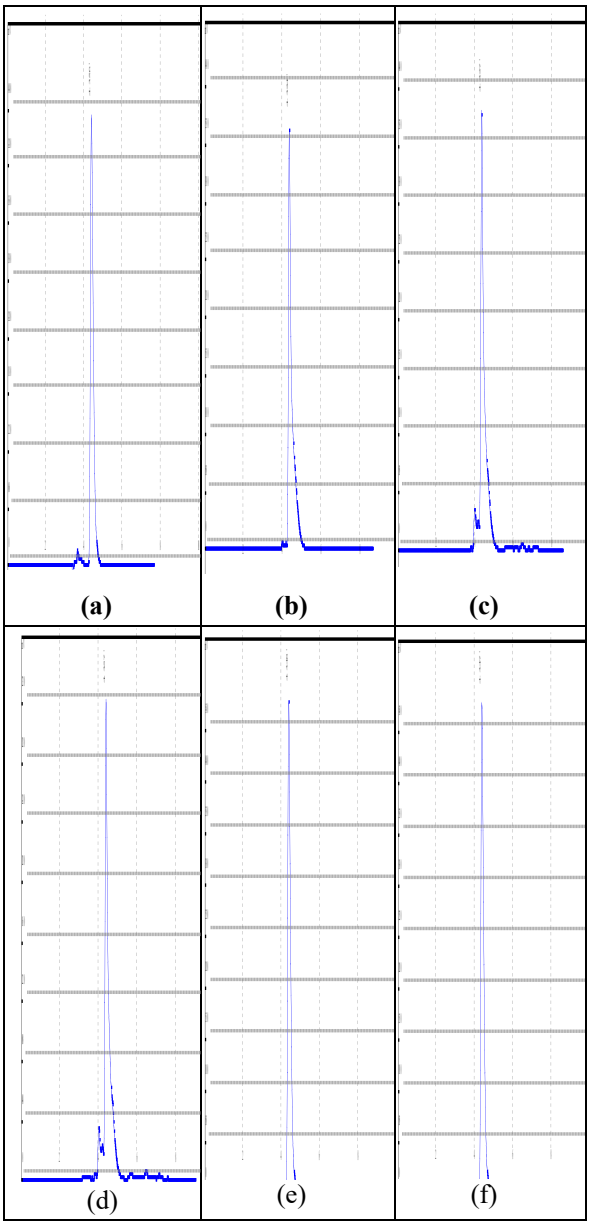


Fig. 6: Chromatograms showing various stress conditions: a) 4-NPTZ without degradation; b) 4-NPTZ after acid degradation.; c) 4-NPTZ after base degradation; d) 4-NPTZ after peroxide-induced oxidative degradation.; e) 4-NPTZ under Photolytic conditions.; f) 4-NPTZ after thermal degradation.

Table 3: Result of Degradation data

Sr. No.	Degradation Type	Area of Standard	Area of degraded sample	% Degradation	R.T. (min)
1	Untreated	6416684	-	0.000%	-
2	Acid Degradation	6416684	5835712	9.05%	4.324
3	Base Degradation	6416684	5602238	12.69%	4.327

4	H_2O_2 Degradation	6416684	6101462	4.91%	4.357
5	Photolytic Degradation	6416684	6302858	1.77%	4.348
6	Thermal Degradation	6416684	6296147	1.88%	4.347

3	3566765	1.18	8009
4	3570420	1.17	8005
5	3580396	1.18	8012
6	3576929	1.19	8004
Mean	3575042	1.18	8007
SD	5362.93	-	-
% RSD	0.150		
-	NMT 2.0 %	NMT 2.0 %	More than 2000

Method Validation:

Method validation is a critical step in the development of an analytical procedure to ensure that the method is scientifically sound, reproducible, and suitable for its intended purpose. It establishes parameters such as specificity, accuracy, precision, linearity, robustness, and detection capability, which are necessary to demonstrate the method's performance and reliability. As per ICH Q2(R1) guidelines[24], the RP-HPLC method developed for the estimation of 4-NPTZ was validated through a series of systematic experiments.

System Suitability Test (SST):

Data analysis shows that the approach satisfies requirements for system suitability. The system appropriateness parameter, therefore, satisfied method validation criteria. The analytical data presented in table 4 are provided for system suitability tests.

Table 4: System Suitability Parameters of the method and Standard Value

Sr. No	Peak Area	Tailing Factor	Theoretical Plates
1	3576180	1.18	8002
2	3579560	1.18	8010

Range:

Based on the linearity evaluation, the method demonstrated a valid working range from 10 ppm to 50 ppm, covering the expected concentration levels of 4-NPTZ in routine analysis.

Level/ Conc.(µg/mL)	Peak areas were recorded for three replicate runs.	Mean area
10	821665	827990
	831014	
	831291	
20	2259664	2290623
	2313762	
	2298442	
30	3548502	3579560
	3606123	
	3584055	
40	4899021	4934098
	4953098	
	4950176	
50	6340514	6416684
	6462526	
	6447012	

Linearity and Range:

Linearity was established across a concentration range of 10–50 ppm by injecting standard solutions at five concentration levels in triplicate. The calibration curve exhibited a strong linear correlation between peak area and concentration, with a correlation coefficient (R^2) was 0.9995, satisfying the ICH requirement. The linearity data and calibration curve are presented in tables 5, and fig. 7, respectively.

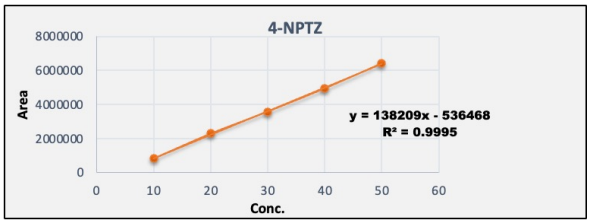


Fig.7: Linearity plot of 4-NPTZ.

Table 5: Result of Linearity

Limit of Detection (LOD) and Limit of Quantification (LOQ):

LOD and LOQ were determined using the signal-to-noise approach and calculated from the standard deviation of the response (σ) and the slope of the calibration curve (S) using the following ICH-recommended formulas:

$$LOD = 3.3 \times \sigma / Slope$$

$$LOQ = 10 \times \sigma / Slope$$

The limit of detection (LOD) and limit of quantification (LOQ) for 4-NPTZ were determined to be 0.8247 µg/mL and 2.4992 µg/mL, respectively.

Accuracy (% recovery):

The accuracy of the RP-HPLC method developed for 4-NPTZ was assessed using the standard addition technique at three concentration levels: 50%, 100%, and 150%. A fixed concentration of 20 ppm test solution was spiked with 10 ppm, 20 ppm, and 30 ppm of standard solution to achieve final concentrations of 30, 40, and 50 ppm, respectively. Each spiked level was analyzed in triplicate, and the percentage recovery was calculated using the following formula:

Recovery (%) = (Mean sample area/Standard area) ×100

All recovery values were found to lie within the acceptable range of 99.7369 to 99.8901, demonstrating the accuracy, reliability, and quantitative suitability of the proposed method for 4-NPTZ. The detailed recovery results are provided in table 6.

Table 6: Accuracy results and statistical data (% recovery) for 4-NPTZ

Recovery Level (%)	Combined Conc. (µg/mL)	Area	Mean Area	Standard Area	Recovery %
50 %	30	3568902	3570143	3579560	99.7369
	30	3570981			
	30	3570546			
100 %	40	4920745	4922179	4934098	99.7584
	40	4922985			
	40	4922807			
150 %	50	6408120	6409634	6416684	99.8901
	50	6410450			
	50	6410332			

Precision:

Precision of the method for 4-NPTZ was assessed in terms of repeatability (intra-day) and intermediate precision (inter-day). The method was tested at 30 µg/mL concentration. Intra-day precision was determined by analyzing three replicate injections at different time intervals on the same day (morning and evening), and the results were expressed as the percentage relative standard deviation (% RSD). To assess inter-day precision, freshly prepared standard solutions were injected in triplicate over three successive days**,** evaluating the method's reproducibility over time. The low % RSD values obtained in both cases confirmed the method's precision and temporal consistency. A summary of the precision data is provided in table 7**.**

Table 7: Precision data for 4-NPTZ (intra-day and inter-day)

Parameter	Intraday precision	Area	Mean area	Interday precision	Area	Mean area
Area	Morning	3579560	3569468	Day 1	3579560	3569468
		3562634			3562634	
		3566210			3566210	
	Evening	3580075	3567618	Day 2	3570804	3572163
		3556508			3558362	
		3566271			3587325	
				Day 3	3569148	3569751
					3578230	

Sr. No.	Parameter	Observations						Limit
		Change in pH			Change in wavelength			
		3	3.2	2.8	329	331	333	
1	Peak area	22906233	22864566	22819336	22874366	22906233	22770255	NMT 2
	%RSD	0.1900285			0.3112272			
2	Theoretical plates	8320	8184	8453	7757	8320	7971	NLT 2000
3	Tailing factor	1.18	1.17	1.18	1.17	1.18	1.17	NMT 2
4	R.T. (min)	4.389	4.387	4.374	4.342	4.389	4.338	-
5	Response Change %	0.00	-0.18	+0.06	-0.14	0.00	-0.59	
						3561875		
% RSD		0.2642		3568543	0.2673			3570460

Assay of 4-NPTZ:

The assay of 4-NPTZ was performed using a validated RP-HPLC method. A working concentration of 30 µg/mL was prepared for both the reference standard and the test solution of 4-NPTZ. The percentage assay was calculated using the following equation:

Assay (%) = (Area of test/Area of standard) ×100

The assay values obtained from replicate injections were found to be consistent and within acceptable limits, confirming the accuracy of the method. The detailed assay results are presented in table 8.

Table 8: Assay results of 4-NPTZ using RP-HPLC method

Sr. No.	% Composition	Area of Standard	Area of sample	% Assay
1	% Assay	3579560	3532729	98.69 %

Robustness:

The robustness of the developed RP-HPLC method for 4-NPTZ was evaluated by introducing minor, deliberate changes to method parameters, including wavelength variation (±2 nm) and mobile phase pH adjustment (±2 units)**.** A 20 µg/mL working solution was used for all robustness trials to ensure consistency in evaluation. The impact of these variations on method performance was assessed by monitoring peak area response**,** retention time**,** theoretical plates**,** and tailing factor. The calculated %RSD values and response changes remained within acceptable limits, confirming the method's reliability under small but deliberate variations. The detailed results are summarized in table 9**.**

Table 9: Result of robustness.

CONCLUSIONS

A robust and reliable RP-HPLC method was successfully developed and validated for the Purity and quantitative analysis of 4-NPTZ using a Quality by Design (QbD) approach. Method optimization through Design of Experiments (DoE) and validation per ICH Q2(R1) guidelines confirmed the method's specificity, linearity, accuracy, precision, robustness, and sensitivity. The method showed excellent linearity ($R^2 = 0.9995$) at a detection wavelength of 331 nm and demonstrated clear separation of degradation products under forced degradation conditions, indicating its stability-indicating capability.

The assay method provided consistent results with a short retention time, making it suitable for routine quality control, regulatory testing, and research applications. The system suitability parameters were within acceptable limits, further affirming the method's analytical reliability. Overall, this validated RP-HPLC method is cost-effective, precise, and fit for the intended purpose of analyzing and monitoring the stability of 4-NPTZ in pharmaceutical development.

ABBREVIATIONS

4-NPTZ stand for 5-(4-nitrophenyl)-1,2,4-triazolidine-3-one, RP-HPLC stands for reversed-phase high-performance liquid chromatography, whereas %RSD refers to percentage recovery. LOD: Limit of detection.

LOQ (Limit of Quantification) QbD means Quality by Design. USFDA refers to the United States Food and Drug Administration, ICH stands for the International Council for Harmonization of Technical Requirements for Pharmaceutical for Human Use, and OPA stands for orthophosphoric acid.

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