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Original Article

RP-HPLC-DAD Method Development and Validation for 1,3,4-Oxadiazole Derivative to Evaluate Its Forced Degradation Behavior and Stability

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Abstract

In the current study, a straightforward high-performance liquid chromatographic (HPLC) approach was developed and validated to identify a novel drug compound called 5-(4-bromophenyl)-N-(2-chloro-4-nitrophenyl)-1,3,4-oxadiazol-2-amine (A3). By putting the compound's solution under hydrolytic, oxidative, and photolytic stress, the method's capacity to detect stability was put to the test. A gradient mobile phase of acetonitrile, orthophosphoric acid, and methanol (90:051:05 v/v), at a flow rate of 1.00 mL/min, was used for the chromatographic separation on a C18 column (Promosil, 5µ, 4.60 250 mm), which was maintained at 40°C and a photodiode array detector was used for detection. At concentrations between 10 and 100.00 µg/mL, Beer's rule was observed. The recovery (99.25–100%, standard deviation [SD] 5%), intraday accuracy and precision (98.62–99.91%, relative standard deviation [RSD] 5%), interday accuracy and precision (96.25–99.91%, RSD 5%), and intermediate accuracy and precision (98.10–99.91%, RSD 5%) all indicated that the developed method was reliable, repeatable, reproducible, and robust. In cases of thermal and moisture deterioration, respectively, the compound's peak resolution and selectivity factors from the nearest resolving peak revealed specificity and selectivity. The selected synthesized compound barely broke down under oxidative and alkaline hydrolytic stress. However, the compound was resistant to photolysis in neutral and acidic environments. The results of this study demonstrate the sensitivity, specificity, and selectivity of the established approach for quality control, stability testing, and preformulation investigations.

Keywords
- 1,3,4-oxadiazole derivative
- column chromatography
- RP-HPLC
- forced degradation

Introduction

A novel derivative 1,3,4-oxadiazole (5-(4-bromophenyl)-N-(2-chloro-4-nitrophenyl)-1,3,4-oxadiazol-2-amine (A3) is a five-membered heterocyclic compound with two nitrogen, one oxygen, and two carbon atoms (►**Fig. 2**). This system is crucial not just in medicinal chemistry but also in pesticide, polymer, and material sciences.[1] Because of its broad range and capacity to target various biological targets, 1,3,4-oxadiazole is a

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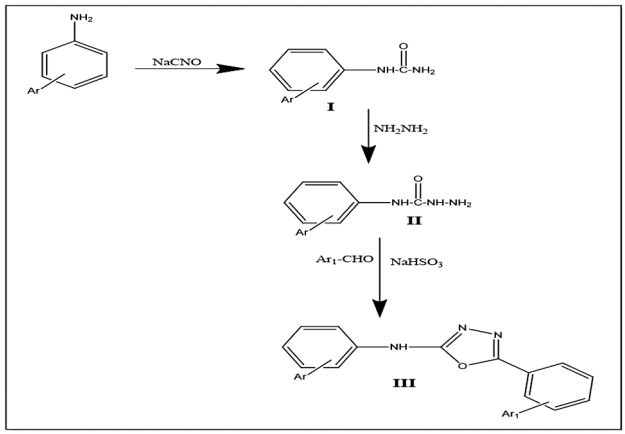

Fig. 1 Chemical structure, infrared (IR), and high-performance liquid chromatographic (HPLC) spectra of pure compound A3.

popular scaffold and an exciting pharmacophore for the development of novel drugs.[2,3] As a result, researchers continued to be interested in molecules having this heterocyclic structure. 1,3,4-oxadiazole derivatives, because of their widespread biological and pharmacological activity, are found to be a fascinating compound for the researchers.[4,5]

In order to accurately measure and identify the analyte in the presence of its degradation products, process contaminants, and excipients, stability-indicating methods are established protocols that may detect changes in the physicochemical properties of a compound. The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) recommendations strongly advocate forced degradation studies on new compounds, active ingredients, and formulations to assess the suitability and applicability of the prescribed analytical techniques in stability testing and routine quality control assessments.[6] In this study, we developed and validated a novel reversed-phase high-performance liquid chromatography (RP-HPLC) method for the A3 compound. In addition, these studies' findings provide crucial details about a molecule's inherent stability properties, which can be used to identify potential degradation products and pathways/mechanisms.[7]

Analytical method development plays a crucial role in the exploration, advancement, and production of pharmaceuticals. RP-HPLC stands out as the most versatile and sensitive analytical procedure, and it possesses the unique ability to handle mixtures with multiple components effortlessly. RP-HPLC is an exceptionally effective technique for purifying a wide array of compounds, including synthetic ones. It is often the preferred method for analyzing and separating intricate mixtures in order to identify specific compounds, consistent reproducibility, and efficient recovery.

Materials and Methods

Chemicals and Reagents

Reagents and chemicals were purchased from Merck, Himedia, India. The mobile phase was composed of HPLC-grade acetonitrile, water (Millipore water), and methanol in the ratio 9:0.5:0.5. Syringe filters were made of 0.22-mm nylon. The method development and validation analysis were carried out by using an HPLC (LC-20AD Prominence) photodiode array detector with quadruplet solvent system (Shimadzu, Japan). The infrared spectra were recorded by using Bruker's alpha attenuated total reflectance (ATR) Fourier transform infrared spectroscopy (FT-IR) spectrometer.

General Method for the Synthesis of 5-substituted-n-aryl-1,3,4-oxadiazol-2-amine analogues[8]: All the new compounds were synthesized as per the reported procedure (►**Fig. 1**–►**Fig. 2**).[9]

5-(4-bromophenyl)-N-(2-chloro-4-nitrophenyl)-1,3,4-oxadiazol-2-amine (A3): Yellow solid. Molecular formula: $C_{14}H_9BrClN_4O_3$; percentage yield: 78%, 122 to 126°C; FT-IR (cm^{-1}): 3,350.02 (NH), 3,059.46 (CH, Ar-H), 1,627.27 (C = N), 1,561.07 (C = C), 1,112.12 (C–O–C), 7,62.96 (C-Br). Lambda max: 235 by HPLC method; retention time (RT): 3.350 minutes.

Physicochemical Properties of the Synthesized Derivatives

Solubility

Solubility of the synthesized compound A3 was performed by dissolving the sample in acetonitrile (ACN) and water (50%). The amount of solid dissolved in specified quantity of solvent will be determined by the RP-HPLC method (purity and RT).

Method Development and Sample preparation

Mobile Phase

ACN, 0.1 N orthophosphoric acid (OPA), methanol in the ratio 90:05:05 is maintained at pH 7.0 and degassed.[7] The working standard solution was prepared by using stock solution, which was diluted with the mobile phase in the range of 10 to 100 µg/mL.

Working Standard

The compound A3 aliquot (10 µL; Launa 5µ 250 4.80 mm) was passed through the column (HPLC 272817-7). The proportions of ACN, OPA, and methanol in the mobile phase were set at 90:05:05. The temperature was kept at 40°C and the flow rate at 1.0 mL/min. Before starting the experiment, the system was also adjusted with the solvent system to obtain a smooth baseline.[10]

System Suitability and Method Validation

Some of the key parameters like theoretical plates (TP; *N*), area under the curve (AUC), height equivalent to the theoretical plate (HETP), and percentage assay were all calculated using the chromatogram.[11] By using ICH methodologies, the technique's accuracy and limit of detection (LOD) were confirmed. Capacity is shown by limit of quantification (LOQ), linearity, precision, robustness, and stability.

Linearity and Beer's Range

By using the chromatographic conditions described earlier, the working standard solutions (10–100 µg/mL) were analyzed in triplicates, independent repeats, and not repetitions at the

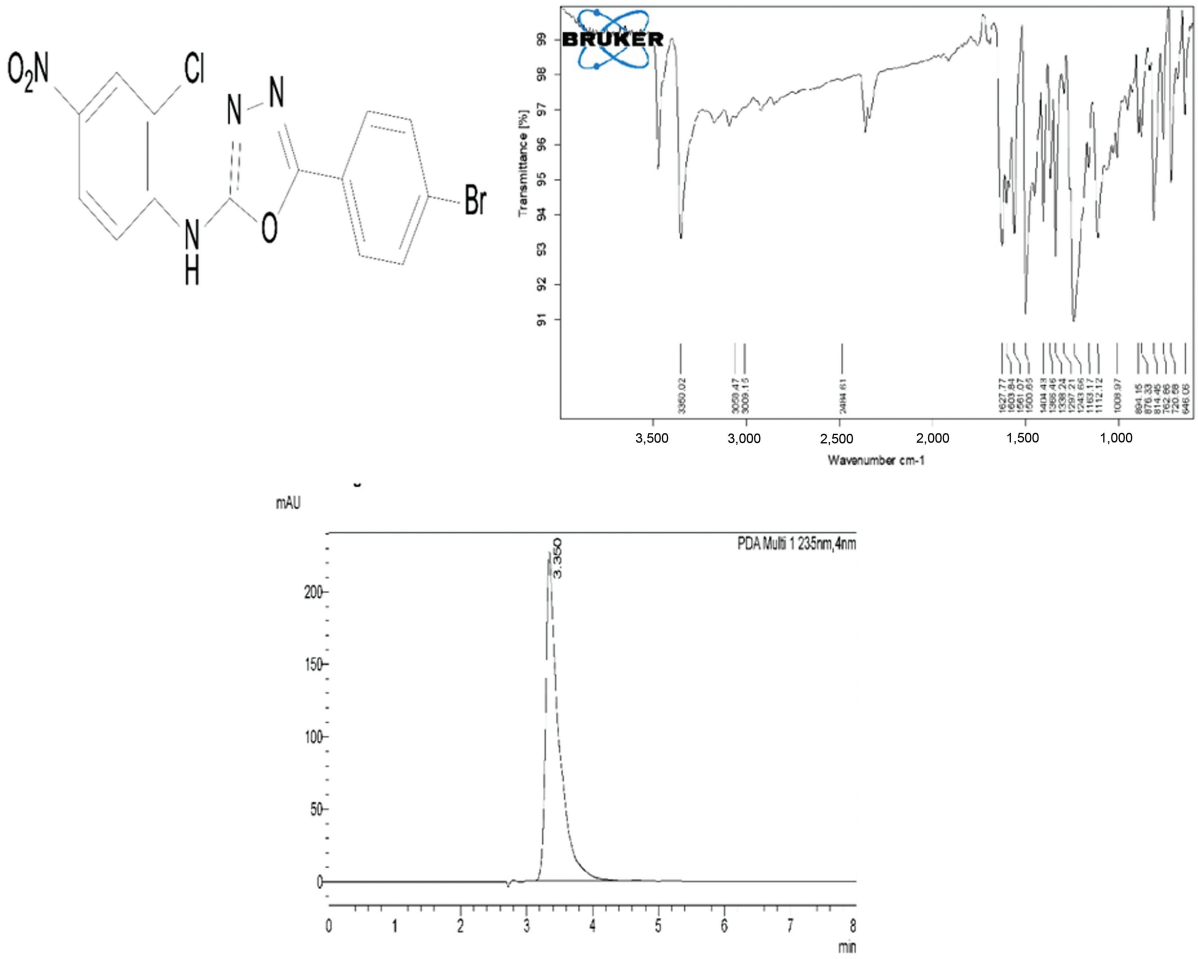

Fig. 2 Route for synthesis of compound A3.

same readings. The model's linearity was visually assessed using the concentration versus peak area (mAU*s) plot and confirmed using the linear regression equation (*I*) and Pearson's correlation coefficient (R^2: 0.990–1.000). The linearity investigations yielded the Beer range:

$$Y = a + bX(I),$$

where *X*, *Y*, *a*, and *b* represent explanatory variable (concentration), dependent variable (peak area), intercept, and slope, respectively.[7,12]

Specificity, Sensitivity, and Selectivity

The two main parameters of specificity are selectivity and forced degradation. The researchers were able to measure selectivity by delivering a 10-µL solution of the test sample solution.[13] It is carried out using the LOQ and LOD. By evaluating a number of solutions with concentrations ranging from 1 to 100 mg/mL, the LOD and LOQ of the sample were evaluated.[14,15]

Forced Degradation

Stress testing is the major use of forced degradation. Different parameters, including heat degradation, humidity, acidic, basic, and oxidative degradation, were used to execute forced degradation. Samples were subjected to thermal deterioration by being held at 60°C for up to 24 hours. The samples' moisture deterioration was held at 35°C for 7 days. While 0.1N NaOH is added to the samples and 0.1N HCl was used for neutralization for basic degradation, they were treated with 0.1N HCl for acid degradation for 5 hours. H_2O_2 is commonly used for oxidative breakdown and the samples were given at 3% H_2O_2 treatment and kept at room temperature for 24 hours.[7,10,14]

Forced degradation studies are essential in the development of analytical methods, specification setting, and formulation design within the quality by design (QbD) framework. Forced degradation is also known as stress testing and intentional degradation.

Accuracy and Precision

The method's precision and accuracy (repeatability, reproducibility, and robustness) were assessed using the same concentration levels used in recovery experiments. By analyzing the solutions three times in 1 day and once every day for 3 days straight,[16] the solution's repeatability and reproducibility (intra- and interday accuracy and precision) were evaluated.[17] The accuracy and precision measurements used were relative standard deviation (RSD) and percent recovery.[18,19]

Stability of Analytical Solution

The stability of the sample was tested by preserving the samples for 0, 24, and 48 hours at room temperature (37 ± 2°C) and monitoring for changes.[20,21]

Filter Interference

This tests whether aliquots can be prepared using the filter accomplished using a 0.45-µm nylon filter and a test sample that has been centrifuged at 5,000 rpm.[22]

Results

To conduct the test, a specific volume of the sample was injected. The RSD was less than 1.0% for the peak and RT area, and the tailing factor (TF) was less than 1.2. Theoretical plates for test samples were 1646. The assessment on system appropriateness is shown in ►**Table 1**. The sample solution was added to the chromatographic apparatus, and an AUC value was noted for each peak. Percent assay was used to calculate the amount.

Using linear regression (R^2) analysis, the calibration's linear standard curve was evaluated. R^2 was $Y = 58{,}607x + 118{,}188$ for the test sample. The observed correlation coefficient (r) for equality was discovered to be 0.9953. With an R-value of 0.9953, ►**Fig. 3** shows how the method is linear under experimental conditions for the concentration range of 10 to 100 µg/mL.

Throughout the specificity analysis, the diluent had no impact on the test sample RT. The stress conditions and absolute% deterioration were computed in relation to the control sample. While heat and humidity degradation revealed 47.58 ± 1.25 and 56.28 ± 2.58 of degradation, respectively, oxidative degradation exhibited 41.58 ± 1.58. Acid and alkali hydrolysis reduced the sample by 29.36 ± 1.25 and 65.28 3.65, respectively, as shown in the ►**Table 2**.

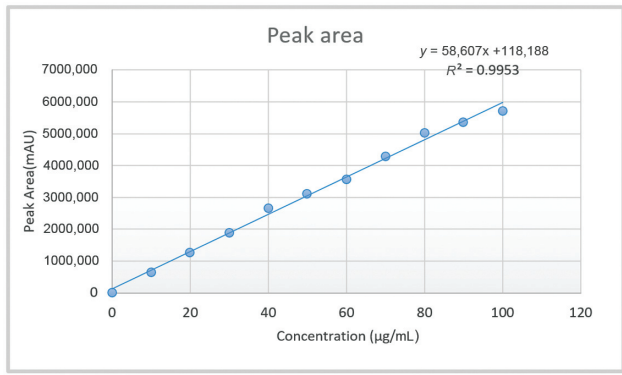

Fig. 3 Calibration curve of compound A3.

The LOD and LOQ were calculated statistically and found to be 0.740 and 0.2242 µg/mL, respectively, between 10.0 and 100 mg/mL of A3. The method was able to determine concentrations of 1 mg/mL with a sufficient amount of accuracy and precision (►**Table 3**). The average recovery for the test sample was calculated to be 96.25% with a precision RSD of 0.632% based on the measurement of recovery at LOQ.

The analysis of intraday and interday precision used the triplicates obtained on the same day (intraday precision) and 3 days later (interday precision). According to ►**Table 4**, the test compound A3 achieved whole% RSD of 98.95 and 98.95%. The sample solution's recovery was found to be within the established limits, and the RSD% results in ►**Table 5** reveal that it was 100, 96.25, and 99.25%. ►**Table 4** shows that there was no change in the filter interference data and no appreciable change in the aliquot's assay result after 2 days. Peak area percentage RSD, TF, TP, and RT all fell within the acceptable limit during the trial, which was 2%.

Table 1 System suitability characteristics derived from the chromatogram of compound A3

Parameters	Values
Retention time	3.35 ± 1.25 min
Capacity factor	0.109
Tailing factor	1.20
Number of theoretical plates (N)	1,646
Height equivalent to theoretical plates	91.138 µm

Discussion

The present study was described to develop the analytical method of synthesized compounds 5-(4-bromophenyl)-N-(2-chloro-4-nitrophenyl)-1,3,4-oxadiazol-2-amine (A3) by the RP-HPLC method. A C18 column was used, which was kept for activation and then stabilized by maintaining the temperature at 40°C with flow rate of 1 mL/min. The optimum mobile phase ratio in the HPLC method (ACN; water;

Table 2 Force degradation study of compound A3

Sl. no.	Stress condition	Stressor	% absolute degradation in assay
1	Control	Not applicable	100
2	Thermal degradation	60°C for 24 h	47.58 ± 1.25
3	Humidity degradation	7 d at room temperature	56.28 ± 2.58
4	Acid degradation	0.1 N HCl	65.28 ± 3.65
5	Alkali hydrolysis	0.1 N NaOH	29.36 ± 1.25
6	Oxidative degradation	3% H_2O_2	41.58 ± 1.58

Table 3 Results of calibration, limit of detection (LOD), and limit of quantification (LOQ) of compound A3

Standard curve	Concentration (µg/mL)	Slope	Intercept
1	10–100	0.8799	0.8250
2	10–100	0.6528	0.7859
3	10–100	0.7852	0.9856
4	10–100	0.6325	0.658
5	10–100	0.7652	0.5362
Mean ($n = 6$)		0.743	0.758
Standard deviation		0.101	0.170
LOD (3.3*SD/S)			0.740 µg/mL
LOQ (10*SD/S)			2.242 µg/mL

Table 4 Solution stability study of compound A3

Time point	%Assay of drug	Cumulative		
		Average	STDEV	%RSD
Day 0	100	NA	NA	NA
Day 1	98.5623	98.4215	0.2589	0.2630
Day 2	99.3514	98.5236	0.6234	0.6327

Abbreviations: RSD, relative standard deviation; STDEV, standard deviation.

Table 5 Recovery, intraday, interday, and intermediate-day accuracy and precision of compound A3

Concentration (µg/mL)	%recovery ± SD ($n = 3$)	Intraday; %RSD ($n = 6$)	Interday; %RSD ($n = 6$)	Intermediate; %RSD ($n = 6$)
10	100 ± 1.25	99.51 ± 3.69	96.25 ± 2.35	99.91 ± 3.69
50	96.25 ± 0.39	98.71 ± 2.58	98.35 ± 1.36	98.52 ± 1.10
100	99.25 ± 1.59	98.62 ± 2.25	98.95 ± 1.99	98.32 ± 2.81

Abbreviations: RSD, relative standard deviation; SD, standard deviation.

methanol) is 90:05:05 and the RT was found to be 3.35 minutes. Once the parameter was optimized, then the developed method was optimized for different parameters such as accuracy, precision, robustness, selectivity and forced degradation, sensitivity stability. Therefore, the purpose of this research is method development by RP-HPLC and validation of the compound A3.

Conclusion

The findings of the present research demonstrated that the method development of compound 5-(4-bromophenyl)-N-(2-chloro-4-nitrophenyl)-1,3,4-oxadiazol-2-amine (A3) was a reliable, economical, reproducible, and simple technique by RP-HPLC. The newly synthesized compound A3 showed lambda max at 235 nm with optimum mobile phase ratio and the RT was found to be 3.35 minutes. The purpose of the study was to show the quality of eluting and separating of compound is efficient in accuracy and shortest feasible run time.

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None.

Conflict of interest
None declared.

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