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Original Article

Integration of Green Analytical Chemistry and Quality by Design Approach for Systematic Development of Stability-Indicating RP-HPLC Method for Estimation of Guanfacine

Kiran Narayan Gaikwad, Mahesh Palled*

Department of Pharmaceutical Chemistry, KLE College of Pharmacy, Belagavi, KLE Academy of Higher Education and Research, Belagavi, Karnataka, INDIA.

ABSTRACT

Background: Guanfacine, a phenylacetyl-guanidine derivative, has gained pharmaceutical significance due to its therapeutic applications. A stability-indicating, Quality by Design (QbD)-based RP-HPLC method was developed and validated for its quantification in bulk drugs to meet its growing demand. Additionally, its environmental sustainability was assessed using the AGREE tool to ensure compliance with Green Analytical Chemistry (GAC) principles. **Materials and Methods:** The RP-HPLC method was optimized using Box-Behnken Design (BBD) with a C-18 column and a mobile phase of methanol and 0.1% ortho-phosphoric acid (60:40 v/v). Key parameters such as specificity, linearity, precision, accuracy, robustness, and ruggedness were evaluated as per ICH guidelines. Stability-indicating capability was confirmed through forced degradation studies under acidic, basic, oxidative, and neutral conditions. The AGREE tool was applied to assess the method's greenness. **Results:** The method exhibited a retention time of 3.96 min, peak area of 2613, tailing factor of 0.18, and 4055.83 theoretical plates. Sensitivity analysis yielded LOD of 0.39 µg/mL and LOQ of 1.19 µg/mL. Precision studies showed %RSD below 0.5%, and linearity was confirmed with R^2=0.9998. The AGREE score of 0.69 indicated moderate greenness, with potential for further improvement. **Conclusion:** The QbD-driven RP-HPLC method was successfully developed, validated, and assessed for environmental sustainability, demonstrating high precision, accuracy, and stability-indicating capability. Future refinements, such as solvent reduction and greener alternatives, could further enhance sustainability in pharmaceutical analysis.

Keywords: Guanfacine, Quality by Design, Green Chemistry, AGREE Tool, RP-HPLC, Box-Behnken Design.

Correspondence:
Dr. Mahesh Palled
Department of Pharmaceutical Chemistry, KLE College of Pharmacy, Belagavi, KLE Academy of Higher Education and Research, Belagavi, Karnataka, INDIA.
Email: mspalled@klepharm.edu

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INTRODUCTION

The growing focus on developing environmentally sustainable analytical methodologies has led to the integration of Quality by Design (QbD) principles with Green Analytical Chemistry (GAC) approaches in modern pharmaceutical analysis. Regulatory organizations like the U.S. FDA and ICH emphasize the importance of robust, efficient, and eco-friendly analytical methods that enhance pharmaceutical quality while minimizing environmental impact.[1,2] Guanfacine, (Figure 1) a selective α_2A-adrenergic receptor agonist, is commonly used to treat Attention Deficit Hyperactivity Disorder (ADHD) and high blood pressure.[3] The need for a validated, stability-indicating method for guanfacine estimation is paramount due to its increasing clinical applications and regulatory requirements for quality control in bulk and pharmaceutical formulations.[4]

RP-HPLC is a extensively used analytical technique for drug quantification due to its high precision, sensitivity, and reproducibility.[5] However, traditional RP-HPLC methods often suffer from excessive solvent consumption, prolonged analysis time, and the use of hazardous chemicals, raising environmental concerns.[6] Green Analytical Chemistry (GAC) principles advocate for methods that reduce solvent usage, minimize waste generation, and employ safer reagents, making them crucial in modern analytical development.[7] The AGREE tool, a recently developed metric, enables a comprehensive assessment of analytical method greenness by scoring various parameters related to reagent toxicity, energy consumption, and sustainability.[8]

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In order to overcome current limitations, this study develops and validates a stability-indicating RP-HPLC method for guanfacine by combining QbD and green analytical techniques. QbD-based analytical method development follows a systematic, risk-based approach to optimize Critical Method Parameters (CMPs) and Critical Quality Attributes (CQAs), ensuring method reliability and robustness.[9] The Box-Behnken Design (BBD), a response surface methodology, was employed to systematically optimize chromatographic conditions such as Mobile Phase (MP) composition, flow rate, and detection wavelength, leading to an efficient and reproducible analytical method.[10]

Furthermore, the study evaluates the greenness of the developed method using the AGREE tool, assessing factors such as solvent type, energy efficiency, waste generation, and overall environmental impact.[11,12] By implementing eco-friendly solvents, reducing reagent consumption, and optimizing chromatographic parameters, this study aims to establish a validated, sustainable, and regulatory-compliant RP-HPLC method for guanfacine quantification. This research highlights the significance of integrating QbD and GAC methodologies in pharmaceutical analysis, paving the way for more sustainable analytical techniques in the industry.

MATERIALS AND METHODS

Chemicals and Reagents

Guanfacine was procured from Unichem Laboratories, Mapusa, Goa, India. The solvents and reagents required for chromatographic analysis were supplied by the KLE College of Pharmacy, Belagavi, ensuring high purity for accurate and reliable assessments.

Instrumentation

An Agilent Technology 1100 Series HPLC system (Germany) equipped with an autoinjector was utilized for chromatographic analysis. Data acquisition and processing were carried out using Chemstation 10.1 software. A C18 column was used for the separation, maintained at an optimized temperature of 27-28°C using a column oven to ensure consistent performance.

The mobile phase comprised methanol along with 0.1% ortho-phosphoric acid, blended in a volumetric ratio of 60:40, flow rate of 1.0 mL/min. The wavelength for detection was established at 223 nm, and the volume of injection was quantified at 5 µL. Forced degradation samples were analyzed using a Photodiode Array Detector (PDA) in scan mode at 223 nm, ensuring peak homogeneity through spectral analysis.

Preparation of Standard and Sample Solutions

Standard Stock Solution

To create a 500 µg/mL stock solution of guanfacine, 10 mg of standard guanfacine was dissolved in 20 mL of methanol, ensuring it was fully dissolved.

Sample Solution

Twenty tablets, each weighing 5.660 g, were finely powdered in a mortar. A quantity equivalent to 0.283 g per tablet was accurately weighed, and the weight corresponding to ten tablets (14.15 mg) was dissolved in 20 mL of methanol, yielding a 500 µg/mL tablet solution. This solution was subsequently diluted to a final concentration of 20 µg/mL for experimental analysis.

Systematic Development of an Integrated QbD and Green Analytical Approach

To develop a stability-indicating RP-HPLC technique for Guanfacine, Green Analytical Chemistry (GAC) concepts were integrated with a Quality by Design (QbD) framework. The method was optimized using a Box-Behnken Design (BBD) as given in Table 1, assessing three critical chromatographic variables: methanol concentration (60%, 61%, and 62%), flow rate (0.8, 0.9, and 1.0 mL/min), and detection wavelength (222, 223, and 224 nm). A total of 17 experimental runs were conducted to evaluate their effects on Retention Time (RT), Theoretical Plates (TP), and Tailing Factor (TF). Response Surface Methodology (RSM) was applied to determine the optimal chromatographic conditions that provided high efficiency (maximized TP), rapid analysis (minimized RT), and acceptable peak symmetry (optimized TF). The final conditions ensured the robustness of the approach for routine pharmaceutical analysis by meeting regulatory standards for linearity, accuracy, and precision.

Method Validation

Method validation ensures the reliability, accuracy, and reproducibility of an analytical procedure in accordance with regulatory guidelines. This study validates the developed RP-HPLC method for guanfacine estimation based on key parameters, including system suitability, linearity, precision, accuracy, robustness, and sensitivity (LOD and LOQ). Statistical analysis of chromatographic data confirms the method's robustness and suitability for routine pharmaceutical analysis. The systematic evaluation aligns with ICH Q2(R1) guidelines, ensuring method reliability for quantifying guanfacine in bulk and formulated drug products.

System Suitability

To assess system suitability, six samples of a guanfacine solution were injected (6 µg/mL) into the RP-HPLC system and RT, TF, TP, and peak area, were determined from the resulting chromatograms. The data obtained were statistically analyzed to

ensure method consistency. The acceptance criteria required the relative standard deviation (%RSD) of the RT, TF, TP, and peak area from six injections to be below 2%.

Specificity and Selectivity

The RP-HPLC peak of the guanfacine (10 µg/mL) solution, sample, and blank chromatogram of the solvent system were obtained to complete the operation. It aids in determining the analyte, peak purity, and interference from other peaks.

Linearity and Range

Guanfacine was used in six different concentrations, ranging from 5 to 25 µg/mL, to test the method's linearity. The usual stock solution was used to make these solutions, which were injected three times. The standard calibration curves were constructed by plotting peak area against analyte concentration, and linear regression analysis was performed to assess the method linearity.

Limit of Detection and Limit of Quantification

The slope of the calibration curve and the standard deviation of the y-intercept were used to statistically determine the method's sensitivity (LOD and LOQ). The LOD and LOQ were calculated using the following formulas:

LOD=3.3×Standard Deviation of y-intercept/Slope of Calibration Curve

LOQ=10×Standard Deviation of y-intercept/Slope of Calibration Curve

Precision and Repeatability Analysis

The intraday and interday precision studies were conducted using three distinct concentrations (10 µg/mL, 15 µg/mL and 20 µg/mL) injected into triplicate. Precision was assessed by calculating the %RSD of repeated injections. The percentage % RSD was calculated to evaluate repeatability and method reproducibility.

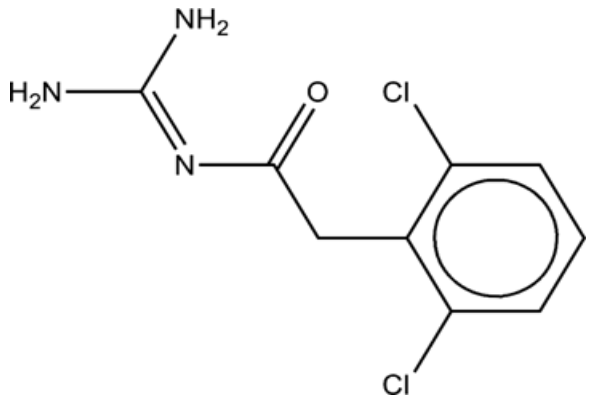

Figure 1: Chemical structure of guanfacine.

Robustness

The robustness was examined by purposefully altering many important chromatographic parameters. To evaluate its effect, the mobile phase flow rate was adjusted from its initial fixed value of 1.0 mL/min to 0.9 mL/min and 1.1 mL/min. Similarly, the mobile phase composition (originally 39% buffer + 61% methanol) was modified to 40% buffer + 60% methanol and 38% buffer + 62% methanol. The detection wavelength, set at 223 nm, was also tested at 222 nm and 224 nm to determine method robustness. The robustness was assessed by calculating the %RSD.

Accuracy

The accuracy of the method was evaluated through a recovery study by spiking a 5 µg/mL guanfacine solution with known amounts of the drug at 80%, 100%, and 120% levels. The proportion of recovery was derived through a comparative analysis of the peak area of the experimental sample against that of the standard reference solution, thereby affirming the methodological dependability for quantitative assessment.

Stability-Indicating Capability and Specificity through Forced Degradation Study

The implementation of forced degradation studies represents a crucial element of method validation designed to evaluate the stability-indicating properties and specificity of an analytical technique. These studies simulate various stress conditions to identify potential degradation pathways, ensuring the method can reliably differentiate the active pharmaceutical ingredient from its degradation products. This study was conducted in accordance with ICH guidelines, to assess the stability of guanfacine under acidic, basic, neutral, and oxidative conditions.

Acid Degradation

A 10 mL volumetric flask was used to prepare the test sample by adding 0.3 mL of guanfacine solution followed by 5 mL of 1.0 N hydrochloric acid. The mixture was immediately neutralized with 0.5 mL of 1.0 N NaOH, which was added dropwise with continuous mixing. The neutralized solution was then diluted to volume with the mobile phase, filtered through a 0.22 µm membrane filter. The overall volume was made up to 10 mL with the mobile phase. Samples were analyzed at 60 and 180 min to observe degradation patterns prior to neutralization.

Table 1: Box-Behnken Design for RP-HPLC Method Optimization.

	Name	Units	Low	Medium	High
A	Methanol	%	60	61	62
B	Flow rate	mL/min	0.8	0.9	1
C	Wavelength	nm	222	223	224

Basic Degradation

A 10 mL volumetric flask was used to prepare the test sample by combining 5 mL of 1.0 N sodium hydroxide with 0.3 mL of guanfacine solution. The mixture was immediately neutralized with 0.5 mL of 1.0 N HCl, added dropwise with continuous mixing. The neutralized solution was then diluted to volume with the mobile phase, filtered through a 0.22 µm membrane filter. The total volume was adjusted to 10 mL with the mobile phase. Samples were analyzed at 60- and 180-min following exposure to assess degradation.

Neutral Degradation

To evaluate hydrolytic stability, 0.3 mL of guanfacine was mixed with 5 mL of water, and the total volume was brought to 10 mL using the mobile phase. Degradation was monitored at 60 and 180 min intervals.

Oxidative Degradation

In oxidative stress condition, a 0.3 mL sample of guanfacine was incorporated with 5 mL of a 3% Hydrogen peroxide (H_2O_2) solution, and the complete volume was calibrated to 10 mL utilizing the mobile phase. Samples were analyzed at 60 and 180 min to determine oxidative degradation

Greenness Evaluation of the RP-HPLC Method Using AGREE Calculation

To ensure environmental sustainability, the validated method was further assessed using the AGREE tool, which evaluates compliance with the principles of GAC. The method's greenness score was determined by analysing the following factors:

1. Use of hazardous solvents: In the proposed study we have used Methanol, a moderately hazardous solvent, and 0.1% ortho-phosphoric acid, a mild acidic reagent, were used.
2. Waste generation: A flow rate of 1.0 mL/min for the mobile phase was consistently maintained to enhance the efficacy of solvent utilization and to reduce the generation of waste.
3. Energy consumption: The Agilent 1100 HPLC system was used, and the short method runtime contributed to lower energy requirements.
4. Operator safety: Proper handling and ventilation measures were required due to the use of methanol.
5. Reagent toxicity and biodegradability: Methanol is slightly hazardous, whereas ortho-phosphoric acid has a relatively lower environmental impact.
6. Method efficiency: The method was optimized for high chromatographic performance, ensuring rapid analysis and minimal solvent usage.

RESULTS

Optimization of Chromatographic Parameters

The optimization of chromatographic conditions was performed using a factorial analysis and Box-Behnken design, evaluating a total of 17 experimental trials. The 3^2 factorial designs and observed values for HPLC technique optimization are shown in Table 2. 3D response surface plots were generated to assess the effect of independent variables on the chromatographic response. These plots provided a visual representation of interactions between flow rate, methanol concentration, and detection wavelength on parameters such as retention time and theoretical plate count (Figure 2).

Method Validation

All the method validation parameters were found to be well within the acceptance range as per regulatory guidelines. Table 3 presents the method validation report of proposed RP-HPLC method.

System Suitability

Six distinct replicates of a guanfacine solution at a concentration of 6 µg/mL were injected into the RP-HPLC to evaluate system suitability. The results showed that the established method was appropriate, with the percentage RSD for the responses being less than 2%.

Specificity and Selectivity

The RT of 2.96 min were observed in thigh-performance liquid chromatography chromatogram of reference and sample Guanfacine. No discernible peak was observed at the retention time associated with Guanfacine in the chromatogram corresponding to the blank sample. The technique was found to be specific and selective for Guanfacine since no visible interference was found at the retention time of the Guanfacine peak in either the standard or sample chromatograms.

Linearity and Range

The calibration curve pertaining to guanfacine exhibited a linear relationship throughout the concentration spectrum of 5-25 µg/mL. The regression equation obtained was

$y = 59.645x+20.852$

With a correlation coefficient (R^2) of 0.9998, confirming excellent linearity. Figure 3 represents the overlay chromatogram of guanfacine with different linear concentrations.

Limit of Detection and Limit of Quantification

By performing a comprehensive statistical assessment, we explored the Detection (LOD) and Quantification (LOQ) limits associated with the recommended methodology, leveraging the standard deviation calculated from the y-intercept and slope of

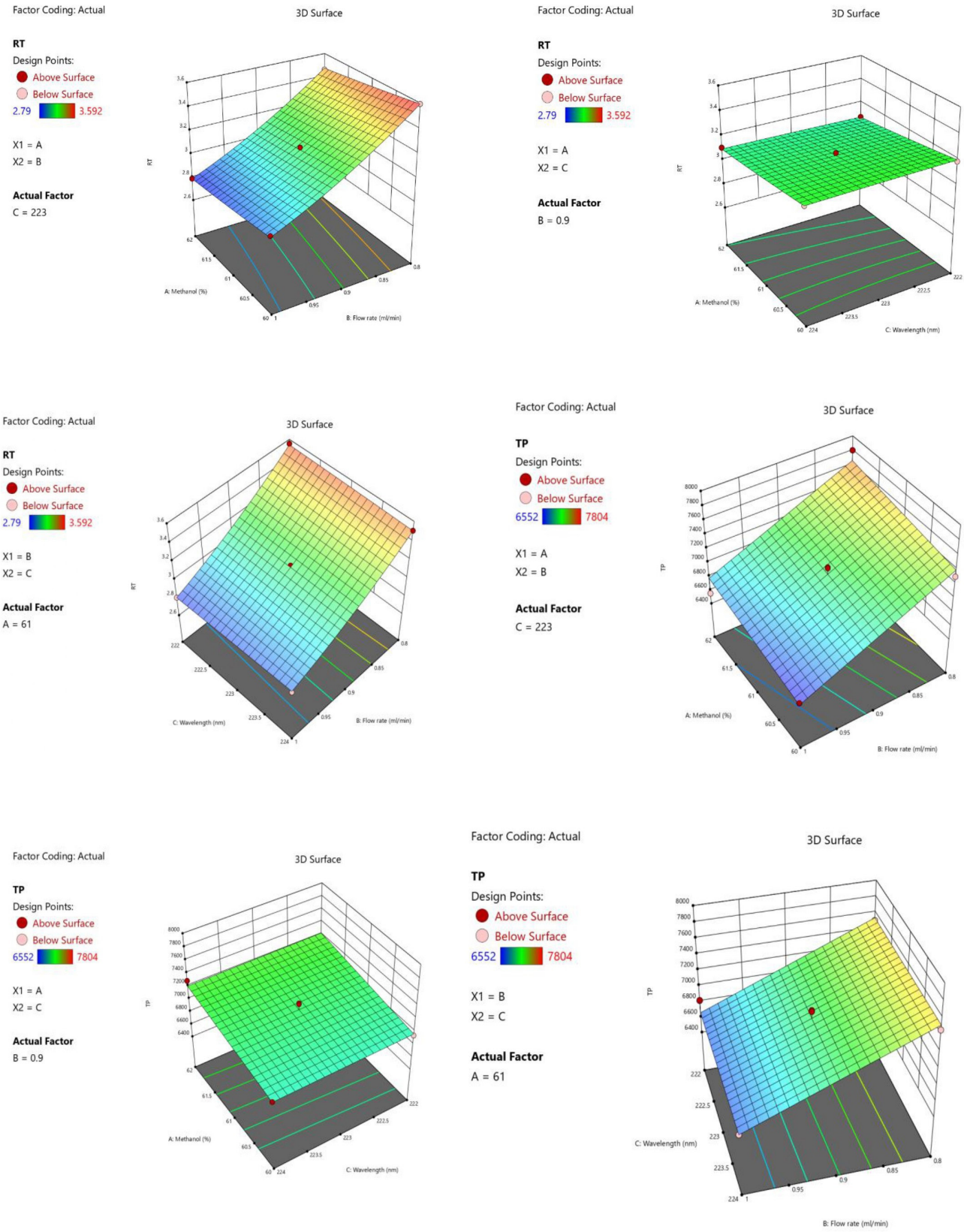

Figure 2: 3D surface plots (a) The impact of flow rate and methanol on RT (b) The effect of methanol and wavelength on RT (c) The effect of flowrate and wavelength on RT (d) The effect of flowrate and methanol on TP (e) The effect of methanol and wavelength on TP (f) The effect of flowrate and wavelength on TP.

the calibration graph. The derived LOD and LOQ figures were noted as 0.39 µg/mL and 1.19 µg/mL respectively.

Precision

Precision was determined by evaluating both intraday and interday variability. Table 3 presents the intra and interday precision values at lower, middle and higher concentration. The low %RSD values obtained at intraday and interday precision studies confirmed the high precision of the method.

Robustness Analysis

The robustness of the method developed by using RP-HPLC was explored through deliberate tweaks in chromatographic variables, particularly the flow rate (when shifted from 1.0 mL/min to 0.9 mL/min and 1.1 mL/min, the %RSD values retained below 2%), mobile phase composition (Variations in methanol concentration resulted in %RSD values less than 2%), and detection wavelength (A slight variation in wavelength resulted in %RSD values less than 2%) demonstrating robustness of the proposed RP-HPLC Method.

Accuracy

The precision of the methodology was assessed utilizing a recovery investigation at spiking levels of 80%, 100%, and 120%. The percentage recovery obtained at these levels was 99.34%, 99.80%, and 100.12%, respectively, confirming method reliability for guanfacine quantification.

Assay

The BBD-driven RP-HPLC analysis of the commercially available formulation of Guanfacine demonstrated remarkable recovery rates. The percentage recovery was determined to be 99.30%. The retention time of Guanfacine in the dosage form remained consistent when compared to the Guanfacine standard. The TF and TP factors were observed to fall within the established acceptance criteria. All sample solution chromatograms exhibited no additional peaks, indicating the absence of interference from dosage form additives with Guanfacine. This outcome highlights the capability of the endorsed RP-HPLC methodology for the in-depth evaluation and inspection of Guanfacine in its natural and formulated forms. The assay chromatogram is illustrated in Figure 4.

Stability-Indicating Capability and Specificity through Forced Degradation Study

Analyses of forced degradation were executed under multiple stress conditions to evaluate the stability of Guanfacine. The percentage of degradation was noted to be below 20% across all assessed levels, thereby signifying the stability of the proposed methodology. Table 4 represents the forced degradation data. Figure 5 illustrates the chromatographic profiles of Guanfacine

Table 2: 3^2 Factorial Design along with the observed values of Responses.

		Factor 1	Factor 2	Factor 3	Response 1	Response 3	Response 4
Std	Run	Methanol (%)	Flow rate (mL/min)	Wavelength (nm)	RT	TP	TF
8	1	62	0.9	224	3.105	7296	0.69
12	2	61	1	224	2.848	6645	0.70
11	3	61	0.8	224	3.508	7466	0.66
17	4	61	0.9	223	3.119	7176	0.69
15	5	61	0.9	223	3.123	7191	0.69
10	6	61	1	222	2.807	6823	0.70
1	7	60	0.8	223	3.592	7309	0.68
13	8	61	0.9	223	3.126	7005	0.69
5	9	60	0.9	222	3.187	6946	0.71
7	10	60	0.9	224	3.190	6961	0.71
2	11	62	0.8	223	3.461	7804	0.68
6	12	62	0.9	222	3.074	7153	0.69
9	13	61	0.8	222	3.557	7504	0.67
16	14	61	0.9	223	3.156	7085	0.68
3	15	60	1	223	2.904	6552	0.70
14	16	61	0.9	223	3.157	7090	0.69
4	17	62	1	223	2.790	6553	0.70

Print of window 47: '3D' Signal Overlay

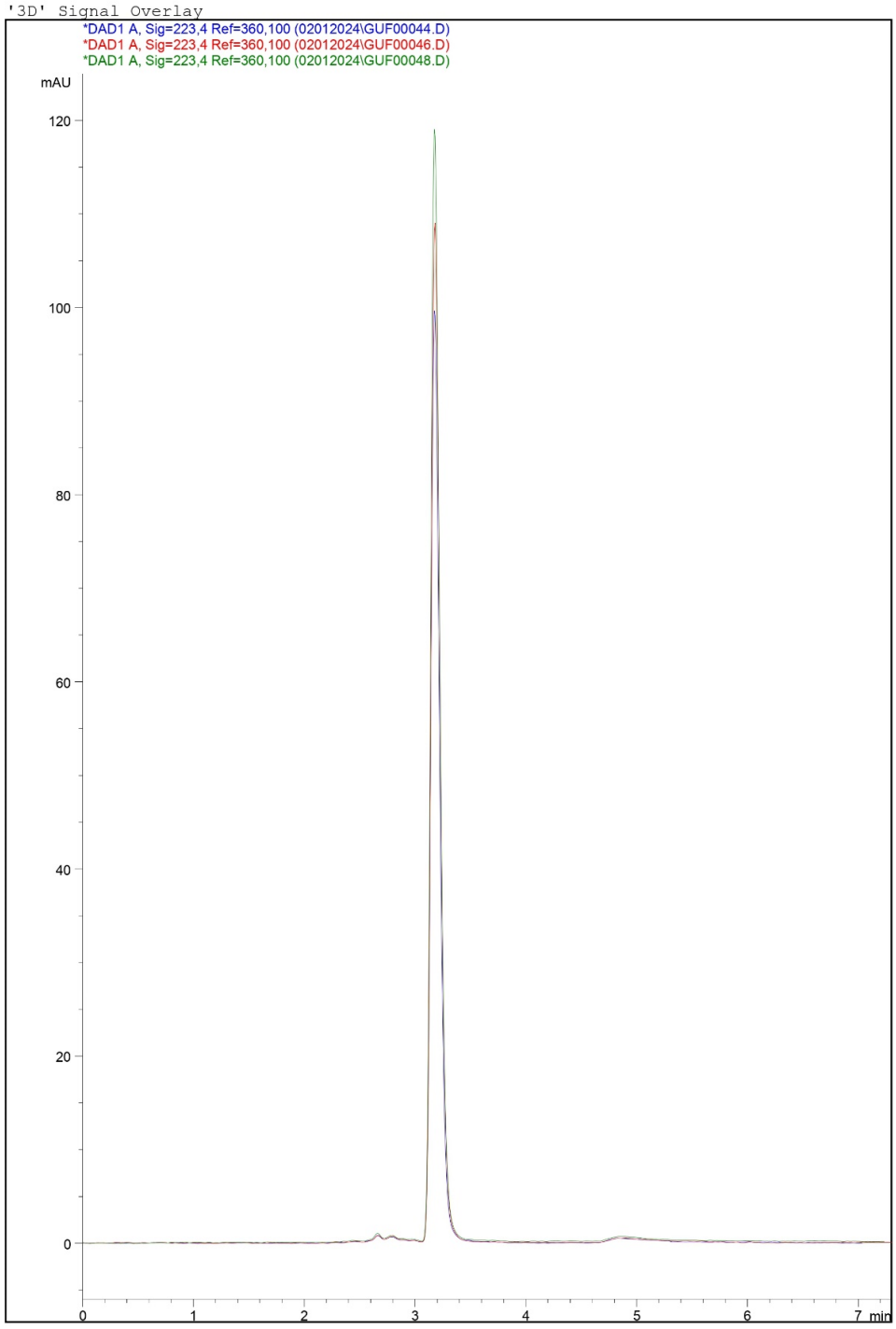

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Figure 3: Overlay Chromatogram of Guanfacine.

Print of window 47: '3D' Signal Overlay

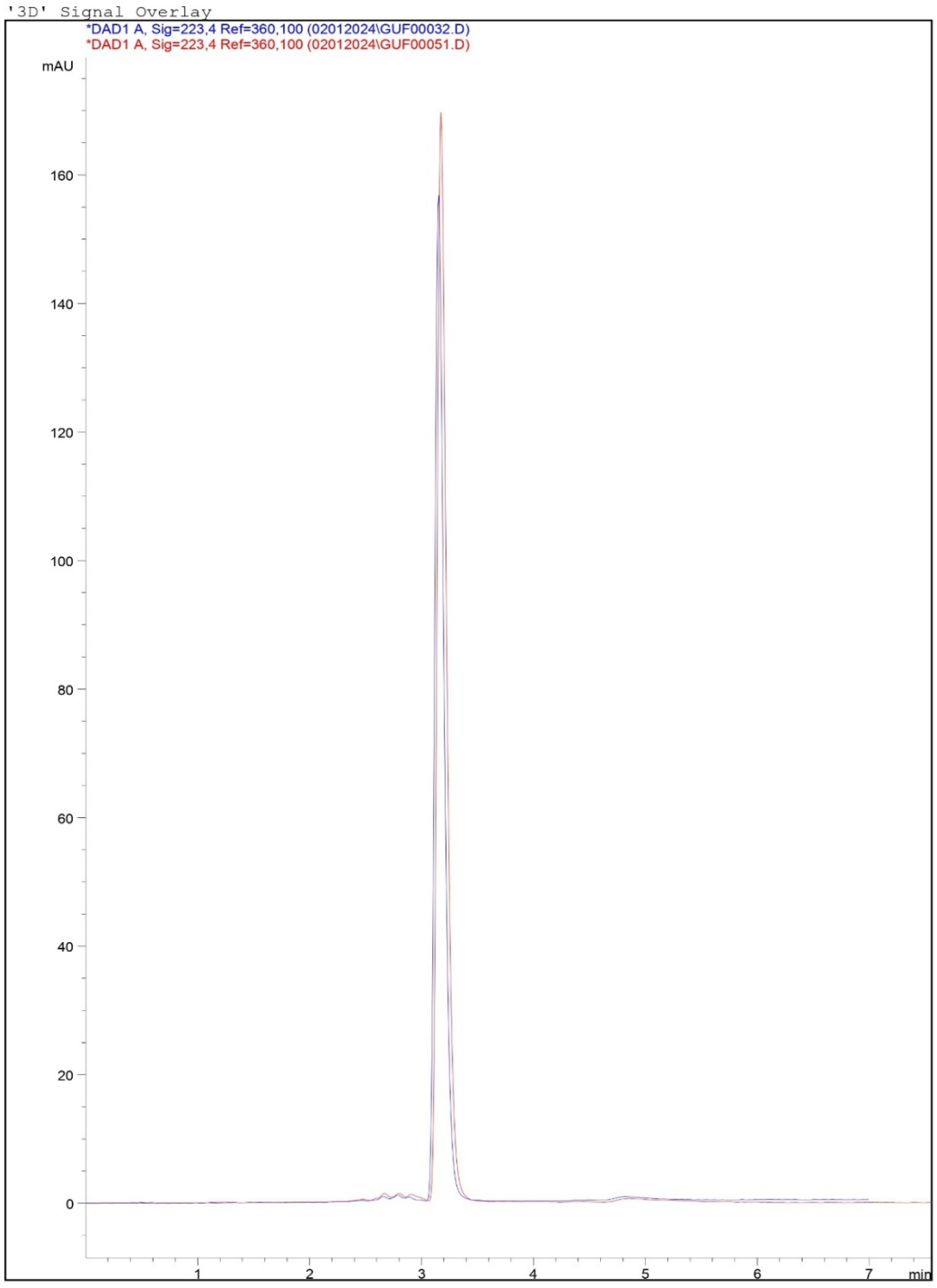

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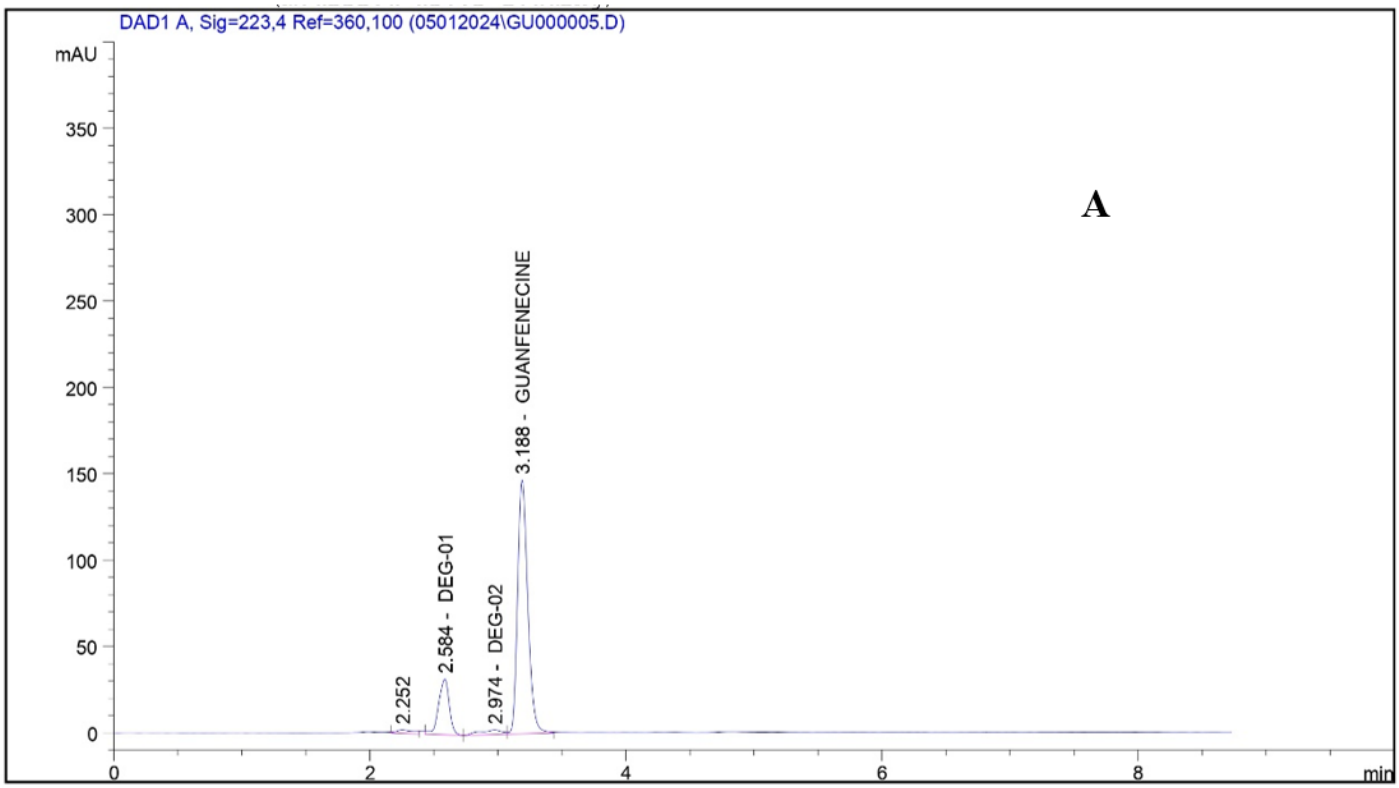
Figure 4: Chromatogram of Guanfacine in Marketed Sample.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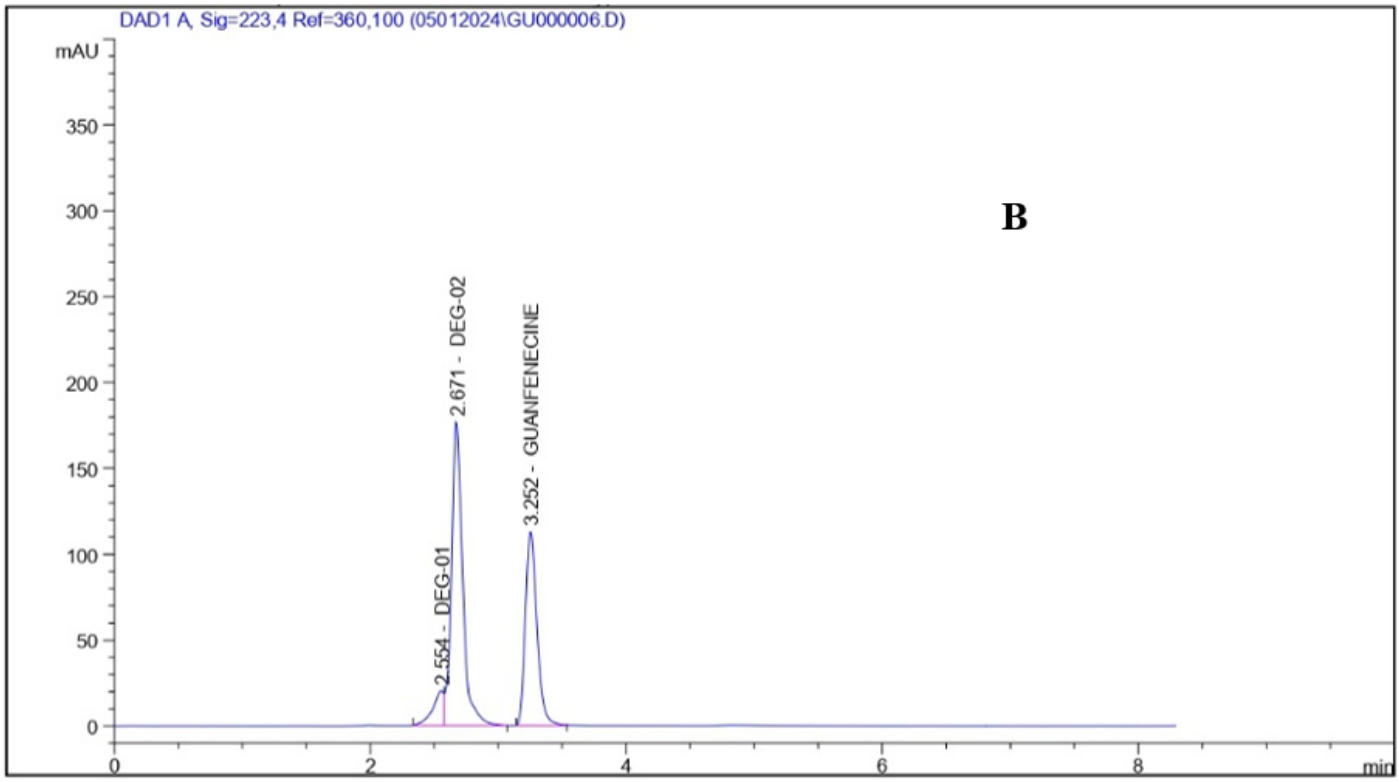
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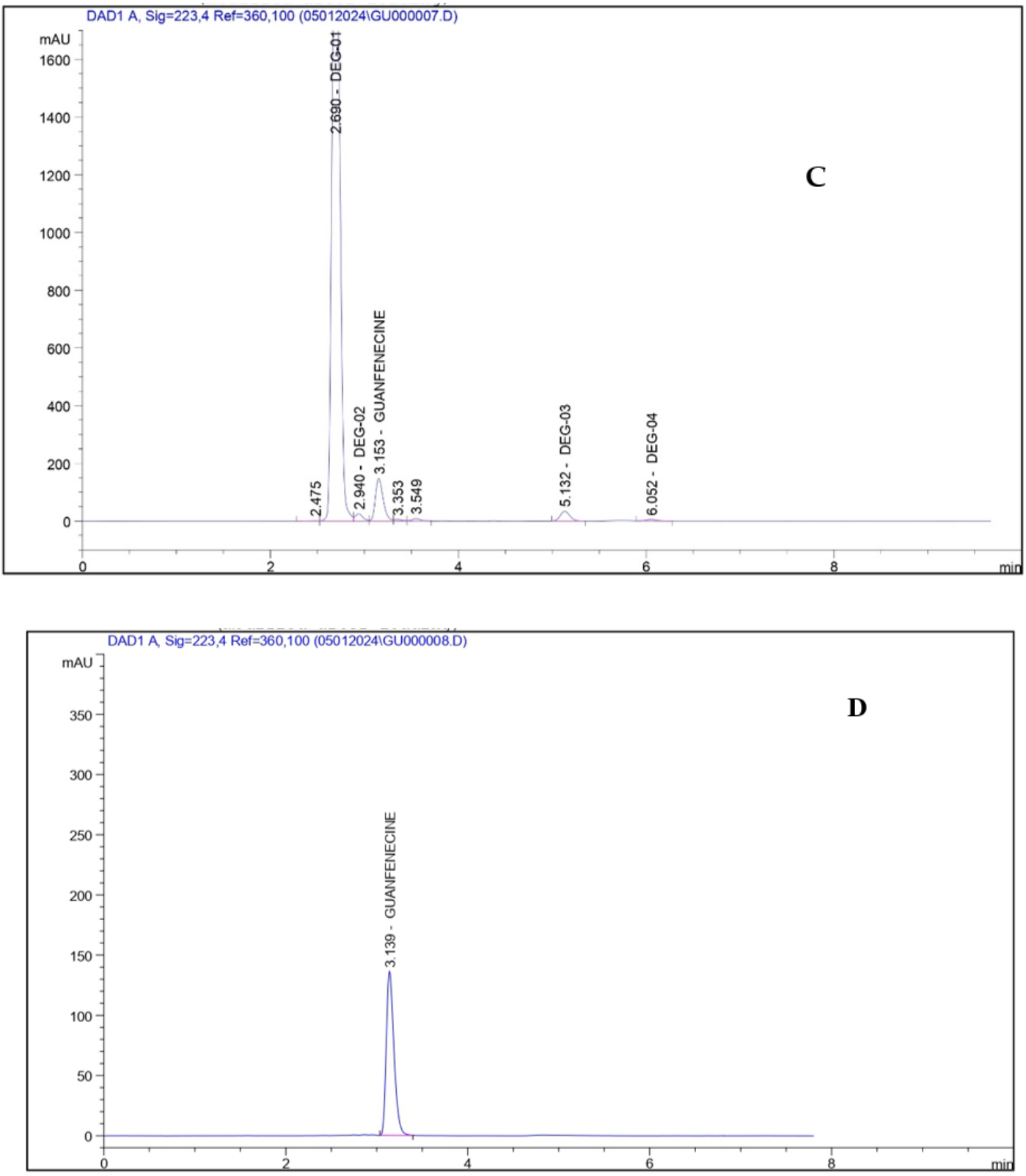

Figure 5: **Forced degradation Guanfacine chromatograms (A) Acid hydrolysis; (B) alkaline hydrolysis; (C) Oxidative degradation; (D) Neutral hydrolysis.**

Table 3: Method Validation Report.

System Suitability				
Parameters	**Concentration**	**Mean**	**SD**	**%RSD**
Retention Time	10 µg/mL	3.950	0.010	0.253
Peak Area	10 µg/mL	2614.666	1.528	0.058
Tailing Factor	10 µg/mL	0.191	0.001	0.524
Theoretical Plates	10 µg/mL	4058.827	6.083	0.150
Linearity and Range				
5 µg/mL- 25 µg/mL		$R^2 = 0.9998$		
Limit of Detection		Limit of Quantification		
0.39 µg/mL		1.19 µg/mL		
Intraday and Interday Precision Data				
Concentration	**% RSD values at Intraday Precision**	**% RSD values at Interday Precision**		
10 µg/mL	0.10%	0.27%		
15 µg/mL	0.13%	0.15%		
20 µg/mL	0.17%	0.06%		
Robustness				
Parameter Count	**Change Level**	**% RSD**		
Flow rate (mL/min)	0.9 mL/min	0.43		
Flow rate (mL/min)	1.1 mL/min	0.37		
Mobile Phase (%)	60% Methanol	0.25		
Mobile Phase (%)	62% Methanol	0.44		
Wave Length (nm)	222 nm	0.31		
Wave Length (nm)	224 nm	0.31		
Accuracy				
Level I	80%	99.34%		
Level II	100%	99.80%		
Level III	120%	100.12%		

after a 2-hr period of degradation under conditions of acid hydrolysis, alkaline hydrolysis, oxidative degradation, and neutral hydrolysis investigation.

GREENSS ASSESSMENT

The AGREE assessment offers an exhaustive appraisal of the environmental sustainability of the formulated RP-HPLC methodology for the quantification of Guanfacine. The justification of score at each principle level is given as below:

Use of Hazardous Chemicals (Score: 0.6)

The mobile phase includes methanol at 60% concentration and ortho-phosphoric acid at 0.1%, representing 40% of the solution's makeup. Methanol is toxic and volatile, leading to a lower score, but its use is necessary for achieving optimal separation. The use of aqueous buffers improves greenness compared to purely organic solvents.

Waste Generation (Score: 0.7)

The method uses a flow rate of 1.0 mL/min, leading to moderate solvent consumption. Compared to conventional HPLC methods, this method has a shorter retention time, reducing total solvent waste. Optimization using Box-Behnken Design (BBD) has minimized waste by selecting the most efficient conditions.

Energy Consumption (Score: 0.8)

The Agilent 1100 HPLC system operates at standard energy consumption levels. The run time is relatively short, reducing power usage per sample. Although HPLC systems require energy, no excessive heating or additional energy-consuming steps were used.

Operator Safety (Score: 0.6)

Methanol exposure poses health risks if inhaled or absorbed through the skin. Proper laboratory handling and ventilation

Table 4: Forced degradation of Guanfacine after 1 and 2 hr.

Degradation	Area of Standard	Area of degraded Sample	Degraded up to %	Actual % degradation
After 1 hr				
Acid Degradation	907.43	851.3	93.81	6.19
Basic Degradation	907.43	740.55	81.61	18.39
H_2O_2 Degradation	907.43	838.25	92.38	7.62
Neutral	907.43	896.79	98.83	1.17
After 2 hr				
Acid Degradation	907.43	817	90.03	9.97
Basic Degradation	907.43	709.6	81.80	19.20
H_2O_2 Degradation	907.43	833.17	91.82	8.18
Neutral	907.43	895.3	98.66	1.34

reduce safety concerns, but the score remains moderate due to the inherent toxicity of methanol.

Reagent Toxicity and Biodegradability (Score: 0.5)

Methanol is classified as hazardous and must be disposed of properly. Ortho-phosphoric acid (0.1%) has minimal environmental impact, improving the score slightly. A higher score would require replacing methanol with greener alternatives like ethanol or acetonitrile-free mobile phases.

Method Efficiency (Score: 0.9)

The method exhibits high resolution, minimal tailing, and optimal retention time, contributing to great efficiency. Shorter analysis time reduces solvent use and enhances method sustainability. This score is the highest among all parameters, indicating strong method optimization.

The method achieves an AGREE score of 0.69, categorizing it as moderately green. The use of methanol lowers the score, but optimized conditions, minimal waste, and high efficiency balance out the impact. The individual scores assigned to different parameters highlight the method's strengths and areas for improvement. This integration of QbD and GAC principles underscores the method's sustainability and regulatory compliance, making it a robust, precise, and environmentally conscious analytical approach for Guanfacine quantification.

DISCUSSION

The project focused on developing an RP-HPLC method distinguished by its precision, accuracy, and strength for assessing guanfacine analytically, based on Quality by Design (QbD) concepts. The Box-Behnken design effectively illustrates the impact of methanol percentage, flow rate, and wavelength on method performance. Flow rate significantly affects retention time and theoretical plates, while methanol concentration has a secondary influence. Wavelength primarily impacts detection without altering chromatographic performance. A consistent tailing factor indicates maintained peak symmetry. The BBD approach optimizes methanol ratio, improving peak area and retention time.[13,14] Similar methodology was applied for captopril detection.[15] Guanfacine degrades under acidic, alkaline, and oxidative conditions, remaining most stable in neutral environments. Basic conditions induce the highest degradation, increasing over time. Oxidative and acidic conditions lead to gradual breakdown. Well-resolved chromatographic peaks confirm clear separation of guanfacine and its degradation products. Managing oxidative stressors and pH is essential for long-term stability. Retention time inconsistencies may arise from minor variations in column condition, mobile phase composition, or flow rate. Detector sensitivity fluctuations are reflected in peak area variations. Low peak values indicate symmetrical, sharp peaks, while tailing factor fluctuations suggest further optimization is needed. High theoretical plate values indicate effective separation, though variability in plate counts suggests potential influences from flow rate, mobile phase, or column temperature. Similar results were reported in evogliptin tartrate estimation.[16] Guanfacine LOD and LOQ values were found to be 0.011 µg/mL and 0.038 µg/mL, respectively. Kumar *et al.*, (2023) confirmed QL precision with a %RSD of 2.17, within the 10% acceptance limit. LOD, LOQ, and ULOL values for guanfacine in another study were 0.05 mg/L, 0.1 mg/L, and 10.0 mg/L, respectively.[17] Low %RSD values confirm method resilience to flow rate variations. Stability across different mobile phase compositions (0.25% and 0.44%) and consistent %RSD at 0.31% indicate reliability. However, significant variations in mean area values between 224 nm (1003.19) and 222 nm (1204.5) suggest wavelength-dependent sensitivity differences. Method robustness was further validated by altering chromatographic conditions, showing %RSD < 2.[18] The comparative data presented in Table 5 indicate that the present method demonstrates favorable sensitivity,[19,20] precision, and robustness parameters relative to previously reported approaches.[21-33]

Greenness assessment highlights the method's sustainability. Methanol usage could be reduced or replaced with ethanol to enhance eco-friendliness. Optimizing solvent consumption,

Table 5: Detailed comparison of analytical methods and validation parameters for guanfacine quantification.

Method/Detection	Matrix/ Formulation	Linearity Range	LOD/ LOQ	Precision (%RSD)	Accuracy/ Recovery (%)	Runtime	Specificity/Notes	Citations
HPLC-MS	Urine	20-2000 ng/mL	LOD: 5 ng/mL; LOQ: 20 ng/mL	<15	>89	<5 min	No interference from drugs/ constituents	(Wolf *et al.*, 2012)
GC-MS	Urine	0.1-2.0 mg/L	LOD: 0.05 mg/L; LOQ: 0.1 mg/L	Not specified	71-93	Not given	Suitable for routine analysis	(Haglock *et al.*, 2008)
LC-MS/MS	Human plasma	0.05-10 ng/mL	LLOQ: 0.05 ng/ mL	1.6-10.5	97.9-103.9	Not given	No ion suppression, robust for bioequiv.	(Wang and Zou, 2022)
LC-MS/MS (Dried Plasma Spot)	Whole blood	0.25-250 ng/mL	Not specified	Not specified	Not specified	Not given	Minimizes hematocrit effect, semi-automated	(Li *et al.*, 2012)
Electron-capture GLC	Plasma, urine	Not specified	LOD: 0.5 ng/mL	Not specified	Not specified	Not given	Derivatization improves sensitivity	(Guerret *et al.*, 1979)
LC-MS/MS (DBS)	Human blood (DBS)	0.05-25 ng/mL	LLOQ: 0.05 ng/ mL	Not specified	Not specified	Not given	Robust, minimally invasive, stable	(Li *et al.*, 2011)
RP-HPLC (Stability)	Bulk, dosage form	30-450 µg/ mL	LOD: 0.011 µg/mL; LOQ: 0.038 µg/ mL	Not specified	99.2-100.5	Not given	Stability-indicating, resolves degradants	(Ahirrao *et al.*, 2011)
LC-MS/MS (DBS, semi-auto)	Human blood (DBS)	0.01-25 ng/mL	LLOQ: 0.01 ng/ mL	Intra: 1.3-8.2; Inter: 4.4-8.7	Inacc: -9.0-7.3	Not given	Online SPE, avoids manual extraction	(Li *et al.*, 2012)
LC-MS/MS	Dog plasma	0.1-20 ng/ mL	LLOQ: 0.1 ng/ mL	<10.8	92.9-108.4	Not given	High recovery, stable, PK studies	(Li *et al.*, 2013)
LC-MS/MS	Rat plasma	0.05-10 ng/mL	Not specified	Not specified	Not specified	Not given	High-throughput, PK studies	(Goparaju *et al.*, 2013)
RP-UHPLC (Stability)	Bulk API	Not specified	Not specified	Not specified	Not specified	Short	Characterizes degradation products	(Kumar and Chalannavar, 2023)
Visible Spectrophotometry	Bulk, formulations	Not specified	Not specified	Not specified	99.7-100.3	Not given	Simple, excipient-tolerant	(NagarjunaReddy *et al.*, 2011)
LC-MS/MS	Oral fluid, serum	Not specified	Not specified	Not specified	Not specified	Not given	Strong correlation oral fluid/serum	(Wohkittel *et al.*, 2021)
RP-HPLC	Bulk API	5-25 µg/ mL	LOD: 0.39 µg/ mL LOQ: 1.19 µg/ mL	Intra: 01 -0.17; Inter: 027-0.06	99.34- 100.12	3.9 min	QBD Method	Kiran Gaikwad et. Al. 2025

column dimensions, and flow rate would further improve sustainability. The AGREE assessment suggests solvent selection and waste reduction strategies can enhance ecological impact while maintaining analytical efficiency.[19,20]

Integrative Approach in RP-HPLC Method Development for Guanfacine

This investigation's comprehensive approach amalgamates principles of green chemistry, Quality by Design (QbD), and advanced analytical methodologies to formulate a dependable, precise, and environmentally sustainable RP-HPLC technique for the quantification of guanfacine. The Box-Behnken Design was employed to systematically evaluate the effects of methanol percentage, flow rate, and wavelength on chromatographic performance. QbD principles enabled method optimization with minimal experimental trials, ensuring high efficiency and reproducibility. Essential parameters including retention time, theoretical plates, and peak symmetry were meticulously optimized, thereby diminishing variability in system suitability. The study incorporates GAC principles to enhance environmental sustainability. AGREE assessment was utilized to evaluate and improve the ecological impact of the method. Potential sustainability improvements include Solvent substitution-Replacing methanol with ethanol or aqueous mobile phases. Reducing solvent consumption-Optimizing flow rate and column dimensions. Energy-efficient detection-Exploring alternative techniques with lower energy requirements.

CONCLUSION

The developed RP-HPLC method for guanfacine quantification successfully integrates Quality by Design principles, ensuring a systematic, reliable, and precise analytical approach. The Box-Behnken design enabled optimal selection of method parameters, improving chromatographic efficiency while maintaining high accuracy and robustness. Stability studies confirmed guanfacine's degradation behavior under various stress conditions, emphasizing the need for pH control and oxidative stress management in pharmaceutical formulations. This method demonstrates excellent precision, accuracy, and specificity, making it highly suitable for routine quality control applications. Sustainability considerations were incorporated through AGREE assessment, identifying areas for enhancing the method's greenness. Future refinements, such as reducing solvent usage, adopting ethanol or water-based mobile phases, and optimizing energy consumption, can further align the method with green chemistry principles. By integrating QbD, analytical performance, and environmental consciousness, this study provides a regulatory-compliant, efficient, and eco-friendly approach for guanfacine estimation, setting a foundation for further advancements in sustainable pharmaceutical analysis.

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ABBREVIATIONS

QbD: Quality by Design; **DOE:** Design of Experiment; **ICH:** International Council for Harmonization; **LOD:** Limit of Detection; **LOQ:** Limit of Quantification; **OPA:** Ortho Phosphoric Acid; **PA:** Peak Area, **QbD:** Quality by Design, **RA:** Risk Assessment **RSD:** Residual Standard Deviation; **RT:** Retention Time; **RP-HPLC:** Reverse Phase-High Performance Liquid Chromatography; **TF:** Tailing Factor; **TP:** Theoretical Plates.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

SUMMARY

Quality by Design-oriented, stability-indicating RP-HPLC methodology was meticulously developed and validated for the quantification of Guanfacine, a phenylacetyl-guanidine derivative possessing notable pharmaceutical relevance. The optimization of the method was conducted utilizing Box-Behnken Design, incorporating a C-18 column alongside a mobile phase composed of methanol and 0.1% ortho-phosphoric acid in a volumetric ratio of 60:40. Validation was executed in accordance with ICH guidelines, which encompassed the evaluation of critical parameters including specificity, linearity, precision, accuracy, robustness, and ruggedness. Investigations into forced degradation substantiated its stability-indicating proficiency under a variety of stress conditions, encompassing acidic, basic, oxidative, and neutral environments. The strategy displayed extraordinary sensitivity, achieving a detection limit of 0.39 µg/mL alongside a quantification limit of 1.19 µg/mL. A retention time of 3.96 min, coupled with exceptional linearity (R^2=0.9998) and precision characterized by a %RSD beneath 0.5%, further corroborated its operational efficiency. The AGREE tool was employed to assess its environmental sustainability, resulting in a moderate greenness score of 0.69. In conclusion, the QbD-driven RP-HPLC method has demonstrated precision, accuracy, and reliability. Future improvements, such as minimizing solvent consumption and integrating more eco-friendly alternatives, may further enhance its sustainability within the field of pharmaceutical analysis.

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