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DEVELOPMENT AND VALIDATION OF A STABILITY-INDICATING RP-HPLC METHOD FOR TIZANIDINE HYDROCHLORIDE USING DOE

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ABSTRACT
Background: Tizanidine hydrochloride (TIZ) is a centrally acting α2-adrenergic receptor agonist widely prescribed for managing spasticity. Given its therapeutic importance, a reliable, stability-indicating analytical method is crucial to ensure both the quality and regulatory compliance of Tizanidine hydrochloride. Existing RP-HPLC methods often lack robustness, sensitivity, or DoE optimization, highlighting the need for an improved approach. **Methodology:** A stability-indicating reverse-phase high-performance liquid chromatography (RP-HPLC) method with stability-indicating properties was developed and validated using a Design of Experiments (DoE) approach. A full factorial design was implemented, optimizing mobile phase composition and flow rate as key method variables. Chromatographic separation was achieved using an Agilent Zorbax Bonus RP column (250 × 4.6 mm, 5 µm) with a mobile phase of 0.1% trifluoroacetic acid (TFA) and acetonitrile (65:35 v/v) at a 0.5 mL/min flow rate. Detection was performed at 228 nm. The method was validated by ICH guidelines, evaluating parameters such as specificity, precision, accuracy, linearity, robustness, and forced degradation. **Results and Discussion:** The method demonstrated excellent linearity (r^2 = 1.00) across concentration levels ranging from 80% to 120% of the target concentration. The LOD and LOQ were 1.00 µg/mL and 3.04 µg/mL, respectively. High precision (%RSD < 2%) and accuracy (99–101% recovery) were observed. Forced degradation studies revealed notable degradation under oxidative (36.08%) and acidic (15.73%) conditions. **Conclusion:** The developed RP-HPLC method is precise, robust, and suitable for the routine quality control and stability assessment of Tizanidine hydrochloride in pharmaceutical formulations.

INTRODUCTION

Tizanidine hydrochloride (TIZ) is a medication that acts as an agonist for the alpha-2 (α_2) adrenergic receptors, similar to clonidine. It is indicated for spasticity management. It is also available under the brand name Zanaflex, among other names. The efficacy appears to be similar to that of baclofen or diazepam (Figure 1). It is intended to be ingested orally [1]. The complex nature of drug compounds makes the development of analytical methods more challenging. Robustness is crucial for

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achieving reliable results. Conventional One-Factor-at-a-Time (OFAT) processes often yield non-optimized methods that lack resilience, resulting in frequent failures. To address these issues, the pharmaceutical industry is utilizing a Design of Experiments (DoE) based approach for analytical method development. This approach enables a more comprehensive understanding of the system, facilitates faster optimization, and enhances the robustness of the developed method [2]. Several liquid chromatography methods have been reported for TIZ in both bulk and medicinal dosage forms, as per a literature study. While existing methods exhibit longer run times and lack DoE optimization, this study aimed to develop a highly efficient method for TIZ screening in bulk drugs and drug products by a DoE approach. The resulting method, validated along with forced degradation studies, offers a shorter run time and increased sensitivity [3]. Although stability-indicating HPLC methods for Tizanidine hydrochloride have been previously reported, most lacked systematic optimization via DoE. Our approach integrates full-factorial DoE, which enhances the method's robustness, reproducibility, and minimizes experimental trials, thereby offering a significant practical advancement for routine pharmaceutical analysis.

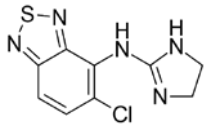


Figure 1: Structure of Tizanidine hydrochloride

MATERIALS AND METHODS

Chemicals and Reagents

Tizanidine hydrochloride was obtained as a complimentary sample from Sichuan Credit Pharmaceutical Co. Ltd. Acetonitrile (HPLC grade) was procured from Thermo Fisher Scientific India Pvt. Ltd., and trifluoroacetic acid (TFA) was sourced from Molychem, India. Type I water used in all experiments was obtained from an internal Milli-Q purification system. Tizan® 2 tablets (Sun Pharma Laboratories Ltd.) were purchased from the market for drug product analysis.

Rationale for HPLC Conditions

The RP-HPLC method was optimized to achieve precise, rapid, and reproducible analysis of Tizanidine hydrochloride. The Agilent Zorbax Bonus-RP column (250 × 4.6 mm, 5 µm) was selected for its high durability and superior peak symmetry. The mobile phase consisted of 0.1% trifluoroacetic acid and acetonitrile (65:35 v/v) to enhance peak resolution, reduce tailing, and maintain stability. The concentration of 0.1% TFA was chosen based on its ability to provide consistent peak symmetry, as lower concentrations (e.g., 0.05%) result in poor resolution, while higher concentrations (e.g., 0.2%) increase column backpressure. The selected mobile phase ensured effective ion-pair formation, optimal elution strength, and symmetrical peak shapes. The chromatographic analysis was carried out using an Agilent 1260 Infinity II HPLC system, equipped with a Diode Array Detector (DAD) to ensure precise peak identification. The method conditions were established based on robustness, reproducibility, and efficiency for routine quality control applications.

REAGENTS AND SOLUTIONS PREPARATION

Stock Solution Preparation

A Tizanidine Standard Stock Solution-I (TSSS-I) was prepared by dissolving 5 mg of Tizanidine hydrochloride in 10 mL of diluent (0.1% TFA: acetonitrile, 65:35 v/v), followed by sonication for 2 minutes to ensure complete dissolution. The volume was adjusted to 10 mL, yielding a final concentration of 500 µg/mL. A working standard solution was prepared by transferring 1 mL of TSSS-I into a 10 mL volumetric flask, adding 5 mL of diluent & vortexing. The volume was made up with diluent to achieve a final concentration of 50 µg/mL [4-5].

Drug Product Sample Preparation for Assay

Ten Tizan® 2 tablets (each containing 2 mg of Tizanidine hydrochloride) were weighed, and the average weight was calculated. The tablets were finely ground using a mortar and pestle to ensure uniformity. A quantity equivalent to 5 mg of Tizanidine hydrochloride was transferred to a 10 mL volumetric flask, followed by the addition of 5 mL of diluent. The solution was sonicated for 5 minutes, and the volume was made up to 10 mL using the same diluent, resulting in a concentration of 500 µg/mL [6-7]. For further dilution, 1.0 mL of the above solution was pipetted into a 10 mL volumetric flask, mixed with 5 mL of diluent, and vortexed. The volume was then made up with diluent to obtain a final concentration of 50 µg/mL.

Chromatographic Conditions

- **Column:** Agilent Zorbax Bonus-RP (250 × 4.6 mm, 5 µm)
- **HPLC System:** Agilent 1260 Infinity II HPLC
- **Detector:** Diode Array Detector (DAD) - G7115A
- **Mobile Phase:** 0.1% Trifluoroacetic Acid:Acetonitrile (65:35 v/v)

- **Flow Rate:** 0.5 mL/min
- **Injection Volume:** 10 µL
- **Oven Temperature:** 30°C
- **Detection Wavelength:** 228 nm
- **Run Time:** 15 minutes
- **Diluent:** 0.1% Trifluoroacetic Acid:Acetonitrile (65:35 v/v)

Data Analysis

Data acquisition and processing were carried out using Agilent OpenLAB CDS software (version 2.8), ensuring accurate peak integration, calibration curve generation, and quantification. Calibration curves were constructed by plotting peak area versus Tizanidine hydrochloride concentration, with a validated linear range of 40–60 µg/mL.

Preparation of calibration standards and quality control samples

Calibration standards were prepared by serial dilution of TSSS-I to obtain concentrations ranging from 80 to 120% (40–60 µg/mL) [8]. The samples were prepared using the following scheme (Table 1):

Table 1: Preparation of Calibration Standards and Quality Control Samples for Tizanidine Hydrochloride

% Level	Tizanidine Conc. (µg/mL)	TSSS-I (mL)	Final Volume (mL)
80%	40	0.8	10
90%	45	0.9	10
100%	50	1.0	10
110%	55	1.1	10
120%	60	1.2	10

Quality control (QC) samples at low (40 µg/mL), medium (50 µg/mL), and high (60 µg/mL) levels were used to assess precision and reproducibility.

Optimization Study

This study aimed to optimize chromatographic performance by identifying the ideal combination of Critical Method Variables (CMVs). Preliminary experiments revealed that the trifluoroacetic acid (TFA) concentration within the mobile phase, along with the flow rate, had a significant impact on peak symmetry and theoretical plate counts. A three-level, two-factor full factorial design was employed to investigate these CMVs. This design explores the combined effects of two variables on targeted response. The actual values for TFA percentage were 70% v/v, 65% v/v, and 60% v/v at high, intermediate, and low levels, respectively. Similarly, the flow rates were 0.55 mL/min, 0.50 mL/min, and 0.45 mL/min. This design is valuable when the relationship between factors and response might be non-linear. The Design-Expert software generated a design matrix plan comprising 13 experimental runs [9].

Statistical Analysis of Data

To assess the accuracy and reliability of the developed RP-HPLC method, a statistical evaluation was performed using a three-level, two-factor full factorial design. The analysis focused on two response variables: theoretical plates (R1) and asymmetry (R2). The quadratic model was applied to establish the correlation between experimental conditions and response variables. The analysis was conducted using Analysis of Variance (ANOVA), where significant F-values confirmed the model's suitability. The adjusted R^2 (R^2adj) values demonstrated a strong correlation between observed and predicted values, indicating the robustness of the statistical model. The model equations representing the theoretical plate number and asymmetry in terms of coded factors are as follows:

$$\boldsymbol{Theoretical\ Plates}\ (\boldsymbol{R1}) = +\mathbf{11678.59} + \mathbf{196.33}\boldsymbol{A} + \mathbf{533.33}\boldsymbol{B} - \mathbf{142.50}\boldsymbol{AB} - \mathbf{377.55}\boldsymbol{A2} - \mathbf{2545.55}\boldsymbol{B2}$$

$$\boldsymbol{Asymmetry}\ (\boldsymbol{R2}) = +\mathbf{1.39} - \mathbf{0.0050}\boldsymbol{A} + \mathbf{0.0700}\boldsymbol{B} + \mathbf{0.0050}\boldsymbol{AB} + \mathbf{0.0012}\boldsymbol{A2} - \mathbf{0.0338}\boldsymbol{B2}$$

Residual analysis was performed to validate the model's accuracy. Normal probability plots of residuals were generated to verify data distribution, confirming that the residuals followed a normal distribution. The residual plots illustrated the agreement between predicted and actual values, ensuring the reliability of the developed model. 3D response surface plots and contour plots were generated to visually depict the effect of mobile phase composition and flow rate on the chromatographic responses. These plots provided a graphical representation of interactions between critical method variables (CMVs) and their impact on peak characteristics. The contour plots illustrated how variations in % TFA concentration and flow rate influenced theoretical plates and asymmetry, assisting in method optimization. The selection of flow rate and TFA concentration was based on preliminary screening, which identified them as the most influential factors affecting retention time and resolution. Other parameters, such as column temperature, injection volume, and wavelength, were kept constant based on established literature values and showed minimal variability in

the response. The statistical validation confirmed that the developed RP-HPLC method was robust, reproducible, and reliable, making it suitable for routine pharmaceutical analysis [10-12].

METHOD VALIDATION [13-17]

Specificity and Assay

A working standard solution and drug product solution (50 µg/mL) were analyzed to confirm peak identification based on retention time. A blank sample was injected to ensure no interference with the central analyte peak. The assay was determined using the formula:

$$\%Assay = \frac{Sample\ Area}{Standard\ Area} \times 100$$

System Suitability and Repeatability

A single sample was injected six times, and system suitability parameters, including retention time, theoretical plates, asymmetry, and peak purity, were evaluated.

Linearity and Range

The method's linearity was confirmed by analyzing five calibration levels from 40 to 60 µg/mL. The correlation coefficient (r^2) was determined to assess the method's accuracy over the concentration range.

Inter-day and Intraday Precision

The stability of the standard solution within a single day was determined by comparing analyses performed at two distinct time points, morning and evening, with the resulting variation quantified as the percentage relative standard deviation (%RSD). The same solution was analyzed again on the following day and compared to the initial morning results. The %RSD was then calculated for this inter-day comparison.

Accuracy

Samples at 80%, 100%, and 120% concentrations were prepared and analyzed in triplicate to evaluate the percentage relative standard deviation (RSD) and percentage recovery.

LOD and LOQ

The limit of detection (LOD) and limit of quantification (LOQ) were calculated using the following formulas:

$$\mathbf{LOD} = \frac{\mathbf{3.3 \times Std.\ Error\ of\ Intercept}}{\boldsymbol{Slope\ of\ calibration\ curve}}$$

$$\mathbf{LOQ} = \frac{\mathbf{10 \times Std.\ Error\ of\ Intercept}}{\boldsymbol{Slope\ of\ calibration\ curve}}$$

Robustness

The robustness of the method was evaluated by altering the column temperature (±2°C) and wavelength (±2 nm), and analyzing the percentage assay variations (Table 2).

Table 2: Robustness Study: Effect of Column Temperature and Wavelength on Method Performance

Condition	Increased	Normal	Decreased
Column Temp.	32°C	30°C	28°C
Wavelength	230 nm	228 nm	226 nm

Forced Degradation Studies

Stress testing was performed under acidic, basic, oxidative, thermal, and UV conditions to evaluate the degradation of Tizanidine hydrochloride (Table 3).

Table 3: Forced Degradation Study of Tizanidine Hydrochloride Under Different Stress Conditions

Condition	Degradation (%)
Acidic (1N HCl, 10 min)	15.73%
Basic (1N NaOH, 10 min)	4.10%
Oxidation (30% H_2O_2, 30 min)	36.08%
Dry Heat (180°C, 5 hours)	4.95%
UV (254 nm, 5 hours)	3.72%

RESULTS

Method Development

For the high-performance liquid chromatography (HPLC) determination of TIZ, a solvent mixture consisting of 0.1% trifluoroacetic acid (TFA) and acetonitrile (ACN) was selected[17]. To optimize performance, a series of preliminary tests were conducted, varying the proportions of the solvent mixture (0.1% TFA: ACN) across the following ratios: 50:50, 55:45, 60:40, 65:35, 70:30, and 75:25.

The sample was examined using ultraviolet-visible (UV-VIS) spectroscopy. The UV absorption profile was generated using a solvent system of 65:35%v/v 0.1% TFA: ACN. This solution was analyzed within the wavelength range of 190 to 400 nm, utilizing the aforementioned solvent mixture as a reference. The peak absorbance of TIZ was identified at a wavelength of 228 nm.

Method development Screening Trials

Initial screening trials were conducted to assess critical method parameters and determine their influence on retention time and peak resolution. These trials served to narrow down optimal chromatographic conditions for final optimization. The relationship between eluent composition and flow rate, and their impact on peak asymmetry as well as theoretical plate number, is shown in Figure 2. Experiment 11, from the initial screening, utilized an eluent system comprising 65% 0.1% TFA, run at a 0.5 mL/min flow rate, and demonstrated promising results. TIZ peak was observed at 4.54 minutes within a 15-minute run time as shown in Figure 3. The method exhibited high efficiency with 11694 theoretical plates, acceptable peak symmetry (1.39, below the threshold of 2.00), and excellent peak purity (1.00). Retention times were confirmed using individual standards.

Optimization Study

To optimize the chromatographic method, a three-level, two-factor full factorial design was employed. Design-Expert software generated a 13-run experimental plan. After conducting all experiments, the data were analyzed using DoE software. Various statistical models were fitted to the data, and the optimal model was selected based on criteria such as a high F-value, a low P-value, and a high R-squared. Two-way ANOVA was then utilized to validate chosen model.

Statistical Analysis of Data

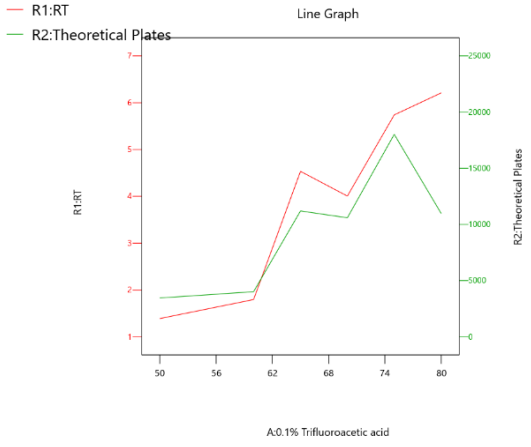
Table 4 presents the statistical evaluation and model fit for both response variables: the number of theoretical plates (R1) and asymmetry (R2). Statistical analysis revealed that a quadratic model effectively represented the data for both response variables. This conclusion is supported by significant F-values of 570.47 and 31.46, respectively. The model's predictive strength, as indicated by the adjusted R-squared (R^2adj) value, demonstrates a robust relationship between the predicted and observed outcomes. The model equations for R1 and R2, expressed in terms of coded factors, are as follows:

$$\boldsymbol{Theoretical\ Plates}\ (\boldsymbol{R1}) = +\mathbf{11678.59} + \mathbf{196.33}\,\boldsymbol{A} + \mathbf{533.33}\,\boldsymbol{B} - \mathbf{142.50}\,\boldsymbol{AB} - \mathbf{377.55}\,\boldsymbol{A^2} - \mathbf{2545.55}\,\boldsymbol{B^2}$$

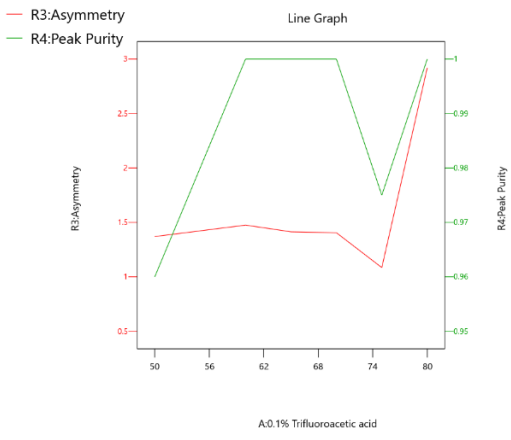
$$\boldsymbol{Asymmetry}\ (\boldsymbol{R2}) = +\mathbf{1.39} - \mathbf{0.0050}\,\boldsymbol{A} + \mathbf{0.0700}\,\boldsymbol{B} + \mathbf{0.0050}\,\boldsymbol{AB} + \mathbf{0.0012}\,\boldsymbol{A^2} - \mathbf{0.0338}\,\boldsymbol{B^2}$$

Table 4: ANOVA results obtained from a three-level, two-factor full factorial design for Theoretical Plates and Asymmetry (Quadratic model)

	Response 1: Theoretical Plates		Response 2: Asymmetry	
Source	**F-value**	**p-value**	**F-value**	**p-value**
Model	570.47	< 0.0001	31.46	0.0001
A-MOBILE PHASE A (0.1% TFA)	25.59	0.0015	0.7098	0.4274
B-FLOW RATE	188.83	< 0.0001	139.12	< 0.0001
AB	8.99	0.0200	0.4732	0.5137
A^2	43.56	0.0003	0.0190	0.8941
B^2	1980.10	< 0.0001	14.92	0.0062
Lack of Fit	34.85	0.0025	4.83	0.0811



A



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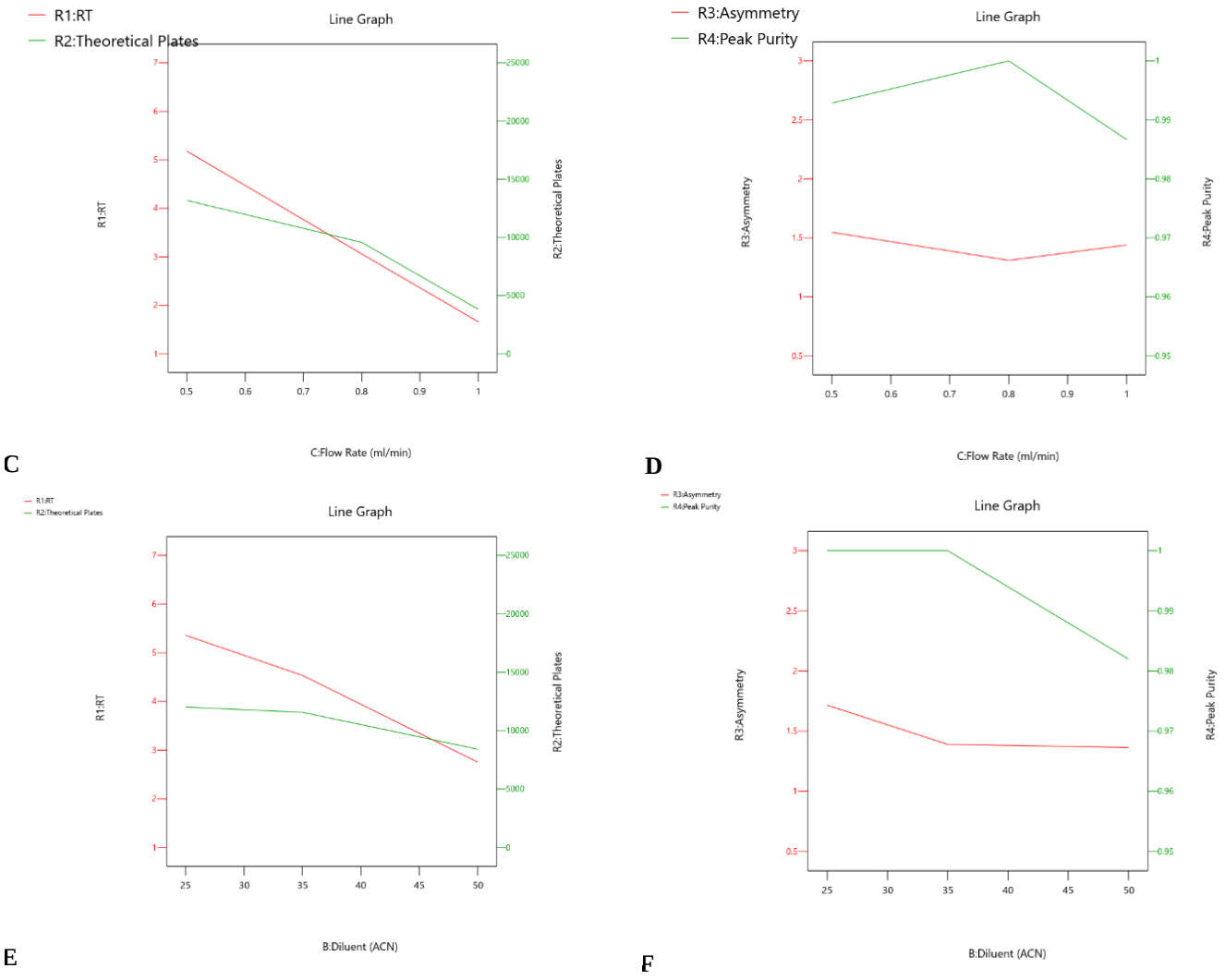


Figure 2: Relationship Plot: Eluent composition, flow rate, and their impact on peak asymmetry, theoretical plate number

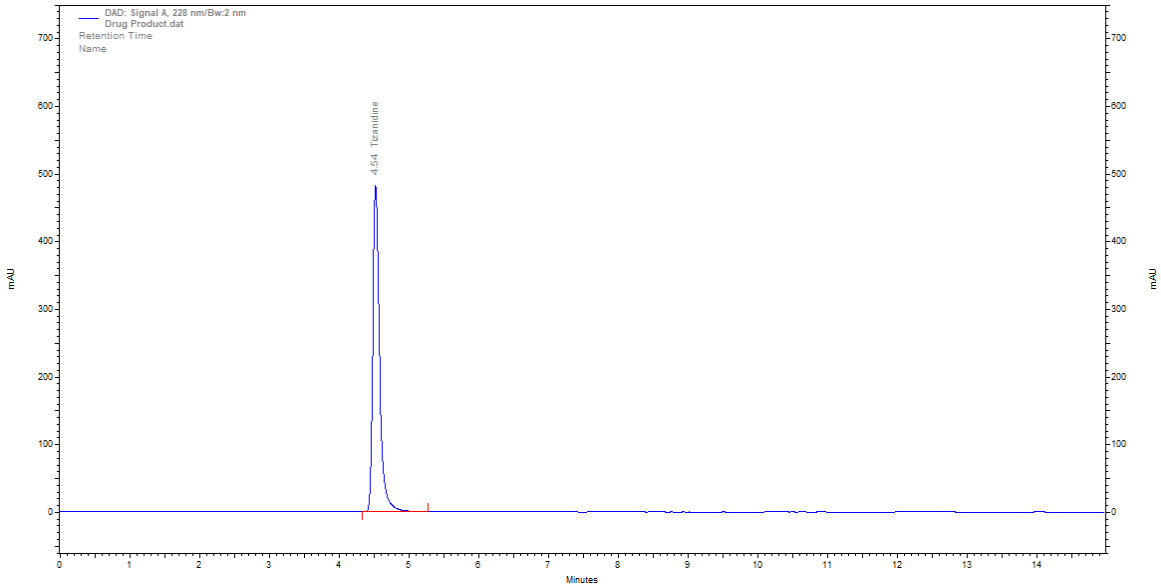


Figure 3: HPLC Chromatogram of Pure Tizanidine hydrochloride (TIZ)

A normal probability plot of residuals for both retention time and asymmetry is presented in Figures 4A and 4B. This plot suggests the statistical accuracy of the model used for retention time prediction. The normal distribution of residuals and the good agreement between predicted and actual values indicate the accuracy and reliability of the model.

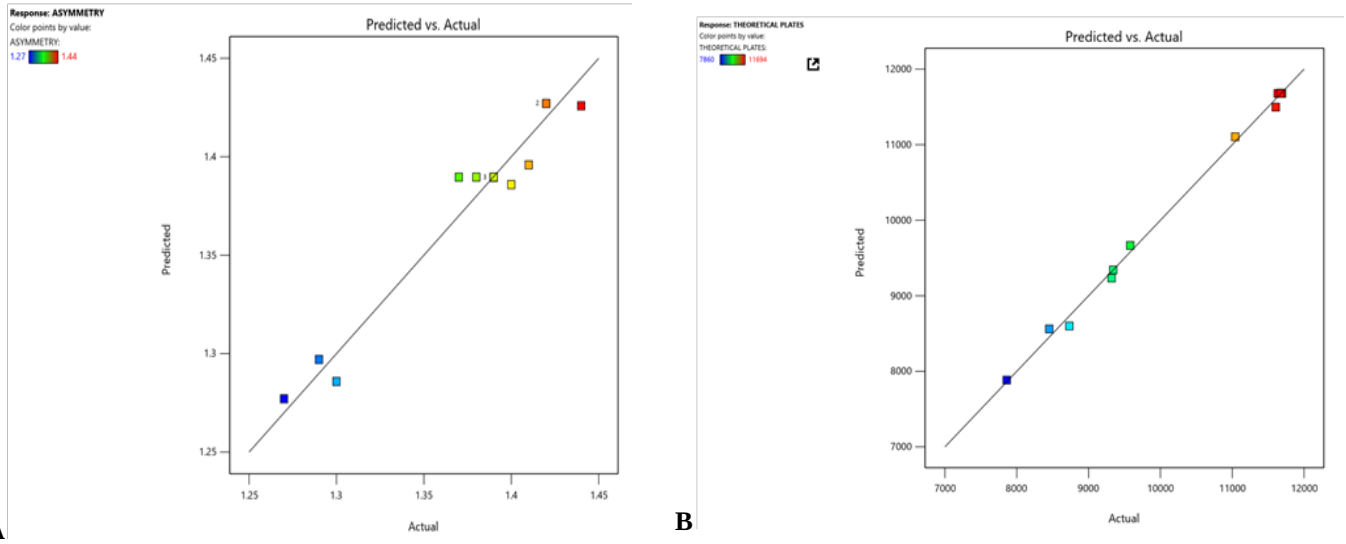
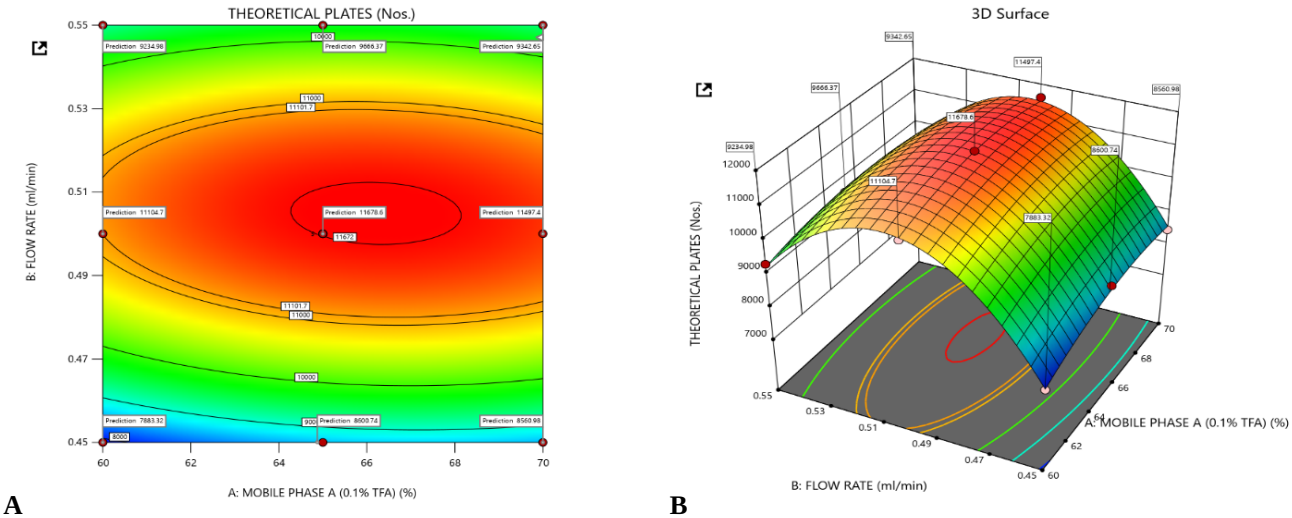
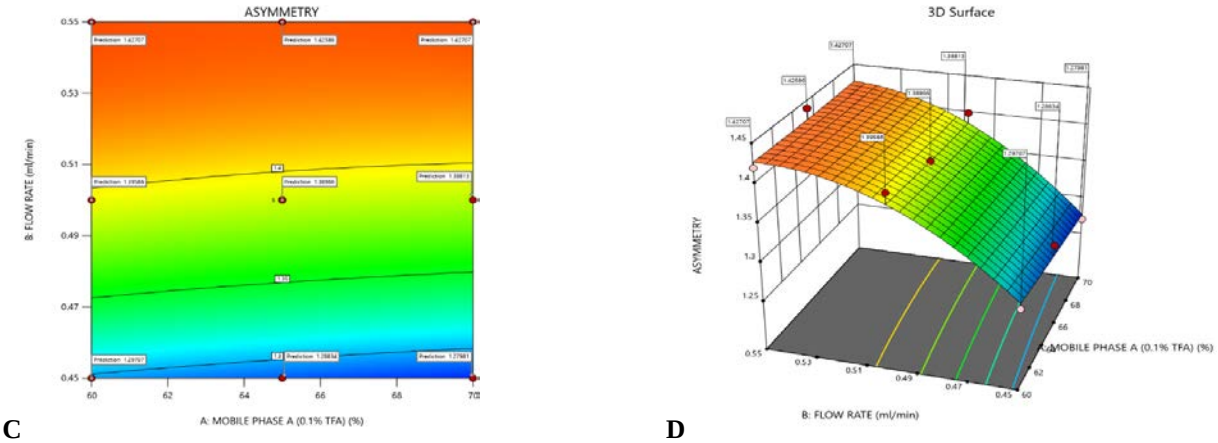


Figure 4: (A) Residual Plot Predicated vs Actual values for Asymmetry and (B) Residual Plot Predicated vs Actual values for Theoretical Plate

A 3D response surface plot visually represents the relationship between two independent variables and a dependent variable (response) in HPLC method development. Three-dimensional response surfaces, shown in Figures 5B and 5D, represent the behaviour of theoretical plate number (R1) and asymmetry (R2). These plots illustrate the impact of critical method variables (CMVs) on the analytical responses. Both Factor A and Factor B exert a positive influence on the number of theoretical plates, as evidenced by the coefficients in the polynomial equation. Conversely, for asymmetry, Factor A has a negative effect, while Factor B exhibits a positive influence, consistent with the polynomial equation coefficients. A counterplot, as shown in Figures 5A and 5C, is a graphical representation used to visualize the response surface of a two-factor, three-level full factorial design. The x-axis represents the %TFA concentration, and the y-axis represents the flow rate. The contour lines connect points of equal response values.

By observing the spacing and direction of these lines, we can understand how the response changes as we vary these factors, which is according to a polynomial equation.





C **D**

Figure 5: Relation Plot: (A and C) Counter plot (B and D) 3D plot for design of experiments trials

METHOD VALIDATION

Specificity and Assay

The retention times of both the API and DP peaks were identical, at 4.54 min. The absence of any blank interference with the main peak confirmed the identification of TIZ in both the API and DP. The assay of the marketed product was 99.89%.

Instrument Precision, Method Precision, Intermediate Precision, and System Suitability

The results of three precision tests (instrument, method, and intermediate) are presented in Table 5, yielding coefficients of variation (%CV) of 0.03%, 0.07%, and 0.06%, respectively. Before these tests, the suitability of the HPLC instrument for validation was confirmed using the optimized parameters.

Linearity

Various levels of linearity were assessed. The graphical representation (Figures 6A and B) showed a linear pattern, as indicated by the correlation coefficient. Linearity was evaluated for TIZ with concentrations ranging from 80% to 120%, and a correlation coefficient of 1.0 was observed.

Robustness

As depicted in Table 6A, changes were implemented in column temperature (±2°C) and wavelength (±2nm) to evaluate the method's sensitivity. The method demonstrates robustness under these conditions, as no significant alterations were observed in the retention time or peak area of either the API or the marketed product.

Accuracy

The accuracy of TIZ was assessed in three replicates, and it was found that the procedure was precise for the test concentration at 80%, 100%, and 120%. The %RSD for the concentrations of 80%, 100%, and 120% were 0.13%, 0.03%, and 0.23% respectively, as shown in Table 6 B. The mean percentage recovery was determined to be between 99% and 101%. Accuracy data indicate that the method can accurately analyze and recover different concentrations of the drug in solution.

Table 5: Instrument precision, method precision, intermediate precision, and system suitability for TIZ

Sample ID	Peak Area		
	Instrument Precision	Method Precision	Intermediate Precision
Repeat 1	6817893	6817596	6820757
Repeat 2	6822059	6821826	6818585
Repeat 3	6821826	6819775	6822059
Repeat 4	6818971	6822562	6822562
Repeat 5	6823406	6812272	6823163
Repeat 6	6818391	6824578	6814174
Average	6820424	6820203	6820108.6
STDEV	2288.29	4754.56	3764.75
%RSD	0.03	0.07	0.06

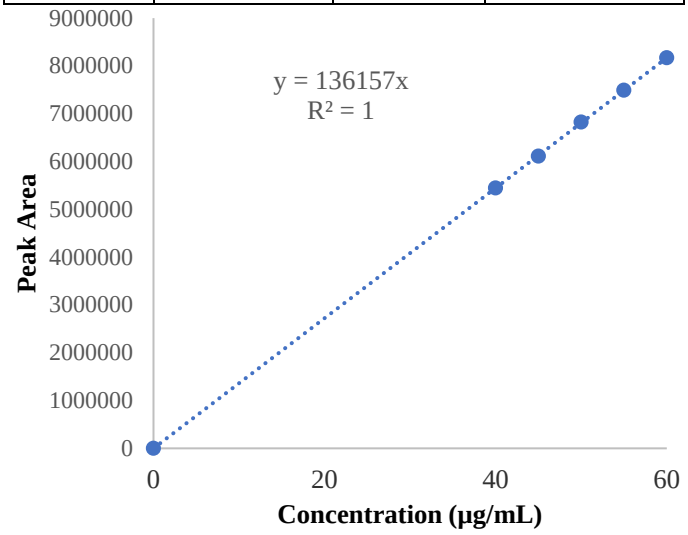


Figure 6 (A): Linearity scale (B) of TIZ standard solution

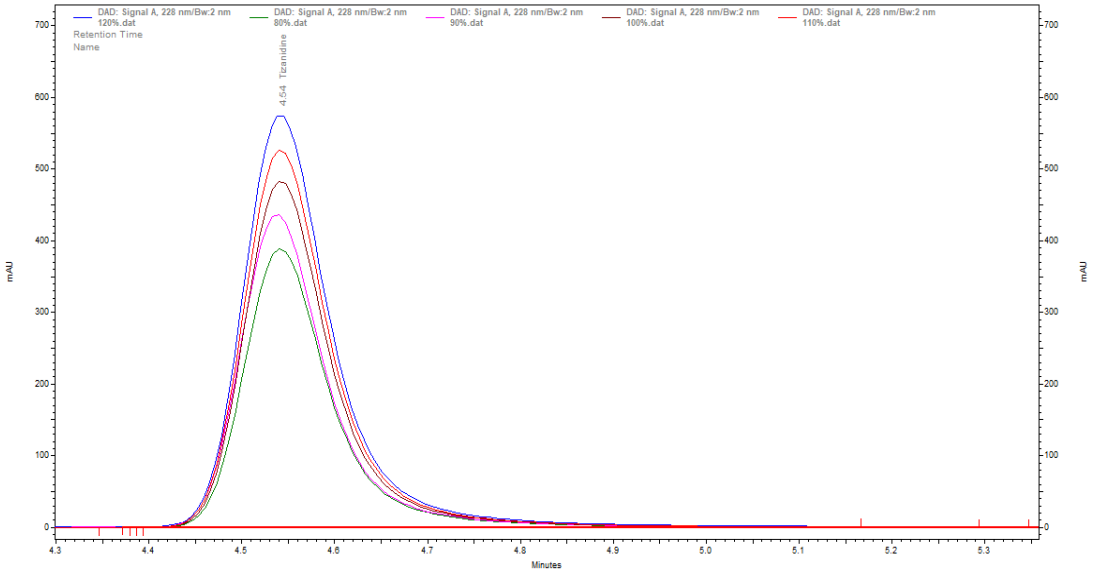


Figure 6(B): Linearity overlay of TIZ standard solution

LOD & LOQ

Linearity data showed remarkably low LOD (1.00 µg/mL) and LOQ (3.04 µg/mL) for TIZ, indicating the method's effectiveness in measuring low drug levels.

Intra- and inter-day precision: Intra- and inter-day precision (%RSD of assay and peak area change for API and marketed product) was 0.03% (Table 6C), which is below the 2% acceptance criterion. This confirms the two-day stability of the working standard solution.

Table 6: Analytical method validation results for Parameters (A) Robustness (B) Accuracy (C) Intra and Inter-Day Precision

Parameters	Sample ID	% Recovery	Average	% RSD
A. Results of robustness study: change in column oven temperature and wavelength				
Column oven temperature change	28°C	99.95	99.95	0.06
	30°C	99.89		
	32°C	100.01		
Change in wavelength (nm)	226 nm	99.92	99.90	0.015
	228 nm	99.89		
	230 nm	99.9		
B. Results of accuracy parameter for tizanidine hydrochloride test method				
80%	Rep 1	99.77	99.85	0.13
	Rep 2	99.99		
	Rep 3	99.77		
100%	Rep 1	99.96	100.00	0.03
	Rep 2	100.02		
	Rep 3	100.02		
120%	Rep 1	99.82	99.62	0.23
	Rep 2	99.46		
	Rep 3	99.78		
C. Intra-day and Inter-day precision				
Day 1	Morning	99.89	99.92	0.042%
	Evening	99.95		
Day 2		99.93	99.92	0.03%

Forced Degradation

The results for forced degradation of TIZ are presented in Table 7. A stress study was conducted, which exhibited high degradation in oxidative conditions, at 36.08% (sample 1) and 29.28% (sample 2). The degradation was 15.73%, 4.10%, 4.95%, and 3.72% in acidic, basic, heat, and photolysis conditions, respectively. Tizanidine hydrochloride showed significant degradation under oxidative and acidic conditions. This can be attributed to the presence of a tertiary amine group and an imidazoline ring, which are susceptible to hydrolytic cleavage and oxidative degradation. Literature reports suggest that oxidative stress leads to N-oxide formation, while acid catalysis may disrupt the heterocyclic system.

Table 7: Results of forced degradation in the tizanidine hydrochloride test method

Sample ID	Condition	% Assay	% Degradation
Working Standard	Control	100.00	-
Acid Hydrolysis Sample	1 mL Stock + 1 mL 1N HCl for 10 min at RT	84.27	15.73
Base Hydrolysis Sample	1 mL Stock + 1 mL 1N NaOH for 10 min at RT	95.90	4.10
Oxidation Sample 1	1 mL Stock + 1 mL 30% H_2O_2 for 30 min at RT	63.92	36.08
Oxidation Sample 2	1 mL Stock + 1 mL 30% H_2O_2 for 10 min at RT	70.72	29.28
Heat degradation Sample	10 mg API + 100˚C for 5 hours	95.05	4.95
UV degradation Sample	10 mg API + 254 nm for 5 hours	96.28	3.72

DISCUSSION

The optimal HPLC method depends on several factors, including the sample characteristics such as its ionic nature, ionizability, molecular weight, as well as its solubility. Since the drug under investigation is polar, RP-HPLC was selected for its simplicity and suitability for separating polar compounds. Changing the pH of the mobile phase will determine if secondary interactions lead to difficulties with peak shape or retention. Including TFA can be advantageous. The cutoff wavelength for 0.1% TFA is 205 nm, allowing analyte detection without interference from the mobile phase. Another factor considered when selecting TFA was the ease of method transfer from RP-HPLC to LC-MS analysis. ACN and methanol are two organic solvents often utilized as mobile phases in HPLC. Compared to methanol, ACN has a low column pressure. ACN has increased elution strength and is compatible with most buffers. The cutoff wavelength for ACN is 190 nm. Agilent Zorbax Bonus RP column measuring 250 x 4.6 mm, with 5 µm particles, exhibits distinct selectivity compared to just alkyl or aryl stationary phases, making it a favorable choice for separating basic, acidic, and highly polar chemicals using RP-HPLC.

As the concentration of TFA increases, the retention time of basic analytes, such as TIZ. This is because TFA acts as an ion-pairing agent, forming ion pairs with the analyte and reducing its interaction with the stationary phase. Whereas, increased ACN concentration results in decreased retention time for TIZ, as ACN is a less polar solvent than water. TFA and ACN concentration balance the retention of the TIZ and the peak broadening effects. Too high or too low TFA concentration leads to reduced plate numbers due to increased peak broadening or insufficient retention. Increased TFA concentration leads to increased asymmetry. Higher TFA concentrations can sometimes lead to increased peak tailing, especially for basic analytes like TIZ. This is because TFA forms ion pairs with the analyte, which can interact more strongly with the stationary phase, causing slower elution of some analyte molecules and leading to tailing. Whereas increased ACN concentration leads to reduced asymmetry. Increasing acetonitrile concentration in the mobile phase can improve peak symmetry for TIZ. An increased flow rate results in a decreased retention time, as the analyte is carried through the column more rapidly. Whereas suboptimal theoretical plates can lead to reduced plate numbers due to increased band broadening. High flow rates lead to increased asymmetry. Very high flow rates can increase peak tailing due to insufficient time for analyte molecules to equilibrate with the stationary phase. Low flow rates reduce asymmetry. However, excessively low flow rates can increase extra-column band broadening, which can also contribute to peak asymmetry.

The optimization study employed a full factorial design to evaluate the effects of TFA concentration and flow rate. The quadratic model provided a strong fit, as confirmed by ANOVA, showing significant influences of these variables on theoretical plates and asymmetry. The normal distribution of residuals

supported the model's statistical validity. The validation data confirmed the method's reliability, with excellent specificity, precision, and robustness. The low %RSD values in precision studies indicated high reproducibility, and the linearity data confirmed the method's suitability for a range of concentrations. The low LOD and LOQ values highlighted its sensitivity for detecting TIZ at low levels. Stress testing revealed that oxidative conditions caused significant degradation, while acidic and basic conditions led to moderate degradation. Heat and photolysis conditions resulted in minimal degradation. These findings indicate the need for careful handling of TIZ under oxidative environments to maintain stability.

A robust and sensitive analytical method was developed and validated, employing reversed-phase high-performance liquid chromatography, and was established and confirmed for the quantification of TIZ. Utilizing gradient elution and UV detection at 228 nm, the method's critical variables were optimized through a three-level, two-factor full factorial design, enhancing robustness. The method demonstrated excellent linearity ($r^2 > 1.0$) within the defined concentration range, and acceptable %RSD values and recovery studies confirmed its precision and accuracy. The method proved specific for tizanidine hydrochloride under stress conditions, in the presence of degradation impurities. This validated method is suitable for routine quality control analysis of tizanidine hydrochloride in pharmaceutical formulations and stability studies, thereby contributing to the assurance of drug product safety and efficacy. Compared to previous RP-HPLC methods reported for Tizanidine hydrochloride, the current method offers a shorter run time of 4.54 minutes. In contrast, earlier studies reported a run time of 10 minutes by Rekha Rani et al. (2023). Additionally, the use of DoE provided enhanced robustness and method reliability by systematically evaluating interactions between variables, unlike traditional OFAT (one-factor-at-a-time) approaches, which are time-consuming and less efficient. A potential limitation of this study is the restricted scope of degradation products evaluated, which may not encompass all possible impurities under diverse storage conditions.

CONCLUSION

A stability-indicating RP-HPLC method for Tizanidine hydrochloride was successfully developed and optimized using a DoE-based approach. The process was validated for specificity, precision, accuracy, linearity, robustness, and sensitivity, confirming that it is suitable for the routine quality control and stability assessment. Forced degradation studies confirmed the method's ability to differentiate the drug from its degradation products, making it reliable for stability testing. The statistical optimization ensured robust method performance, minimizing variability while enhancing reproducibility. This validated RP-HPLC method is efficient, precise, and stability-indicating, making it an essential tool for the pharmaceutical industry in the quality control and regulatory assessment of Tizanidine hydrochloride formulations.

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FINANCIAL ASSISTANCE

Nill

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTION

Prasad Gunjal collected data and performed experiments. Janki Patel conducted the analysis. Prasad Gunjal wrote the first draft of the manuscript, and all authors reviewed and revised previous versions. All authors contributed to the study's conception and design and gave final approval.

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