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INTEGRATION OF BOX–BEHNKEN DESIGN AND QBD PRINCIPLES FOR OPTIMIZING RP-HPLC ANALYSIS OF IMEGLIMIN HYDROCHLORIDE

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Abstract
The development and validation of analytical methods are critical components in ensuring the quality, safety, and efficacy of pharmaceutical products. This study presents a comprehensive approach to developing and validating an analytical method for Imeglimin Hydrochloride, a novel anti-diabetic agent with a unique dual mechanism targeting mitochondrial bioenergetics. Emphasis is placed on the integration of Quality by Design principles to enhance method robustness, accuracy, and reproducibility. A systematic approach was adopted for method development utilizing reverse-phase high-performance liquid chromatography (RP-HPLC). The Box-Behnken Design was utilized to optimize critical method parameters, and method validation was conducted in accordance with the ICH Q2 (R1) guidelines. The developed methods were systematically evaluated for linearity, accuracy, precision, specificity, robustness, ruggedness, and stability.The HPLC method, employing a mobile phase consisting of methanol and water in a 60:40 (v/v) ratio, demonstrated excellent system suitability and precision, with detection performed at 239 nm. Method is effectively used to assay marketed formulations, validating their suitability for routine quality control. Furthermore, risk assessments and Quality by Design tools such as design space and control strategies were employed to ensure method reliability throughout the drug's lifecycle. This work highlights the significance of adopting a science-based and regulatory-compliant strategy for analytical method development, particularly for novel pharmaceutical compounds like Imeglimin Hydrochloride. The validated methods offer reliable tools for quality assurance in both research and industrial settings, supporting regulatory submissions and advancing diabetes therapeutics

(Keyword: Quality by Design, Reverse-Phase HPLC, Box-Behnken Design, Imeglimin Hydrochloride.)

Introduction:

Analytical techniques are utilized to quantify the active pharmaceutical ingredient in commercial products, ensuring quality assurance and adherence to regulatory guidelines (1).analytical chemistry undeniably serves as a foundational and enabling science for various fields such as medicinal research, life sciences, environmental monitoring, and materials science (2). Ongoing advancements

in analytical chemistry, particularly through the development of sophisticated instruments such as high-performance liquid chromatography (HPLC), ultraviolet-visible (UV-Vis) spectrophotometry, and mass spectrometry, have markedly improved the precision and reliability of pharmaceutical analysis. Effective method development facilitates the optimal use of laboratory resources while fulfilling the intended objectives across different stages of drug development(3). Regulatory authorities mandate method validation at specific stages of the drug approval process, which involves demonstrating that analytical techniques are appropriate for their intended application (4,5).In recent years, there has been an increasing focus on adopting systematic and scientifically-driven approaches in the development of analytical methods. Quality by Design (QbD) is one such framework that focuses on incorporating quality throughout each phase of the method lifecycle. By applying QbD principles, a comprehensive understanding of critical method parameters (CMPs) and critical quality attributes (CQAs) is achieved, enabling risk-based optimization and control strategies that enhance method robustness, consistency, and adherence to regulatory standards(6,7).This concept emphasizes a systematic, science-driven strategy to ensure quality is built into products from the initial stages of development (8).

Imeglimin Hydrochloride(R)-6-imino-N,N,4-trimethyl-1-1,4,5,6-tetrahydro-1,3,5-triazin-2-amine hydrochloride a novel oral antidiabetic agent, represents an emerging therapeutic option with a dual mechanism of action targeting insulin secretion and sensitivity. Given its unique pharmacological properties and potential clinical applications, the development and validation of analytical methods for its quantification in both bulk drug substances and dosage forms is imperative(9). This study centers on the development and validation of a reverse-phase HPLC method for Imeglimin Hydrochloride, utilizing Quality by Design (QbD) principles and conforming to ICH guidelines to ensure accuracy, precision, and consistency in quality control[10].

Within the Quality by Design (QbD) framework, the application of statistical Design of Experiments (DoE) is utilized to define the design space, thereby facilitating the development of a robust and reliable analytical method. The design space delineates the range of experimental conditions within which variations in procedural parameters have a minimal impact on the analytical performance or outcome. This approach strengthens the method's robustness throughout the development process[11]. The Food and Drug Administration (FDA) advocates for the adoption of Quality by Design (QbD), which focuses on embedding quality into both the product and process via a systematic, scientific, and risk-based approach during development, rather than relying solely on post-production quality testing. The International Conference on Harmonization (ICH) has published several guidelines to facilitate and standardize the implementation of the Quality by Design (QbD) framework [12]. Recently, the FDA has issued several important publications detailing comprehensive strategies for the application of Quality by Design (QbD) principles to analytical measurements and the development of HPLC methods [13,14].The present investigation was carried out with the objective of developing and thoroughly validating a reverse-phase HPLC method for the analysis of Imeglimin HCl, in alignment with the principles of Quality by Design (QbD).

Experimental Work

1) Apparatus:

Double beam UV visible spectrophotometer (UV1900, SHIMADZU, Japan). Electronic balance (SR Electronics, Mumbai, India). Hot air oven (Prerana Ent Mumbai).Sonicator (CD 4820, Digital ultra sonic cleaner, Mumbai, India). Digital pH meter (Digital systronic, Mumbai, India).FTIR spectrophotometer (FTIR-4600, type A, JASCO). HPLC (Agilent 1100 series)

2) Materials:

The reference standard for Imeglimin Hydrochloride was obtained from Zydu Healthcare Pvt Ltd, Gujarat, India. The chemicals used in the study included Sodium Hydroxide, Hydrochloric Acid,

Hydrogen Peroxide, Acetonitrile (HPLC grade), Methanol (HPLC grade), and purified distilled water.

3) Chromatographic condition:

Column - Repro Q, C18 150mm × 4.6 mm
Mobile phase - Methanol: Water (60:40 v/v)
Injection volume - 20 µL
Wavelength - 239 nm
Column température - 40°C
Run time - 10 min
Pump - Reciprocating pump

4) Selection of Detection Wavelength:

An accurately weighed 50 mg portion of the standard drug was transferred to a 100 ml volumetric flask to prepare a stock solution with a concentration of 500 µg/ml. A further dilution was performed to achieve a final concentration of 15 µg/mL, with the iso-absorptive point determined at 239 nm.

5) Development of an HPLC technique using the QbD approach:

Analytical QbD developed the following HPLC procedure:
The HPLC analytical method was developed and optimized within the framework of Quality by Design (QbD). Flow rate (A) and mobile phase composition (B) were identified as the critical method parameters (CMPs), whereas peak area and retention time (RT) were designated as the critical quality attributes (CQAs). To methodically examine the impacts and interactions among these variables and their influence on the analytical results, a design of experiments (DoE) technique was used.
The interaction effects between variables on CQAs were graphically interpreted using 3D surface plots created with statistical software (Design-Expert). These graphs offer insights into strong technique conditions and make it easier to grasp the ideal analytical region.

6) Selection of quality target product profile:

The establishment of the Quality Target Product Profile (QTPP) is essential for identifying the factors influencing its parameters. In the developed HPLC method, retention time, theoretical plate count, and peak asymmetry were selected as the key QTPP attributes.

7) Determine critical quality attributes:

Critical Quality Attributes (CQAs) are method parameters that directly affect the Quality Target Product Profile (QTPP). Among these factors, buffer pH and mobile phase composition were recognized as crucial variables that must be controlled to ensure the QTPP remains within the desired response range.

8) The Validation of the Box-Behnken Design:

Fifteen experimental trials were designed based on grid search results and carried out using the selected compositions. These runs were evaluated for critical quality attributes (CQAs), including tailing factor (TF), assay, and the number of theoretical plates (TP). Response data were then used to generate linear correlation plots by comparing the predicted and observed values.

Development and Validation of a Reverse-Phase HPLC Analytical Method:

1) Selection of Mobile Phase:

Various mobile phase compositions were evaluated to identify the optimal combination. The selected mobile phase consisted of HPLC-grade methanol and water in a 60:40 v/v ratio.

2) Preparation of mobile phase:
The mobile phase was prepared by combining HPLC-grade methanol and water in a 60:40 (v/v) ratio. The resulting solution was subjected to sonication for 15 minutes to effectively remove any dissolved gases.

3) Preparation of Diluents:
Distilled water and the selected mobile phase were used as diluents in the study. The solution was degassed by sonication to eliminate dissolved gases.

4) **Preparation of Standard Stock Solution:**
An accurately weighed quantity of 50 mg of the standard drug was transferred to a 100 mL volumetric flask and diluted appropriately to obtain a stock solution with a concentration of 500 µg/mL. One milliliter of the stock solution was transferred to a 100 ml volumetric flask, diluted appropriately, and sonicated for 5 minutes. The volume was then adjusted to the mark with the diluent to achieve a final concentration of 5 µg/ml. Finally, 20 µL of the prepared solution was injected into the chromatographic system for analysis.

5) Preparation of Sample Solution:
Powdered samples from twenty tablets, sourced from three different manufacturers, were carefully weighed. A quantity equivalent to 50 mg of Imeglimin Hydrochloride (IMG HCl) was transferred into a 100 mL volumetric flask, initially dissolved in 50 mL of distilled water, and subsequently sonicated for 5 minutes to facilitate dissolution. The volume was then adjusted to the mark with distilled water to obtain a solution with a concentration of 500 µg/mL. Subsequent dilutions were performed to yield concentrations falling within the validated linearity range for analytical evaluation.

Method Validation Parameters:
1.System suitability test:
The system suitability test (SST) is an essential pre-analysis step in HPLC method development that verifies the chromatographic system is operating properly and capable of delivering reliable results. This process involves the evaluation of critical parameters, including USP plate count, USP tailing factor, USP resolution, and percentage relative standard deviation (%RSD), to ensure the system's efficiency, precision, and reproducibility. The SST is conducted by injecting a standard solution multiple times and assessing these parameters against predefined acceptance criteria, as per ICH and USP guidelines. A well-optimized SST ensures that the system can provide accurate, precise, and reproducible data, minimizing variability and potential errors in sample analysis. If any parameter falls outside the acceptable range, troubleshooting is required before proceeding with the actual sample analysis.

Sr. No.	Parameter	Acceptance Criteria
1	USP plate count	NLT 2000
2	USP tailing	NMT 2.0
3	USP resolution	NLL 2.0
4	% RSD	NMT 2.0

Acceptance criteria for evaluation of experimental result

2.Specificity:
To assess potential interference, the method was applied to analyze the chromatograms of the mobile phase blank, standard solution, and tablet formulation separately. According to the acceptance criteria, no detectable peak should be observed at the analyte's retention time in either the blank or standard solutions. Furthermore, the peaks from the standard and sample solutions

should be well-resolved and distinct. The purity of each peak was evaluated to ensure accurate identification and quantification of the analyte, without interference from excipients or other components.

3.Linearity :

The linearity of the method was assessed by preparing standard solutions at five concentration levels: 5, 10, 15, 20, and 25 µg/ml. Linear regression analysis was performed to evaluate the relationship between analyte concentration and the corresponding analytical response. The coefficient of determination (r^2) was required to be ≥ 0.999, demonstrating a strong linear correlation between concentration and absorbance.

4.System precision:

To assess system precision, a standard solution at 15 µg/ml was prepared and injected six times under the same conditions. The relative standard deviation (RSD) was calculated to assess the consistency of peak responses.

The RSD value should be ≤ 2.0%, ensuring minimal variation in repeated measurements.

5.Method precision:

Method precision was assessed by preparing six independent sample solutions at a concentration of 15 µg/ml, following the established test procedure. Each sample was analyzed using consistent chromatographic conditions to assess the method's reproducibility.

The RSD should be ≤ 2.0%, ensuring the method produces reproducible results across multiple sample preparations.

6.Accuracy :

The accuracy of the method was assessed through a percent recovery study conducted at three concentration levels: 50%, 100%, and 150% of the nominal concentration. Known amounts of the standard drug were added to previously analyzed samples, and the recovered drug quantity was measured to assess the method's accuracy in determining the true concentration. The percent recovery should fall within 98% to 102%, with the RSD not exceeding 2.0%, ensuring the method's reliability in quantifying the drug accurately.

8. Ruggedness:

The method's robustness was evaluated using an IMG HCl concentration, with two analysts analyzing aliquots from a uniform batch under the same operational and environmental conditions.

Acceptance criteria: RSD < 2%

9. Robustness:

The robustness of the method was evaluated by making slight changes to the flow rate, mobile phase composition, and wavelength, and then observing the effects of these variations on the results obtained.

a) Changing flow rate by ± 2.

b) Changing the composition of mobile phase by ± 2%.

c) Changing column temperature by ± 2° C. System suitability was done for each condition.

Acceptance criteria:

System suitability criteria shall be passed.

Overall % RSD should be less than 2.0.

10.Solution stability:
The stability of the Imeglimin (IMG) solution was assessed by storing standard samples at room temperature and under refrigerated conditions between 2 and 8 °C. The study revealed that the drug solution remained stable for a duration of two days when maintained under these specified storage environments. The acceptance criterion for stability was defined as a percentage difference in results not exceeding 2.0%.

11. LOD & LOQ:
The limits of detection (LOD) and quantitation (LOQ) were determined based on the standard deviation of the intercept and the slope of the calibration curve.
Acceptance criteria: Detection limit= >2 times base line
Quantification limit= Signal to Noise =10.1
$LOD = 3.3\ Sa \div b$
$LOQ = 10\ Sa \div b$

Result:
QbD-Based Optimization of HPLC Method Using Flow Rate and Mobile Phase Composition

Run	Factor 1 A: Flow Rate (ml)	Factor 2 B: Mobile Phase Ratio (ml/min)	Response 1 Area	Response 2 Retention Time
1	1.1	60:40	1680.35	3.62
2	0.9	70:30	1878.5	3.84
3	**0.9**	**60:40**	**1973.84**	**4.115**
4	1.1	80:20	1582.99	3.458
5	1	80:20	1727.8	3.783
6	1	60:40	1735.97	3.6653
7	1.1	70:30	1592.62	3.244
8	0.9	80:20	2043.67	4.041
9	1	70:30	1739.63	3.559

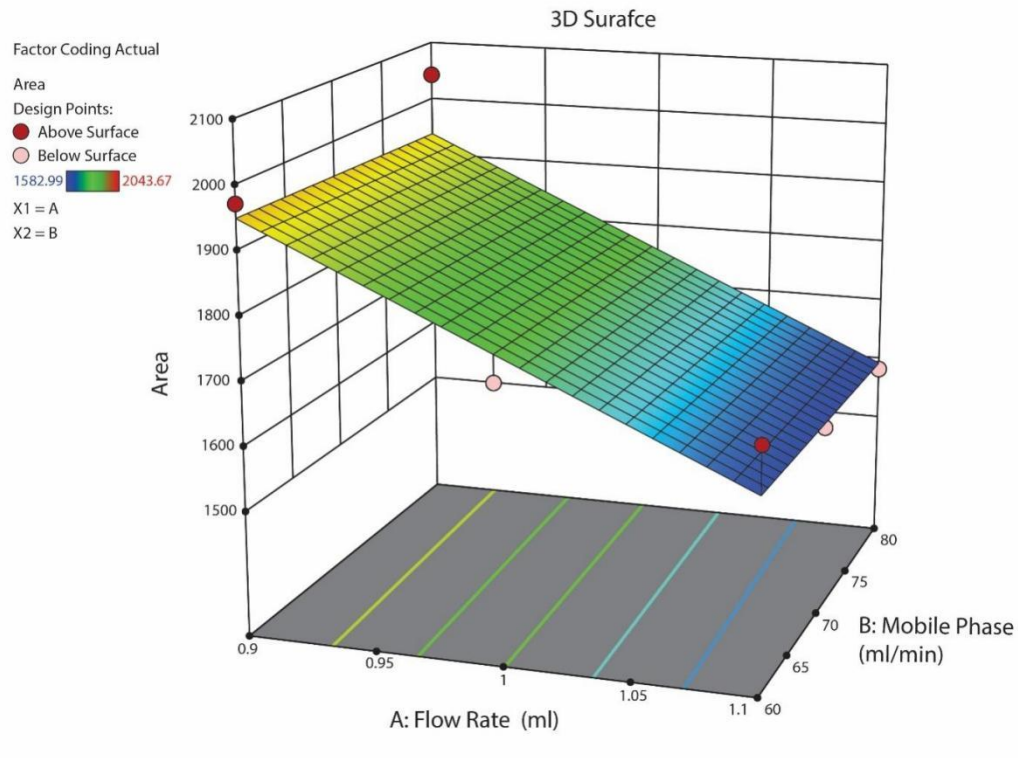
A)Analysis of Variance (ANOVA) for Selected Factorial Model – Response: Area
A factorial ANOVA was conducted to evaluate the influence of Flow Rate (Factor A) on the chromatographic response, specifically the peak area. The analysis was performed using a Type II Classical Sum of Squares approach, with the factor coding being Coded. The corresponding results are presented in Table 16.

Model Summary
Analysis of variance (ANOVA) results indicate that the model is statistically significant, evidenced by a Model F-value of 28.64 and a p-value of 0.0009. The low p-value ($p < 0.05$) signifies that the variation in area response is predominantly influenced by changes in flow rate, with only a 0.09% probability that this effect is attributable to random variation.

Source	Sum of Squares	df	Mean Square	F-value	p-value	Significance
Model	1.869E + 05	2	93451.51	28.64	0.0009	Significant
A- Flow Rate	1.869E + 05	2	93451.51	28.64	0.0009	Significance
Residual	19578.48	6	3263.08			
Total	2.065E + 05	8				

Interpretation of 3D Surface Plot for Area

3D surface plot illustration of the variation in peak area



Significance of Factor A (Flow Rate):

The flow rate is identified as a statistically significant factor impacting the area, with a p-value of 0.0009, well below the conventional threshold of 0.05. This confirms that changes in the flow rate have a substantial effect on the peak area response.

The 3D surface plot demonstrates how the peak area varies in response to changes in flow rate (A: 0.9, 1.0, 1.1 ml/min) and mobile phase ratio (B: 60, 70, 80 ml of organic component).

Observations:

1. The maximum peak area was recorded at a lower flow rate of 0.9 ml/min and a reduced mobile phase composition of 60 ml.
2. As flow rate increases, peak area decreases, indicating dilution and peak broadening effects.
3. At higher mobile phase ratios (80 ml), the area decreased significantly across all flow rates, possibly due to inadequate interaction time for analyte retention.
4. The red bars indicate actual experimental values above the predicted surface, while the pale red dots represent values below the model surface, helping to validate model predictability.

Interpretation:

These observations indicate that reducing the flow rate and decreasing the mobile phase ratio enhance the detector response (area) by improving retention and interaction between the analyte and the column.

Conversely, an increased concentration of the organic phase accelerates compound elution, leading to a decrease in peak area. Therefore, optimal conditions for maximizing peak area were identified as a lower flow rate (0.9 ml/min) combined with a mobile phase ratio between 60 and 70 ml

D)Analysis of Variance (ANOVA) and Model Fit for Retention Time

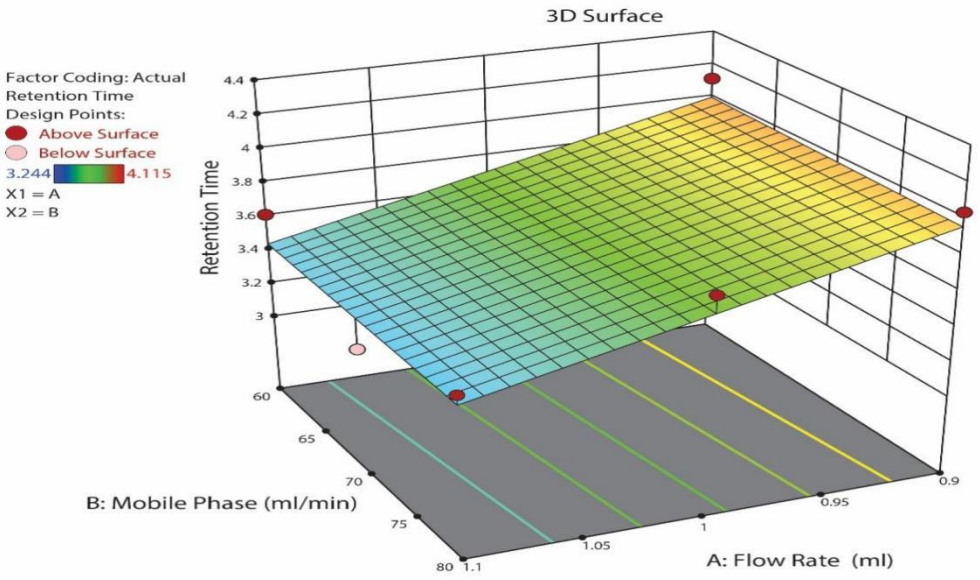
Analysis of Variance (ANOVA) was performed to evaluate the significance of both the model and its individual factors. Additionally, various fit statistics were examined to evaluate the adequacy of the model in explaining the observed variation in Retention Time.

Source	Sum of Squares	df	Mean Square	F-value	p-value	Significance
Model	0.4722	2	0.2261	10.36	0.013	Significance
A- Flow Rate	0.4722	1	0.4722	20.61	0.0043	Significance
A- Flow Rate^2	0.2361	1	0.2361	10.36	0.0113	
Residual	0.1367	6	0.0228			
Cor Total	0.6089	8				

A Model F-value of 10.36 indicates that the model is statistically significant. The probability of obtaining such a high F-value by random chance is just 1.13%, suggesting a low probability of error. The p-values for the model and the individual factors (A-Flow Rate and A-Flow Rate^2) are both less than 0.0500, indicating their statistical significance. Specifically, the p-value for the linear Flow Rate term is 0.0043, and the quadratic term has a p-value of 0.0113. This indicates that both the linear and nonlinear effects of Flow Rate significantly influence Retention Time within the studied range.

A)Interpretation of 3D Surface Plot for Retention Time

3D surface plot illustration of the variation in retention time



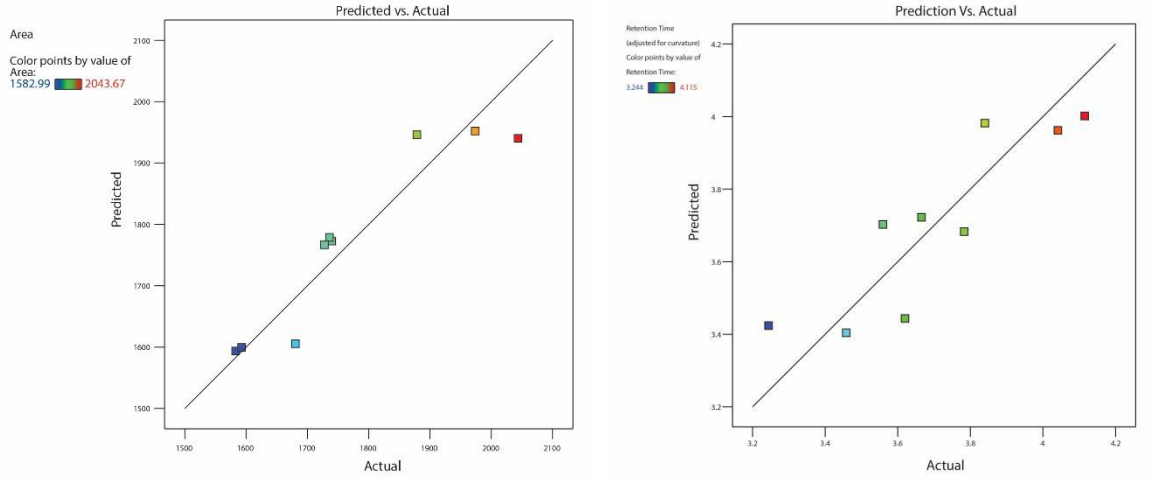
The 3D surface plot illustrates retention time (RT) in relation to flow rate and mobile phase composition.

Observations:

An increase in retention time (RT) was observed at a lower flow rate of 0.9 ml/min and a decreased mobile phase ratio of 60 ml. An increase in flow rate to 1.1 ml/min resulted in a decreased retention time, indicating that analytes elute more rapidly at higher flow rates. Similarly, increasing the mobile phase organic content from 60 to 80 ml decreased RT, due to reduced interaction time with the stationary phase.

The surface shows a consistent trend, validating that retention time is inversely proportional to flow rate and organic content.

Predicted and actual value for response- Area, Retention time



Following the execution of the Design of Experiments (DOE), a multiple regression model was constructed to predict the peak area of Imeglimin Hydrochloride (IMG HCl). The model's validity was assessed using various diagnostic plots and statistical parameters. Figure 7 displays the plot of predicted versus actual peak areas. As illustrated, the data points are closely aligned with the diagonal line, indicating a strong correlation between the predicted and observed values. The model's R-squared value of 0.95 further confirms its effectiveness in explaining the variability in peak area.

OPTIMIZATION OF BATCH:

Compound name	Concentration (µg/ml)	Retention time (min)	Area (µ.v.sec)	Height	Theoretical plates	Tailing
Imeglimin HCL	400	2.697	1503.540	133.58	3123	0.356

Development and Validation of an RP-HPLC Analytical Method for Drug Quantification
Development of an Analytical Method Using Reverse-Phase HPLC

a) Selection of mobile phase

Table No: Chromatographic behaviour at various compositions.

Sr. No.	Mobile Phase	Remark
1	Acetonitrile: Water (20:80)	No sharp peak, broad peak
2	Acetonitrile: Water (70:30)	No sharp peak
3	Methanol: Water (70:30)	No sharp peak
4	Methanol: Water (80:20)	No sharp peak
5	Methanol: Water (60:40)	Resolve peak & Sharp

Discussion: The results demonstrate that employing a solvent system of Methanol and Water (60:40% v/v) at 239 nm yielded an appropriate retention time of 3.518 minutes with a sharp peak shape, achieving 2595 theoretical plates for IMG HCl.

(b)Validation of developed analytical method by using RP-HPLC

1.System Suitability Test:

Table : Results of System Suitability Test IMG HCl

Sr. No.	Parameter	Acceptance Criteria	IMG Values
1	USP Plate	NLT2000	2595
2	USP Tailing	NMT 2.0	1.516
3	% RSD	NMT 2.0	0.231

System suitability was assessed by calculating the plate count and tailing factor through the injection of the standard solution. The corresponding chromatogram is presented in Figure 8.

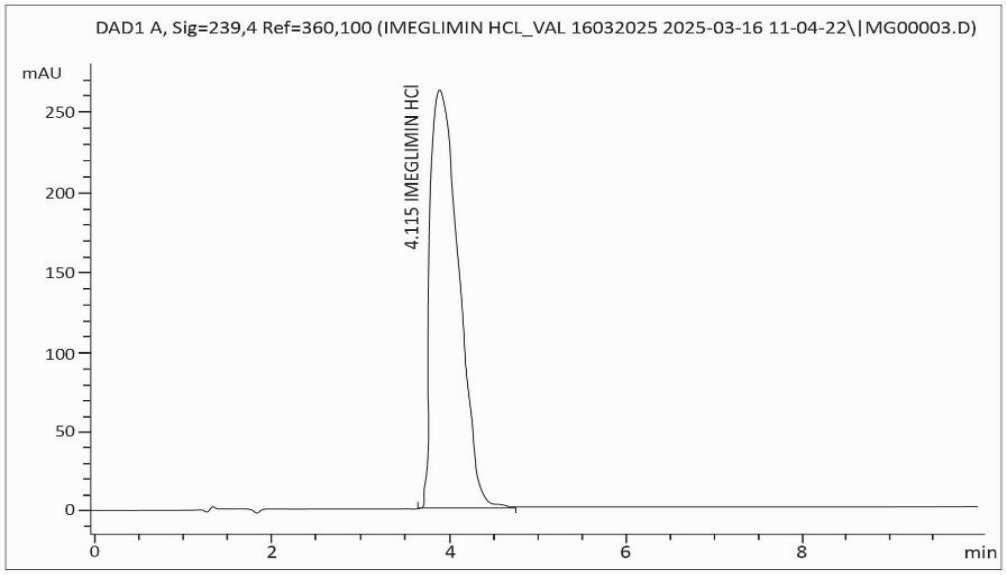


Figure No. 8 Chromatogram of Standard

Table No. 19 Result of Standard Area of Drug

Parameter	Mean Value	% RSD	Acceptance Criteria
Retention Time (min)	4.115	0.41%	% RSD < 1%
Peak Area	1971.28	0.52%	% RSD < 2%
Tailing Factor	1.10	-	≤ 2

2. Specificity

To check the interference, the method analyzes the chromatograms of mobile phase blank, std solution, and tablet separately. The Figure below demonstrates that the active components were properly separated from the excipients, and there was no interference with IMG RT. Hence the method was specific. [4]

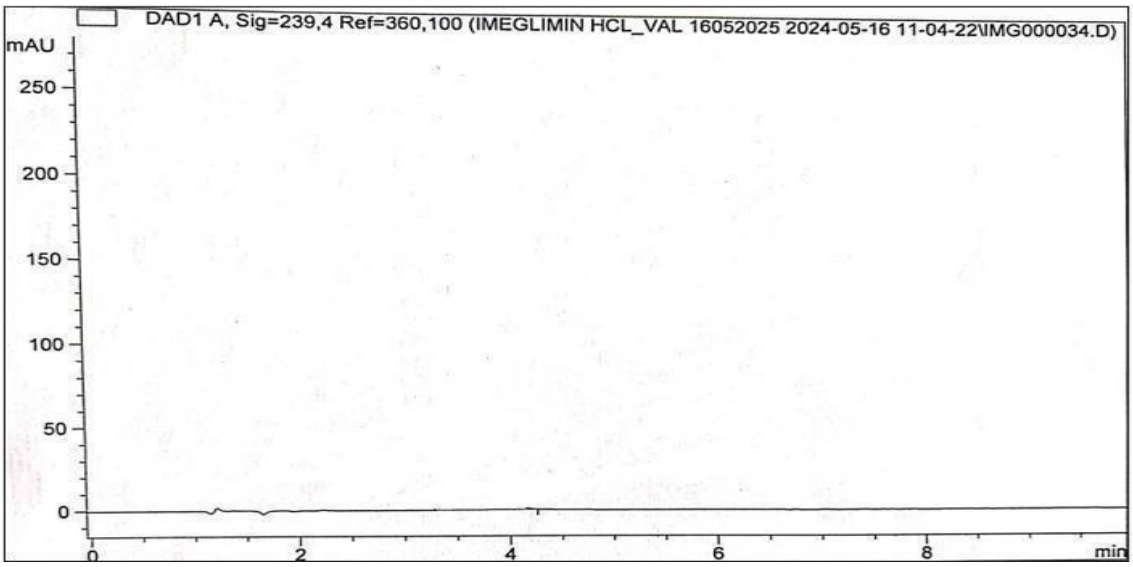


Fig. No.29: Chromatogram of Blank

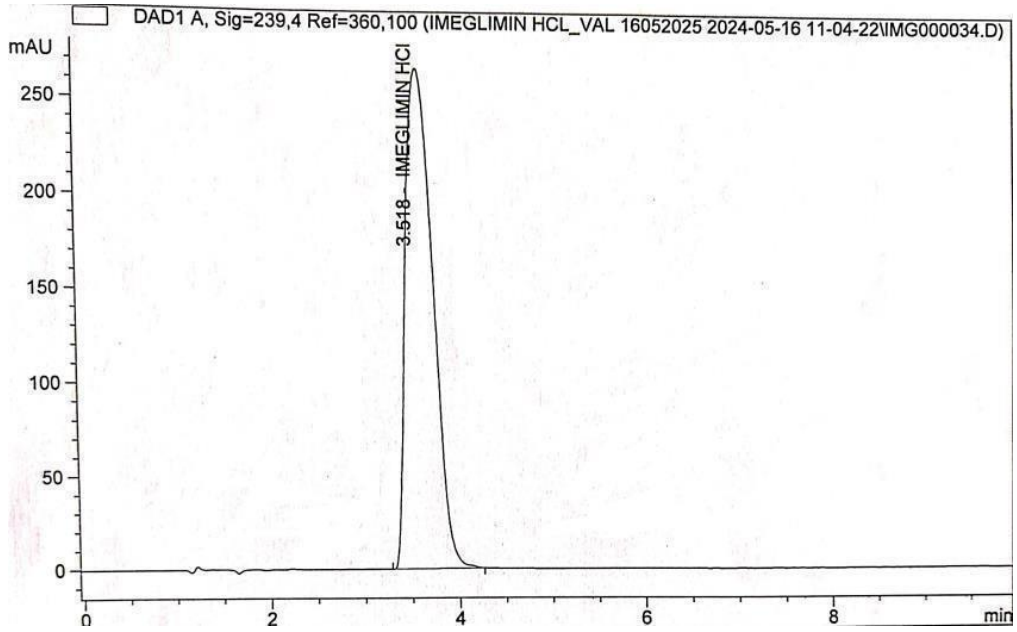


Fig. No.30: Chromatogram of standard

Table No. 38: Results of standard formulation

RT (min)	Area
3.518	5322.660

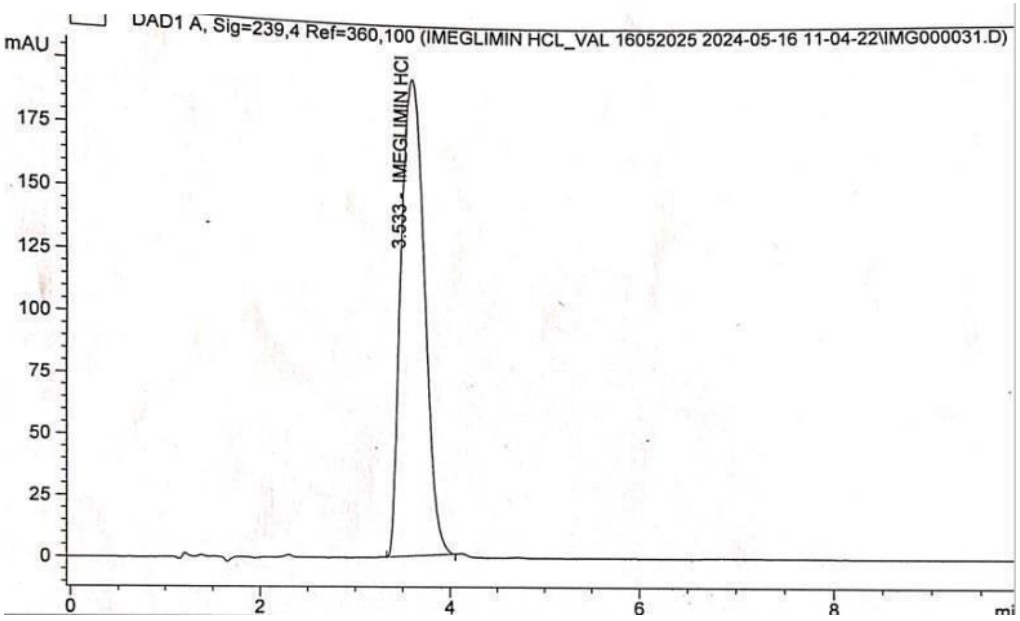


Fig. No.31: Chromatogram ofImeglyn (sample1)

Table No. 39: Results of Imeglyn formulation

RT (min)	Area
3.533	3405.721

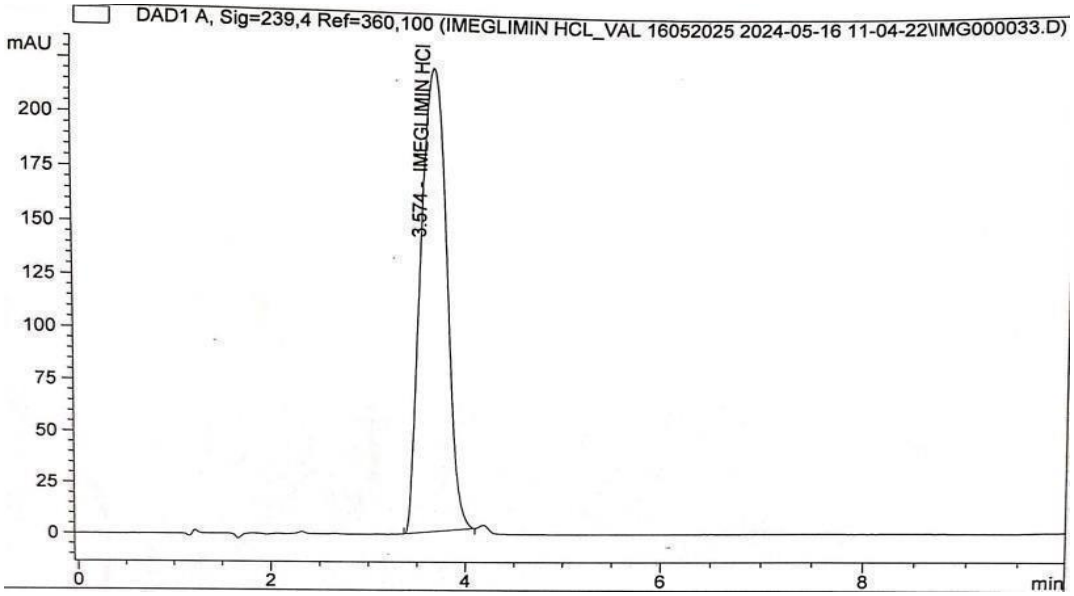


Fig. No.32: Chromatogram of Imezemic (sample 2)

Table No. 40: Results of Imezemic formulation

RT (min)	Area
3.574	4076.808

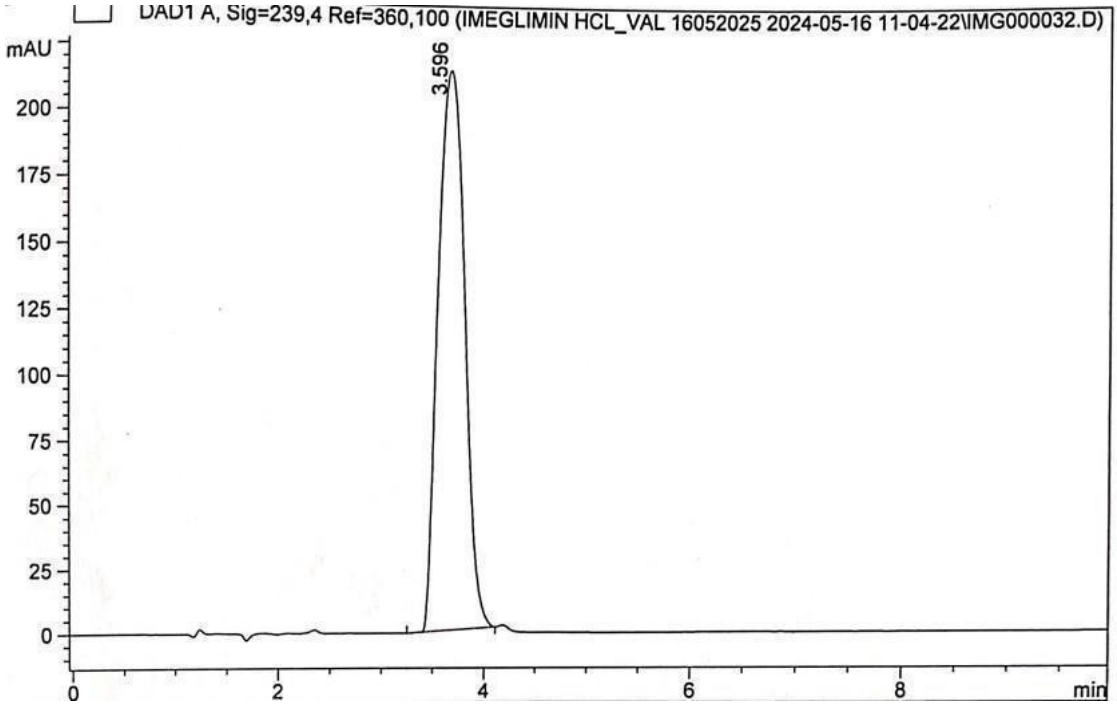


Fig. No.33: Chromatogram of Lupimeg (sample 3)

Table No. 41: Results of Lupimeg formulation

RT (min)	Area
3.574	4076.808

3. Linearity

The samples were analyzed over a concentration range of 5–25 µg/ml using a wavelength of 239 nm. Each concentration was analyzed in triplicate, and the average peak was plotted against concentration.

Concentration (µg/ml)	Peak Area			Mean Area	+-SD	%RSD
	1	2	3			
5	429.88	432.45	434.42	432.25	2.27	0.52%
10	807.90	813.61	809.81	810.44	2.88	0.35%
15	1221.12	1226.32	1227.24	1224.89	3.15	0.26%
20	1580.45	1586.38	1582.20	1583.01	2.99	0.195
25	1970.43	1972.35	1971.82	1971.20	0.96	0.05%

Regression Equation: y= 197.45x + 58.92
Correlation Coefficient (R2): 0.9985

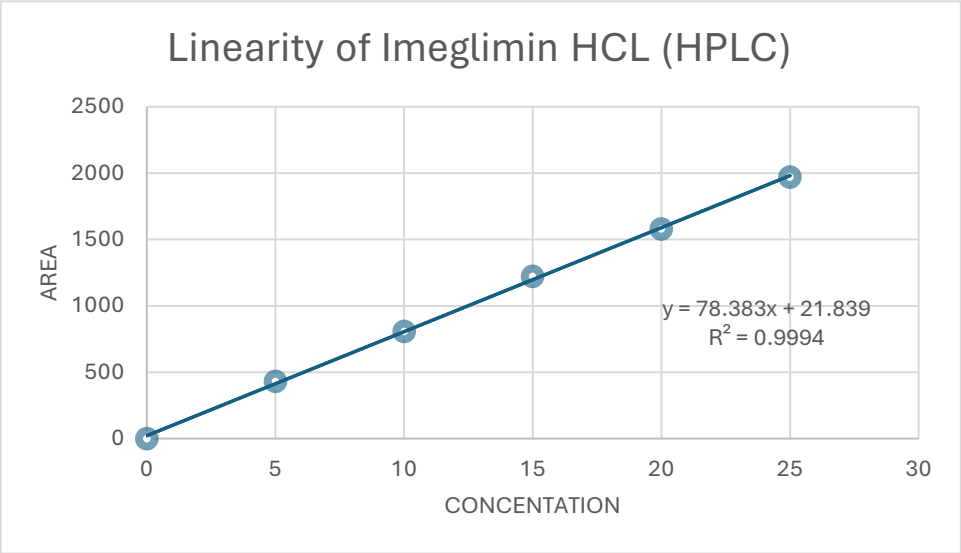


Figure X: Linearity of the IMG HCl

4.Accuracy (% Recovery)

The recovery study was carried out at three distinct percentage :80%, 100%, and 120%.

Level %	Amount Added (µg/ml)	Trial 1	Trial 2	Trial 3	Mean Recovered	% Recovery	SD	%RSD
80	8	7.91	7.96	7.95	7.94	99.25%	0.03	0.38%
100	10	9.97	9.99	9.98	9.98	99.80%	0.01	0.10%
120	12	12.07	12.04	12.03	12.05	100.42%	0.02	0.17%

5.Precision

a. Intra-day Precision (Repeatability)

Sr. no.	Conc. (µg/ml)	Peak Area Readings (µV. Sec)	Mean Area (µV. Sec)	SD	%RSD
1	40	1971.34	1972.30	0.68	0.034
2	40	1973.24			
3	40	1972.94			
4	40	1971.88			
5	40	1972.10			

Area SD: 0.68, %RSD: 0.034%

The intra-day precision study for Imeglimin HCl at a concentration of 40 µg/ml showed excellent repeatability. The peak area measurements across three replicates demonstrated a %RSD of 0.034%, which is within the acceptable limit of <2.0% as per ICH guidelines. This confirms the high reproducibility and reliability of the developed HPLC method under same day conditions.

b. Interday-day Precision

Conc. (µg/mL)	Day	Trial 1	Trial 2	Trial 3	Mean Area (µV.Sec)	SD	%RSD
40	Day 1	1971.34	1972.84	1970.92	1971.70	0.96	0.05
	Day 2	1973.24	1972.90	1971.89	1972.68	0.69	0.03
	Day 3	1970.85	1971.38	1972.04	1971.42	0.59	0.03
	Day 4	1973.42	1972.92	1972.45	1972.93	0.48	0.02
	Day 5	1971.97	1972.54	1972.10	1972.20	0.29	0.01

Area SD: 0.602, %RSD: 0.025%

The concentration selected for this evaluation was 40 µg/ml, and the study was conducted across five consecutive days, with each day's analysis performed in triplicate.
The peak area responses for each day showed consistent readings with minimal variation. The mean area values ranged from 1971.42 to 1972.93 µV·sec, indicating a high degree of stability in the detector response and method performance over time. The standard deviation (SD) values across days ranged from 0.29 to 0.96, and the corresponding %RSD values were exceptionally low, ranging between 0.01% and 0.05%.
The overall SD and %RSD for all five days were found to be 0.602 and 0.025%, respectively. These values are significantly below the acceptable limit of 2.0% as specified by ICH Q2(R1) guidelines for method precision, thereby confirming the method's excellent reproducibility under varying time conditions.
Such low variability over an extended duration affirms the method's temporal stability and robustness, making it highly suitable for routine analysis in quality control environments. The minimal deviation across days suggests that external factors such as analyst variation, ambient conditions, or instrument drift had negligible influence on the method's performance.

6.LOD and LOQ

Based on the slope (S = 197.45) and SD (σ = 1.65):

LOD

$$LOD = \frac{3.3 \times 1.65}{197.45} = \frac{5.445}{197.45} \approx 0.0275\ \mu g/ml$$

LOQ

$$LOQ = \frac{10 \times 1.65}{197.45} = \frac{16.5}{197.45} \approx 0.0836\ \mu g/ml$$

7. Range

The analytical range was determined based on the results obtained from linearity, accuracy, and precision studies.
Discussion:
a) The method demonstrated linearity over the concentration range of 5–25 µg/mL for the finished product.
b) Themethod for found to be accurate in the range of 80%-120% for finished products.

8.Ruggedness:

Repeatability of the test results was evaluated by comparing the same sample under various conditions, including different analysts, laboratories, and instruments.

Table No. 58: Result of Ruggedness of IMG HCl

Drug	Conc	Area	Mean	±SD	%RSD
IMG HCl (Analyst 1)	15	1325.426	1315.54	9.4	0.714
	15	1310.412			
	15	1327.808			
IMG HCl (Analyst 2)	15	1324.856	1321.341	6.5	0.499
	15	1325.426			
	15	1313.741			

Discussion: The % RSD was found to be within the limit of acceptable criteria. Hence method was found to be rugged for IMG HCl.

9. Robustness:

A method's robustness refers to its capacity to stay unaffected by little intentional changes in parameters. To assess the robustness of the suggested technique, minor but intentional modifications to the optimum method parameters were made. The study examined how changing mobile phase composition, flow rate, and column temperature by ± 2°C affects drug peak retention time and tailing factor.

a) Changing flow rate by ± 0.2 ml.
b) Changing the mobile phase composition by ± 2%.
c) Changing column temperature by ± 2° C.

1) Change in 0.8 ml flow

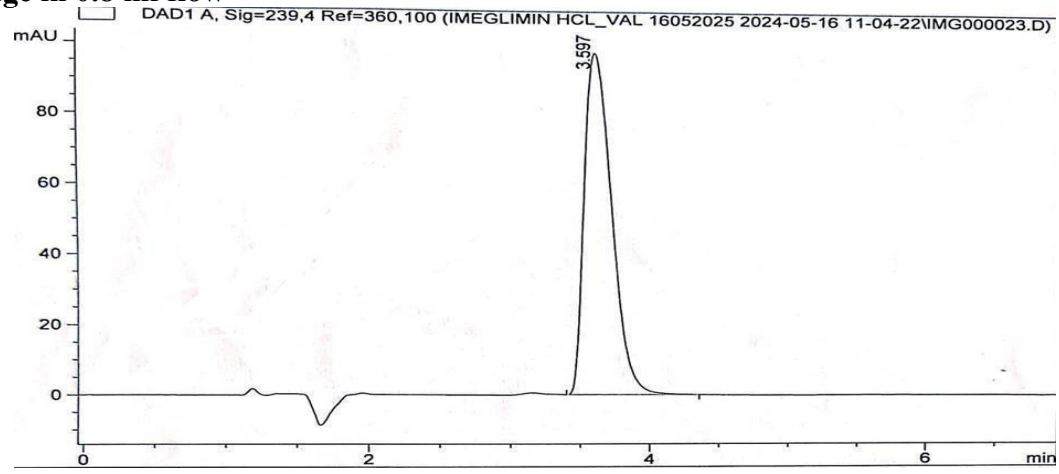


Fig. No.44: Chromatogram of change in flow (0.8 ml)

Table No. 59: Results of flow change (0.8 ml)

RT (min)	Area
3.597	1338.588

2) Change in flow rate 1.2

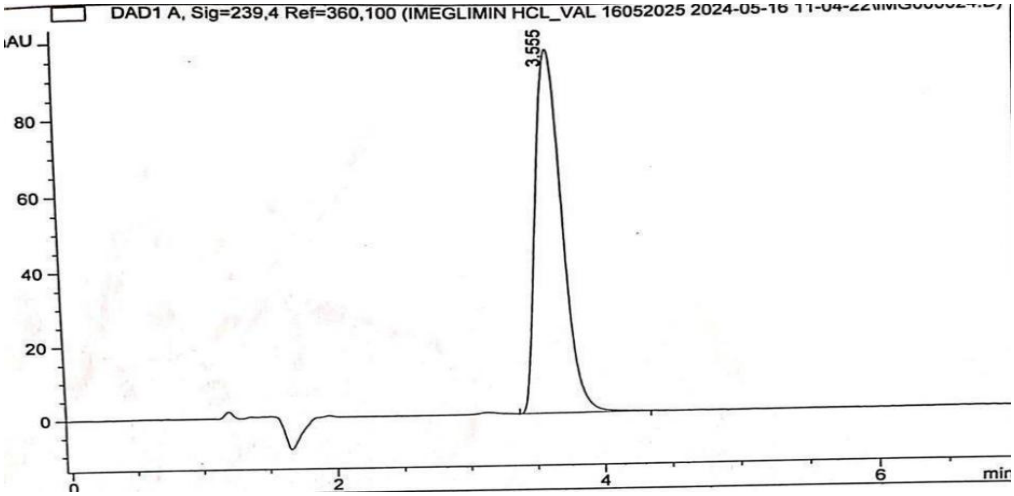


Table No. 59: Results of flow change (1.2 ml)

RT (min)	Area
3.555	1330.581

3) Change in Mobile phase composition (88 Methanol: 12Water)

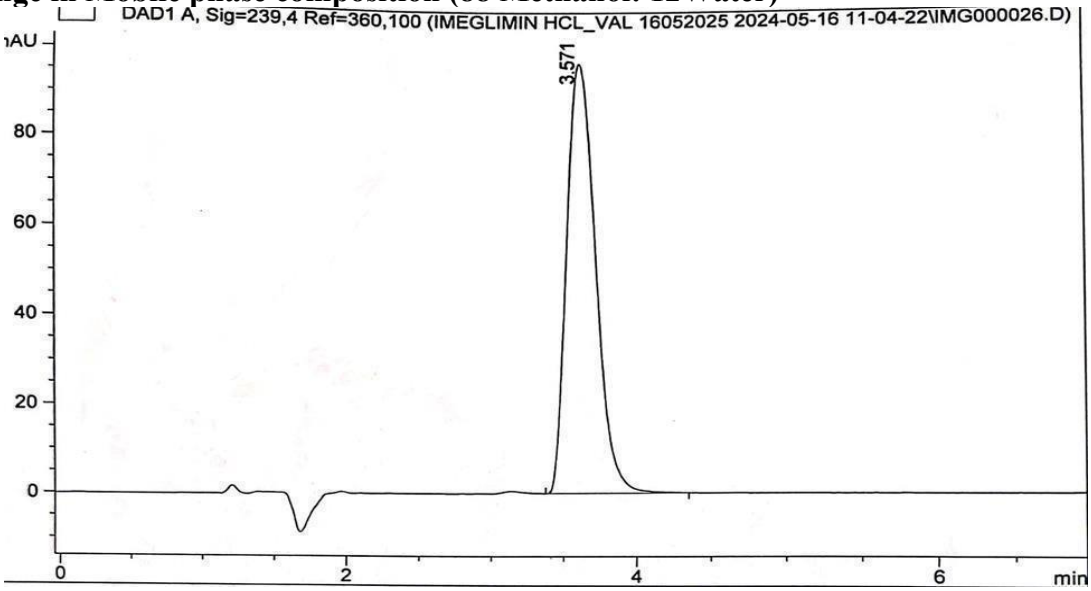


Fig. No.46: Chromatogram of MP composition (88 Methanol: 12Water)

Table No. 61: Results of MP composition (88 Methanol: 12Water)

RT (min)	Area
3.571	1341.335

4) Change in Mobile phase composition (92 Methanol: 08Water)

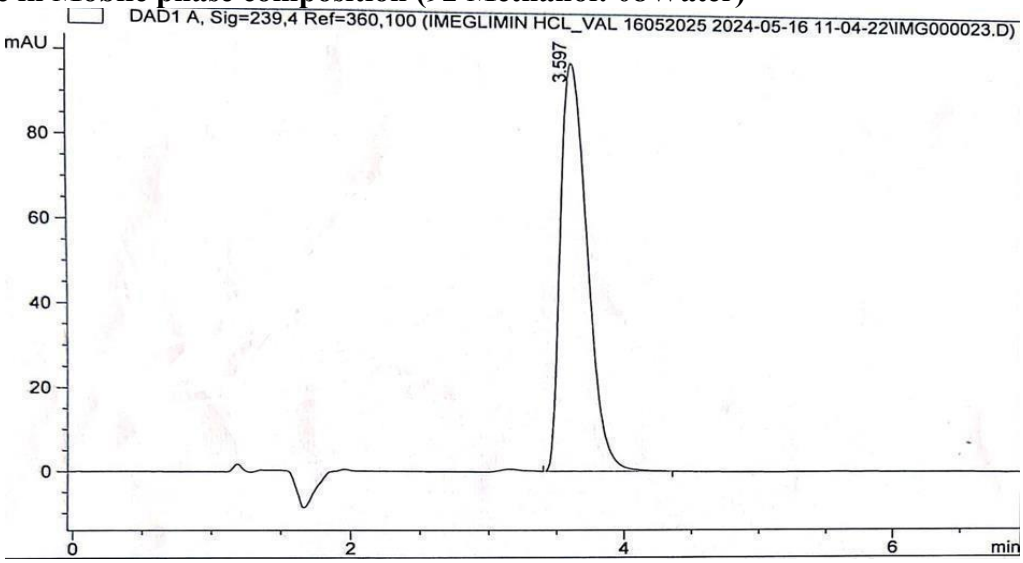


Fig. No.47: Chromatogram of MP composition (92 Methanol: 08Water)

Table No. 62: Results of MP composition (92 Methanol: 08Water)

RT (min)	Area
3.597	1336.154

5) Change in column temperature by ± 2° C (38° C)

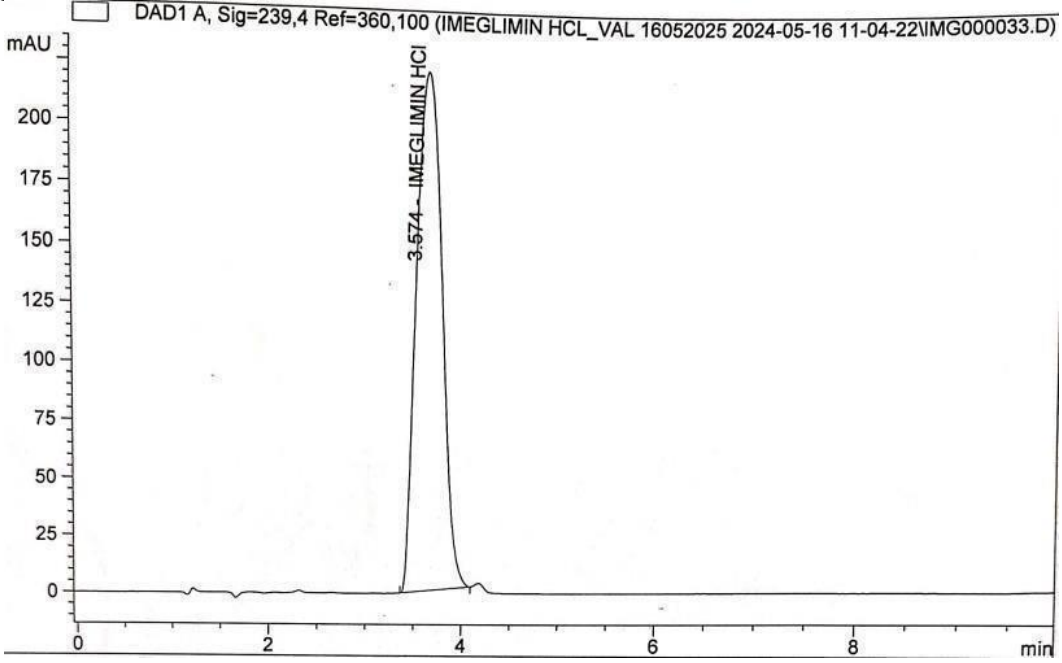


Fig. No.48: Chromatogram of change in column temperature (38° C)

Table No. 63: Result of Change in column temperature (38° C)

RT (min)	Area
3.574	1319.050

6) Change in column temperature by ± 2° C (42°

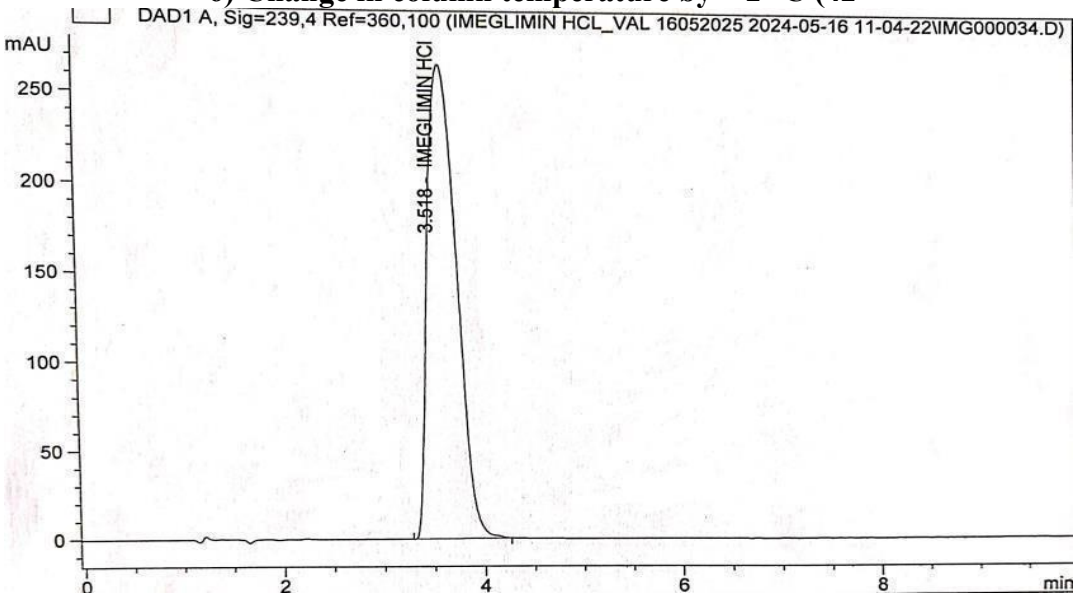


Fig. No.49: Chromatogram of change in column temperature (42° C)

Table No. 64: Result of Change in column temperature (42° C)

RT (min)	Area
3.518	1310.154

Table No. 65: Results of Robustness study of IMG HCl

Parameters	Conc. (µg/m	Amount of detected (mean ± RSD)
Chromatogram of changing flow rate by ±0.2 (0.8ml)	15	1338.588±1.936
Chromatogram of changing flow rate by ±0.2 (1.2ml)	15	1330.581±1.6
Chromatogram of changing mobile phase composition by ±2	15	1341.335±1.9
Chromatogram of changing mobile phase composition by ±2	15	1336.154±1.8
Chromatogram of changing column temperature by ± 2° C (38° C)	15	1319.050±1.2
Chromatogram of changing column temperature by ± 2° C (42° C)	15	1310.154±0.8

Discussion: The % RSD was found to be within the limit of acceptable criteria. Hence for purposeful changes method was found to be robust. Above Table No. 65 shows RSD value less than two.

10. Solution Stability

The stability of IMG was evaluated by placing std samples at room temperature between 2 and 8 degrees Celsius. The experimental investigation demonstrated that, drug solution was stable for two days under the specified storage conditions.

Table No. 66: Results of IMG stability

	Stability at RT	Stability at 2-8°C
	% Assay	% Deviation
Initial	100 %	00
8 hours	99.7	0.3
16 hours	99.6	0.4
24 hours	99.3	0.7

Discussion:

An HPLC method based on the Analytical Quality-by-Design (AQbD) approach was developed for the estimation of Imeglimin HCl in pharmaceutical formulations. The analytical target profile encompassed parameters like retention time, mobile phase composition, and peak asymmetry. Of the variables studied, mobile phase composition and flow rate were identified as critical quality attributes (CQAs) affecting these key parameters.

Risk assessment played a crucial role in identifying the variables that significantly influence the analytical target profile. During chromatographic analysis, variables such as column selection, instrument configuration, and injection volume were held constant, whereas parameters including mobile phase composition, flow rate, and column temperature were systematically varied and assessed as part of the robustness study. The AQbD strategy successfully led to the development of an optimized reversed-phase HPLC (RP-HPLC) method for Imeglimin HCL analysis. The optimized method employed a Repro Q C18 column (150 mm × 4.6 mm) and a mobile phase consisting of methanol and water in a 60:40 (v/v) ratio. Under these conditions, Imeglimin HCl exhibited a retention time of 4.115 minutes.

The method exhibited linearity within the concentration range of 5–25 µg/mL, with a correlation coefficient (R^2) of 0.999. Precision studies yielded relative standard deviation (%RSD) values below 2% for repeatability, intraday, and interday analyses, thereby confirming the method's reliability and precision.The limit of detection (LOD) was determined to be 0.0275 µg/mL, while the limit of quantification (LOQ) was established at 0.0836 µg/mL. The recovery of spiked samples ranged from 99.57 ± 1.47% to 100.79 ± 1.73%, thereby meeting the acceptance criteria specified in the ICH guidelines. The method development and validation adhered to the standards set by ICH.

Conclusion:

An HPLC method was successfully developed employing a Quality-by-Design (QbD) approach, guided by well-defined objectives specified within the Analytical Target Product Profile (ATPP). Key chromatographic variables, including mobile phase composition and flow rate, were systematically examined through the application of experimental design methodologies. The Quality-by-Design (QbD) framework was employed to optimize the HPLC method for Imeglimin HCl through a multivariate study of two critical factors—mobile phase ratio and flow rate—each evaluated at three levels using a central composite design.

The interdependencies between these variables were investigated, allowing for optimization across various conditions.This approach improved insight into the factors influencing chromatographic performance and facilitated the creation of a robust method that reliably meets its analytical goals. The validated method satisfied all predetermined acceptance criteria and was demonstrated to be linear, precise, accurate, specific, robust, and rugged for the quantification of Imeglimin HCl. Applying the QbD methodology facilitated a deeper understanding of critical method variables, reducing the risk of failure during validation or transfer.Utilizing Design Expert software for automated method development led to a more efficient and robust process than conventional manual approaches. Statistical analysis of the results validated the method’s reliability, selectivity, and reproducibility. The optimized method is therefore suitable for routine quality control applications in the pharmaceutical industry.

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