ORIGINAL ARTICLE

Development, Optimization, and Validation of a Quality by Design-assisted Reverse-phase High-performance Liquid Chromatography Method for Impurity Profiling of Imeglimin Hydrochloride "Quality by Design Assisted Impurity Profiling of Imeglimin Hydrochloride"

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Abstract

Introduction: The newly-approved tetrahydrotriazine-based antidiabetic drug imeglimin hydrochloride (IMGH) might be contained process- and degradation-related impurities, which require sensitive and robust analytical control in compliance with regulatory requirements. In this paper, a Quality by Design (QbD)-supported Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC) technique of comprehensive impurity profiling of IMGH in pharmaceutical dosage forms is designed, optimized, and validated. **Materials and Methods:** A QbD-enabled systematic method based on the central composite design was employed to establish the influence of key technique parameters, such as mobile phase composition and pH, on chromatographic responses, such as retention duration, theoretical plates and percent recovery. To obtain optimum separation, a Phenomenex ODS C18 column (250 × 4.6 mm, 5.0 mm) was used with 10 mM acetate buffer: ACN (30:70) as the mobile phase and 1.0 ml/min flow rate, column temperature of 30°C, and ultraviolet detection at 240 nm. **Discussion and Conclusion:** The optimized method was validated in accordance with ICH Q2(R1) and Q3B guidelines, demonstrating excellent linearity (R^2 = 0.999), precision, accuracy, specificity, robustness, and sensitivity. IMGH and its associated impurity, N2, N2,6-trimethyl-1,3,5-triazine-2,4-diamine were found to have limits of detection of 2.158 µg/mL and 0.04117 µg/mL, respectively, and limits of quantification of 6.021 µg/ml and 0.125 µg/ml, respectively. Recovery studies confirmed the accuracy of the method, with recoveries within acceptable regulatory limits. In order to ensure product safety and consistency, the suggested QbD-based RP-HPLC approach provides a straightforward, reliable, and legally compliant analytical instrument for routine quality control and impurity monitoring of IMGH in pharmaceutical formulations.

Key words: ICH guidelines, imeglimin hydrochloride, impurity profiling, quality by design, RP-HPLC

INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a chronic metabolic disorder that is characterized by persistent hyperglycemia. It is the most prevalent disease worldwide and has been rising steadily over the last several decades, along with impaired metabolism, obesity, and sedentary lifestyles as risk factors. The current data suggest

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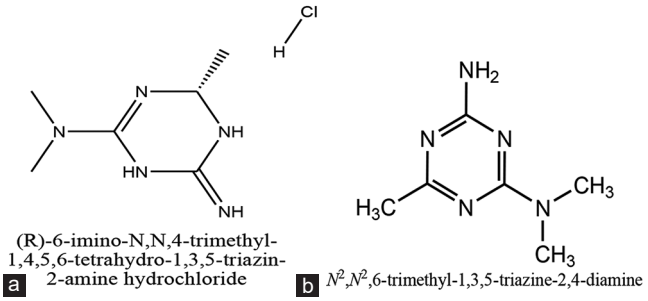
that more than 380 million people worldwide suffer from T2DM, which is expected to increase to more than 592 million people by 2035.[1] Over 95% of individuals have T2DM, which was previously limited to adults but is now more common in children. According to the WHO in 2021, 1.6 million deaths occurred before the age of 70 years due to diabetes and 530000 kidney disease deaths were caused by diabetes.[2] According to the International Diabetes Federation, approximately 415 million adults aged 20–79 years had DM in 2015.[3]

Imeglimin hydrochloride (IMEG.HCl), a tetrahydrotriazine-containing newly approved oral antidiabetic agent that comes under the "glimins" category, is used to treat T2DM.[4] Chemically, imeglimin hydrochloride (IMGH) is designated as (R)-6-imino-N,N,4-trimethyl-1,4,5,6-tetrahydro-1,3,5-triazin-2-amine hydrochloride [Figure 1].[5] IMGH enhances mitochondrial function, reduces hepatic gluconeogenesis, and improves glucose homeostasis by reducing mitochondrial free radicals.[6,7] IMGH increases glucose-stimulated insulin release from β cells, possibly by increasing the synthesis of nicotinamide adenine dinucleotide.[8] Impurities in imeglimin like IKI (6-(dimethylamino)-4-methyl-1,3,5-triazin-2(1H)-one),[6] N2,N2,6-trimethyl-1,3,5-triazine-2,4-diamine[9] comes from synthesis, by product, or storage and may interfere in the quality of the product, and the presence of impurity at elevated level poses several toxicological effects. Therefore, regular monitoring of impurities for any formulation to identify and quantify the impurities present within the product is the crucial step.

Quality by Design (QbD) is a structured technique that methodically focuses on finding and controlling variability during method development. After method development, the performance of the developed method was assessed according to the International Council for Harmonization (ICH) validation guidelines.[6] Several factors, such as solvent polarities, different buffers, mobile phase ratio, injection volume, and flow rate, are chromatographic parameters that contribute to the variability in the analytical method.

Various methods, including Liquid Chromatography Quadrupole Time-of-Flight Mass Spectrometry and nuclear magnetic resonance,[9] Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC),[10] ultra-performance liquid chromatography-atmospheric pressure chemical ionization-tandem mass spectrometry (UPLC-APCI-MS/MS),[11] liquid chromatography-tandem mass spectrometry (LC-MS/MS),[12] and ultraviolet (UV)-visible spectroscopy,[13] are available for the determination of IMGH but not for impurities. Therefore, the current study's objective was to develop a trustworthy RP-HPLC technique for the quantitative determination of contaminants that was improved using a QbD methodology and verified in compliance with ICH requirements.



a (R)-6-imino-N,N,4-trimethyl-1,4,5,6-tetrahydro-1,3,5-triazin-2-amine hydrochloride

b N^2,N^2,6-trimethyl-1,3,5-triazine-2,4-diamine

Figure 1: Chemical structure of (a) imeglimin and (b) impurity N2,N2,6-trimethyl-1,3,5-triazine-2,4-diamine

EXPERIMENTAL

Materials

IMGH API and related impurity (N2,N2,6-trimethyl-1,3,5-triazine-2,4-diamine) were procured from Pharma Affiliates. Imeglyn 500 tablets, marketed by Zydus Healthcare, labeled to contain 500 mg of Imeglimin hydrochloride in each film-coated tablet, were purchased from the market. HPLC-grade chemicals were used for method development.

Chromatographic system

Chromatographic separation was achieved using HiQSiL C18 Columns (250 × 4.6 mm, 5 µm), with a mobile phase of 10 mM acetate buffer: ACN (30:70, v/v) and a flow rate of 1.0 mL/min at ambient column temperature.[14] For chromatographic separation, the selected wavelength was 240 nm, and the injection volume was 5 µ. HPLC method validation was performed according to ICH guidelines, and optimization using a central composite design (CCD) was performed for many parameters, including pH and mobile phase, two variables at three different levels.

Analytical characterization

The solubility of IMGH in various solvents was analyzed. Attenuated Total Reflectance Fourier Transform Infrared (FTIR), Lumos, Bruker USA, was used for the functional group identification present in the IMGH and related impurity N2, N2,6-trimethyl-1,3,5-triazine-2,4-diamine.

Optimization of analytical condition using design of experiment (DoE) software

The established approach was optimized using CCD; the independent variables pH and mobile phase ratio were investigated due to their influence on the dependent variables. For the separation procedure, an experimental design was created that included independent variables, including temperature, the pH of the mobile phase, and the kind of column utilized. The number of theoretical plates (Y2), retention time

(Y1), and compound recovery (Y3) were used to gauge how well the separation worked. Desirability functions and an overlay plot were used to get the best composition predictions. The expected analytical conditions were found using the DoE software. The experimental design results were statistically analyzed using the QbD-based Design-Expert-13® software. To determine the model's appropriateness and reliability, statistical validation was performed, which recognizes the distribution of the correlation coefficient F-value (R2), predicted R-squared (R2 Pred), adjusted R-squared (R2 Adj), PRESS statistics, and adequate precision produced by the analysis of variance (ANOVA) provision.[15,16]

Determination of λ max

The mobile phase of 10mM acetate buffer: ACN (30:70) was prepared, maintained pH 4.64, sonicated for 10 min, and filtered through a 0.45 m membrane filter paper. The IMGH and impurity N2,N2,6-trimethyl-1,3,5-triazine-2,4-diamine standard stock solutions (1 mg/mL) were further diluted for concentration of 1000 µg/mL, and dilutions of impurities ranging from 0.2 to 1.2 µg/mL were made in a similar pattern as IMGH. Both prepared dilutions were scanned over the UV range of 200–400 nm to detect the wavelength.[17]

HPLC method development and validation

Mobile phase and chromatographic conditions selection

IMGH and related impurities were used as working standards for chromatographic separation. The introduction of different mobile phase ratios at different pH levels leads to system suitability characteristics. A total of nine trials of different mobile phases were performed. High resolution and adequate peak characteristics were obtained using a mobile phase 10 mM acetate buffer: ACN in 30:70 v/v at a 1 mL/min flow rate.

Chromatograms and system suitability parameters

Validation of analytical methods

Validation provides a high level of assurance for method specificity and is written proof that the procedure's performance characteristics satisfy the requirements for the intended analytical application.

Linearity

Six different concentrations of IMGH and impurity N2,N2,6-trimethyl-1,3,5-triazine-2,4-diamine ranging from 20–120 µg/mL to 0.2–1.2 µg/mL, respectively, were selected for linearity. The observed peak area versus drug concentration was interpreted by linear least square regression analysis, and the calibration curve of drug concentration and peak area results the slope and the correlation coefficient.[18]

Precision

The closeness of successive samplings of the same solution was used to gauge the developed method's precision. By injecting three replicates for each concentration, it was observed in terms of % RSD.[19]

Limit of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ were calculated using the standard deviation of the response and the slope of the calibration curve in accordance with the ICH Q3 criteria. While the formula for LOQ was $10 \times \sigma/S$, the formula for LOD was $3.3 \times \sigma/S$.[20]

Robustness

The robustness of the developed method was assessed by purposefully modifying the system suitability parameters, such as the mobile phase ratio, flow rate, and pH.[21]

RESULTS AND DISCUSSION

FTIR study

Functional group identification of the IMGH and the impurity N2,N2,6-trimethyl-1,3,5-triazine-2,4-diamine was achieved by introducing powdered samples of both imeglimin and the impurity N2,N2,6-trimethyl-1,3,5-triazine-2,4-diamine between two KBr discs. The results are shown as peaks of different vibrations in the graph [Figure 2 and Table 1].

Determination of λ max

The mobile phase of 10 mM acetate buffer: ACN (30:70) was prepared, sonicated for 10 min, and filtered via 0.45 m membrane filter paper. The IMGH and impurity N2,N2,6-trimethyl-1,3,5-triazine-2,4-diamine standard stock solutions (1 mg/mL) were further diluted to obtain a concentration of 1000 µg/mL, and dilutions of impurities ranging from 0.2 to 1.2 µg/mL were made in a similar pattern as IMGH. Both prepared dilutions were scanned over the UV range of 200–400 nm to detect the wavelength.[17] The maximum wavelength for Imeglimin was found 240nm as shown in figure 3.

Analytical condition optimization for the development of the RP-HPLC technique for IMGH and impurity N2,N2,6-trimethyl-1,3,5-triazine-2,4-diamine

Experimental design

The developed method was optimized by evaluating the theoretical plates, retention time, and % recovery, followed by the CCD and quadratic design model, as presented in Table 2. For optimization, nine trial runs using a response surface study type, randomized subtype, CCD, and quadratic

Table 1: Fourier Transform Infrared interpretation of imeglimin hydrochloride (IMGH) and its impurity N2, N2,6-trimethyl-1,3,5-triazine-2,4-diamine

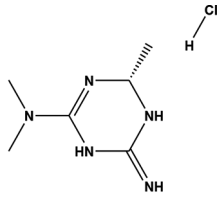
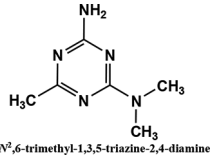
Name	Structure	Functional groups/types of vibrations	Reference value	Observed value
IMGH	 (R)-6-imino-N,N,4-trimethyl-1,4,5,6-tetrahydro-1,3,5-triazin-2-amine hydrochloride	N-H	3500–3100	3383.6
		C=N	1690–1640	1652.2
		C-N	1350–1000	1374
		C-H	3150–3050	2924.1
Impurity	 N^2,N^2,6-trimethyl-1,3,5-triazine-2,4-diamine	C=N	1690–1640	1687.91
		C-N	1350–1000	1328.04
		N-H	3500–3100	3421.87
		C-H	3150–3050	3051.52

Table 2: Experimental runs and response variables imeglimin hydrochloride impurity N2, N2,6-trimethyl-1,3,5-triazine-2,4-diamine

Standard	Run	Factor 1	Factor 2	Response 1	Response 2	Response 3
		A: Mobile phase (Content of Acetonitrile) (%)	B: pH	Retention time	Theoretical plates	Recovery
		%		Min	Numbers	%
6	1	98.28	3.5	6.89	4950.14	88.61
8	2	70	5.6	5.89	4858.13	97.89
3	3	50	5	5.11	4824.14	96.53
5	4	41.71	3.5	4.89	4572.84	99.41
4	5	90	5	6	4847.72	98.99
9	6	70	3.5	6.12	4758.21	97.81
1	7	50	2	5.13	4516.98	96.51
7	8	70	1.37	6.11	4750.97	97.86
2	9	90	2	6	4881.14	99.99

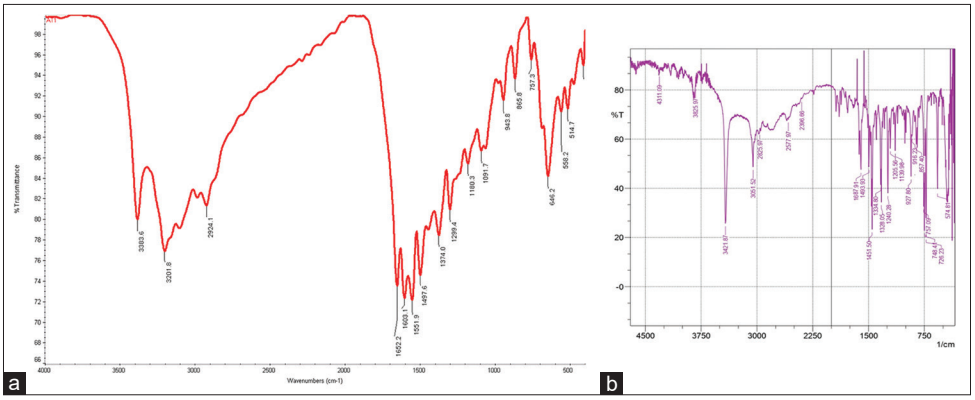


Figure 2: Fourier Transform infrared data of (a) imeglimin hydrochloride and (b) impurity

design model were employed.

Retention time, theoretical plates, and recovery values were entered into the DoE software design expert-13 trial version following nine trial runs, yielding predicted retention time, theoretical plate, and recovery percentage values [Figure 5]. Using the actual and predicted values, the percentage error was calculated to obtain the outcome of predictability; a low

Table 3: Predicted values and experimental results of response variables Retention time, Theoretical plates, and Recovery

Run	Retention time			Theoretical plates			Recovery		
	Actual value	Predicted value	% Error	Actual value	Predicted value	% Error	Actual value	Predicted value	% Error
1.	6.89	6.60	4.39	4950.14	4936.23	12	98.64	99.31	0.75
2.	5.89	5.73	2.79	4858.13	4848.54	6	97.32	97.36	0.79
3.	5.11	5.18	−1.35	4824.14	4796.50	0	96.39	96.21	0.12
4.	4.89	4.98	−1.81	4572.84	4610.49	−5	95.81	95.88	−0.53
5.	6.00	6.33	−5.21	4847.72	4856.54	16	99.16	98.64	−0.08
6.	6.12	5.79	5.70	4758.21	4773.36	−1	97.15	97.59	−1.01
7.	5.13	5.26	−2.47	4516.98	4519.89	−18	96.41	96.55	−1.67
8.	6.11	5.85	4.44	4750.97	4698.18	−9	98.11	97.83	0.79
9.	6.00	6.41	−6.40	4881.14	4920.51	−3	99.35	98.97	0.81

% Error=(Actual-Predicted) ×100/Predicted

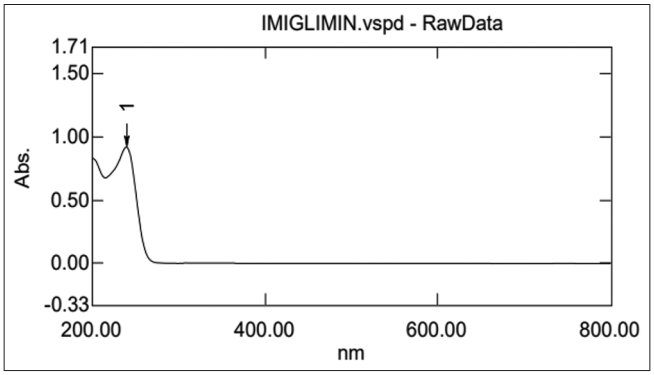


Figure 3: Ultraviolet spectra of imeglimin hydrochloride

% error suggested that the model had good predictability [Table 3].

A correlation analysis was conducted between the responses and independent variables. Quadratic polynomials were used to represent each response, and the ANOVA statistical parameters were determined [Table 4]. A highly significant level of significance was found for each model (P = 0001). An excellent match between the model and responses (P = 0.0001 in every case) was indicated by both residual mean squares and mean squares of regression being bigger than F in every instance [Table 5].

Impact of mobile phase ratio and pH on retention time

The chromatographic performance was enhanced and improved by changing the ratio of the mobile phase and pH, followed by the QBD approach. Chromatographic performance was assessed by analyzing the retention time, theoretical plates, and % recovery. After introducing various combinations of mobile phases, result indicated that a mobile phase ratio of 30:70 (10 mM acetate buffer: ACN) and a pH level of 4.6 showed a retention time of 5.6 min, theoretical plates of 4806.51, and 97.26% recovery. These calculations suggested that optimum results might be obtained using a mobile phase ratio of 10 mM acetate buffer: ACN (30:70) [Figure 4, 6].

RP-HPLC of IMGH and impurity N2,N2,6-trimethyl-1,3,5-triazine-2,4-diamine chromatogram and system suitability parameters

The chromatographic column was saturated with the prepared mobile phase (10 mM acetate buffer: ACN (30:70)). A working standard solution of IMGH and impurity at a concentration of 10 µg/mL and 1.2 µg/mL, respectively, was introduced into the Shimadzu LC-20 AD [Figure 7 and Table 6].

Linearity

Six different concentrations of standard stock solutions of Imeglimin and Impurity N2,N2,6-trimethyl-1,3,5-triazine-2,4-diamine, ranging from 10–60 µg/mL to 0.2–1.2 µg/mL, respectively, were added to create the calibration curve for the linear relationship between concentration and peak area, as indicated in Tables 7 and 8 [Figure 8 and 9].

Range

The range in the analytical method refers to the upper and lower analyte concentrations in the sample for which the analytical process has been shown to have sufficient precision, accuracy, and linearity. The approach appears to follow Beer's law, according to a linearity investigation with concentration ranges of 20–120 µg/ml for IMGH and 02–1.2 µg/mL for contaminants.

Precision

Three replicates of IMGH at three distinct concentrations (25, 50, and 75 µg/mL) were examined in a day for intraday investigation [Tables 9 and 10] and on 3 consecutive days for interday investigations. The same procedure was followed for the impurity N2,N2,6-trimethyl-1,3,5-triazine-2,4-diamine

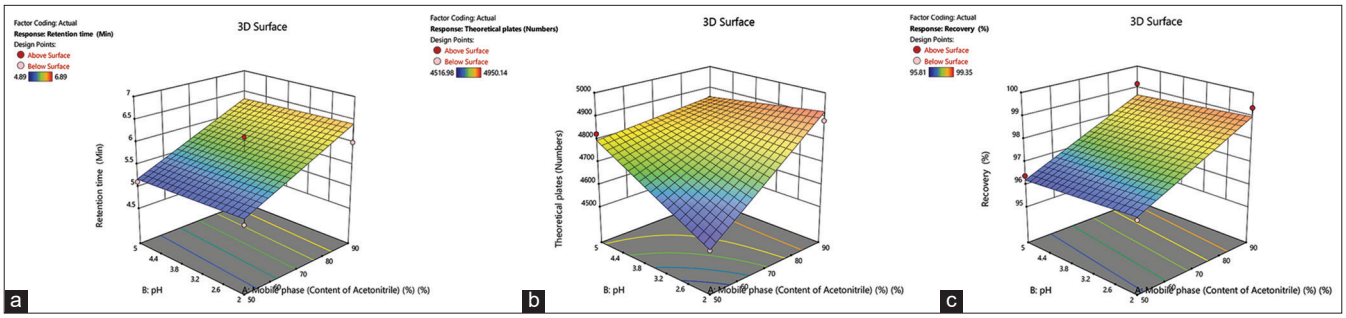


Figure 4: 3D surface plot for the effect of mobile phase ratio and pH on (a) retention time, (b) theoretical plates, and (c) recovery

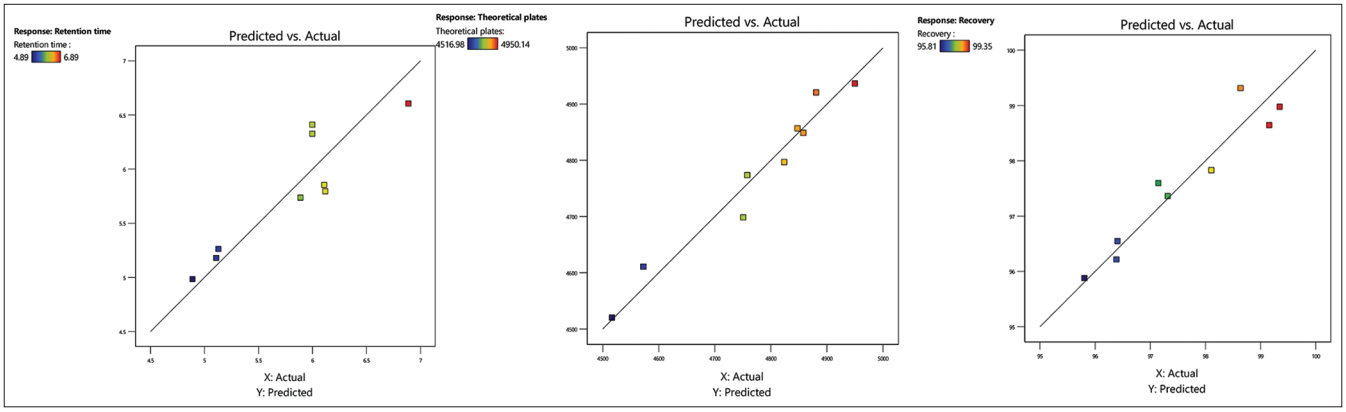


Figure 5: Actual and predicted values plot of retention time, theoretical plates, and % recovery

Table 4: Analysis of variance-based model adequacy and precision statistics for critical method attributes

Statistical Parameters	Retention time	Theoretical plates	Recovery
Standard deviation	0.3114	37.74	0.4456
Mean	5.79	4773.36	97.59
C.V. %	5.38	0.7905	0.4566
R^2	0.8197	0.9568	0.9098
Adjusted R^2	0.7596	0.9309	0.8797
Predicted R^2	0.5974	0.8352	0.7965
Adeq Precision	9.0219	16.5498	13.3464

[Tables 11 and 12] at three distinct concentrations (0.3, 0.6, and 0.9 µg/mL). The % RSD was calculated after intraday and interday analysis.

Accuracy

A drug recovery study was performed to assess the accuracy of the developed method. Three distinct levels (50, 100, and 150%) of pure IMGH were added to a basic concentration of 50 µg/mL of the sample solution, and Impurity N2,N2,6-trimethyl-1,3,5-triazine-2,4-diamine was spiked at different concentrations of 0.4, 0.8, and 1.2 µg/mL for the accuracy content determination of impurity N2,N2,6-trimethyl-1,3,5-triazine-2,4-diaminefrom the spiked sample [Tables 13 and 14].

The LOQ and LOD

LOQ of IMGH and impurity N2,N2,6-trimethyl-1,3,5-triazine-2,4-diamine were found as 6.021 µg/mL and 0.125 µg/mL, respectively. Similarly, the LOD was calculated for IMGH and impurity N2,N2,6-trimethyl-1,3,5-triazine-2,4-diamine, which were found to be 2.158 µg/mL and 0.04117 µg/mL, respectively.

Robustness

Robustness of the method was determined by using the three different variations of pH, Flow rate, and Wavelength which impact the peak area, obtained results were indicated in Table 15.

Assay

Ten 500 mg Imeglyn tablets were weighed, crushed, and ground into a powder. After weighing and transferring 10 mg of crushed powder into a 10 mL volumetric flask, the flask was filled to capacity with diluent and sonicated for 30 min. A 0.45 µm filter was used to filter the sample solution following sonication. After being further diluted with diluent to reach a concentration of 100 mg/mL, the filtrate (1 mL) was loaded onto a chromatograph. Six measurements were made from the homogenous mixture to calculate the percentage content of IMGH and the principal impurity N2,N2,6-trimethyl-1,3,5-triazine-2,4-diamine from the linearity equation.

Table 5: Analysis of variance for the linear model

Response	Source	Sum of squares	df	Mean square	F-value	*P*-value	
R1	Model	2.65	2	1.32	13.64	0.0059	significant
	A-Mobile phase (Content of Acetonitrile) (%)	2.63	1	2.63	27.13	0.0020	
	B-pH	0.0137	1	0.0137	0.1413	0.7199	
	Residual	0.5820	6	0.0970			
	Cor Total	3.23	8				
R2	Model	1.577E+05	3	52570.59	36.92	0.0008	significant
	A-Mobile phase (Content of Acetonitrile) (%)	1.061E+05	1	1.061E+05	74.51	0.0003	
	B-pH	22608.64	1	22608.64	15.88	0.0105	
	AB	28998.68	1	28998.68	20.37	0.0063	
	Residual	7119.69	5	1423.94			
	Cor Total	1.648E+05	8				
R3	Model	12.01	2	6.01	30.24	0.0007	significant
	A-Mobile phase (Content of Acetonitrile) (%)	11.79	1	11.79	59.38	0.0003	
	B-pH	0.2202	1	0.2202	1.11	0.3329	
	Residual	1.19	6	0.1986			
	Cor Total	13.20	8				

Table 6: Chromatographic parameters and system suitability results for imeglimin hydrochloride (IMGH) and associated impurity N2, N2,6-trimethyl-1,3,5-triazine-2,4-diamine

Name	RT (Min)±RSD	Concentration (µg/mL)	Area	Plates	Asymmetry
IMGH	11±0.8	10	710152	10542	1.08
Impurity	6.30±0.6	1.2	13543	6085	0.98

Table 7: Linearity study of imeglimin hydrochloride (IMGH)

Replicates	Concentrations of IMGH					
	10 µg/mL	20 µg/mL	30 µg/mL	40 µg/mL	50 µg/mL	60 µg/mL
	Peak area					
1	710152	1428237	2260138	3058153	3779335	4535721
2	710642	1415038	2254132	3118042	3719125	4486153
3	720121	1431238	2219021	3028150	3678335	4561633
4	710156	1423920	2234193	3147143	3719236	4525428
5	720458	1428121	2231138	3052358	3617337	4461532
6	710852	1409138	2220135	3028163	3789865	4483371
Mean	713730.2	1422615.3	2236459.5	3072001.5	3717205.5	4508973.0
Standard deviation	5089.3	8683.6	17187.4	49372.8	64236.9	37910.8
%RSD	0.7	0.6	0.8	1.6	1.7	0.8

DISCUSSION

To estimate IMGH and related impurities in pharmaceutical formulations simultaneously, the current work used a QbD-based RP-HPLC approach. QBD Analytical methods reported previously, such as LCMS,[12,22] RP-HPLC,[23] and UV-visible spectroscopy,[13] solely focused on the API estimation of IMGH, with limited attention on the impurity profiling of. For instance, Chandarana *et al*. developed a green bioanalytical LC-MS/MS method for the estimation

Table 8: Linearity study of impurity N2, N2,6-trimethyl-1,3,5-triazine-2,4-diamine

Replicates	ConcentrationsofImpurityN2, N2,6-trimethyl-1,3,5-triazine-2,4-diamine					
	0.2 µg/mL	0.4 µg/mL	0.6 µg/mL	0.8 µg/mL	1 µg/mL	1.2 µg/mL
	Peak area					
1	3873	5938	7786	9787	11367	13543
2	3931	5896	7694	9898	11436	13568
3	3860	5912	7875	9769	11442	13456
4	3883	5799	7889	9796	11369	13749
5	3823	5813	7785	9789	11398	13653
6	3812	5882	7868	9888	11584	13457
Mean	3863.67	5873.33	7816.17	9821.17	11432.67	13571.00
Standard deviation	43.20	55.55	75.18	56.44	80.71	114.39
%RSD	1.12	0.95	0.96	0.57	0.71	0.84

Table 9: Intra-day precision study of imeglimin hydrochloride

Concentration (µg/mL)	Area	SD	%RSD
25	1368237	25120.84	1.80
25	1388129		
25	1418138		
50	3258153	10000.00	0.31
50	3278153		
50	3268153		
75	4676326	27537.85	0.58
75	4721326		
75	4726326		

Table 10: Inter-day precision study of imeglimin hydrochloride

Concentration (µg/mL)	Area	SD	%RSD
25	1388133	3666.14	0.26%
25	1388129		
25	1388132		
50	3238281	14436.64	0.44%
50	3238279		
50	3263285		
75	4676229	20002.50	0.43%
75	4696236		
75	4656231		

Table 11: Intra-day precision study of impurity N2, N2,6-trimethyl-1,3,5-triazine-2,4-diamine

Concentration (µg/mL)	Area	SD	%RSD
0.3	5518	65.18	1.17
0.3	5607		
0.3	5645		
0.6	9349	33.05	0.35
0.6	9403		
0.6	9343		
0.9	11735	214.27	1.85
0.9	11638		
0.9	11325		

Table 12: Inter-day precision study of impurity N2, N2,6-trimethyl-1,3,5-triazine-2,4-diamine

Concentration (µg/mL)	Area	SD	%RSD
0.3	5689	51.03	0.90
0.3	5593		
0.3	5671		
0.6	9325	61.45	0.66
0.6	9362		
0.6	9645		
0.9	11818	25.89	0.22
0.9	11779		
0.9	11769		

of IMGH.[12] The UPLC–APCI–MS/MS method, which is reported for genotoxic impurity identification are high operational complexity and costly, which may not be appropriate for the routine analysis.[11] By combining IMGH and its impurities into a single chromatographic run, the proposed RP-HPLC method offers a more effective and thorough analytical procedure.

Method optimization was performed using a quality-by-design–based response surface methodology employing CCD with nine experimental runs to evaluate the impact of mobile phase composition and pH on critical analytical attributes. ANOVA results identified the mobile phase acetonitrile content ($P < 0.05$) as the main factor affecting retention time and recovery, while pH had no discernible impact on these responses. According to the model, this

Table 13: Recovery study of imeglimin

Level	Concentration (µg/ml)		Area	% Recovery	Mean±SD	%RSD
	Sample	Standard				
50%	50 µg	25 µg	222356	99.00	100.01±1.383	1.38
			221360	99.45		
			216686	101.59		
100%	50 µg	50 µg	331467	101.48	100.02±1.895	1.91
			333985	100.71		
			343642	97.88		
150%	50 µg	75 µg	456892	99.33	100.01±1.315	1.31
			457563	99.18		
			446989	101.53		

Table 14: Recovery study of impurity N2, N2,6-trimethyl-1,3,5-triazine-2,4-diamine

Level (%)	Concentration (µg/mL)		Area	% Recovery	Mean±SD	%RSD
	Sample	Standard				
50	0.8 µg	0.4 µg	58736	100.29	100±0.257	0.25
			59025	99.80		
			58964	99.91		
100	0.8 µg	0.8 µg	70869	100.39	100±0.337	0.33
			71253	99.85		
			71308	99.77		
150	0.8 µg	1.2 µg	84869	98.89	100±0.997	1.0
			83253	100.81		
			83658	100.32		

Table 15: Robustness study of the imeglimin hydrochloride (IMGH) and Impurity N2, N2,6-trimethyl-1,3,5-triazine-2,4-diamine

Drug	% RSD found for robustness study								
	pH			Flow rate (1 mL/min)			Wavelength (nm)		
	4.5	4.6	4.7	0.9	1.0	1.1	239	240	241
IMGH	0.4396	0.2795	0.5538	0.0825	0.2378	0.0998	0.186	0.2093	0.2597
Impurity	0.6817	0.3498	0.8307	0.6713	0.3588	0.7629	0.7635	0.5457	0.9236

strategy can achieve optimal chromatographic performance across multiple criteria with a composite desirability value of 1. An optimized mobile phase allowed for well-resolved and sharp peaks to be obtained. IMG exhibited a retention time of 11 min, comparatively higher than previously reported values of 2.5 min,[4] 3.38 min,[1] and 5.7 min, and its impurity N2,N2,6-trimethyl-1,3,5-triazine-2,4-diamine showed a retention time of 6.30 min. IMGH and its impurities were accurately quantified over a wide concentration range using the developed method, which showed excellent linearity, with correlation coefficients (R^2) of 0.99902 and 0.9991, respectively. As the intraday and interday %RSD values were below 2%, the precision and accuracy of the method were confirmed, and recovery studies at 50%, 100%, and 150% demonstrated accuracy, with recoveries within 97.88–100.71% for IMGH and 98.89–100.81% for its impurity, in accordance with ICH Q2(R1).To confirm the method's sensitivity, the LOQ values for IMGH and its impurity N2,N2,6-trimethyl-1,3,5-triazine-2,4-diaminewere found to be 6.021 µg/mL and 0.125 µg/mL, respectively, and the LODs values for IMGH and its impurity N2,N2,6-trimethyl-1,3,5-triazine-2,4-diaminewere found to be 2.158 g/mL and 0.04117 g/mL, respectively. The robustness of the method was demonstrated by intentionally varying the wavelengths and column oven temperatures, supporting its suitability for routine quality control. Moreover, the developed, optimized,

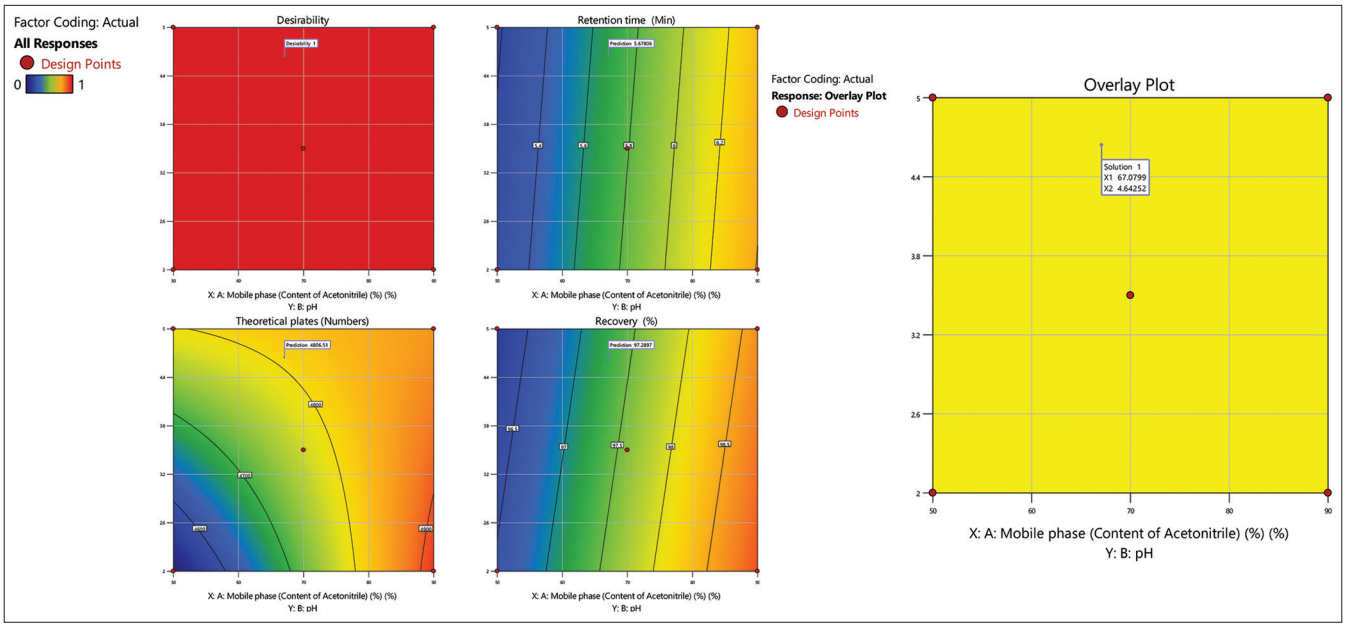


Figure 6: Mobile phase ratio 10 mM acetate buffer and ACN (30:70) and pH 4.6 provides a retention time of 5.6 min, theoretical plates 4806.51, and a 97.26% recovery with a desirability value of 1

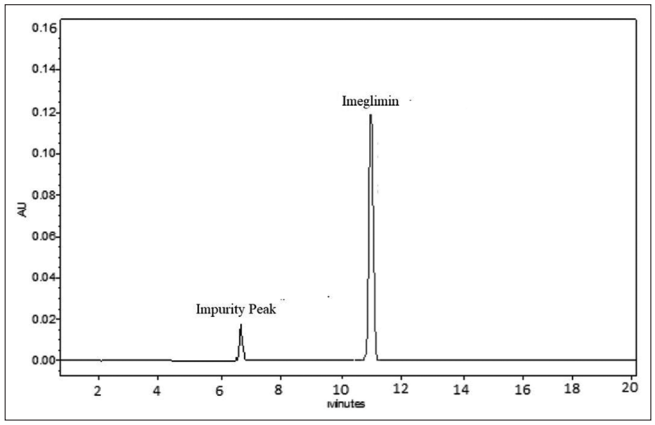


Figure 7: Chromatogram of standard imeglimin hydrochloride and impurity

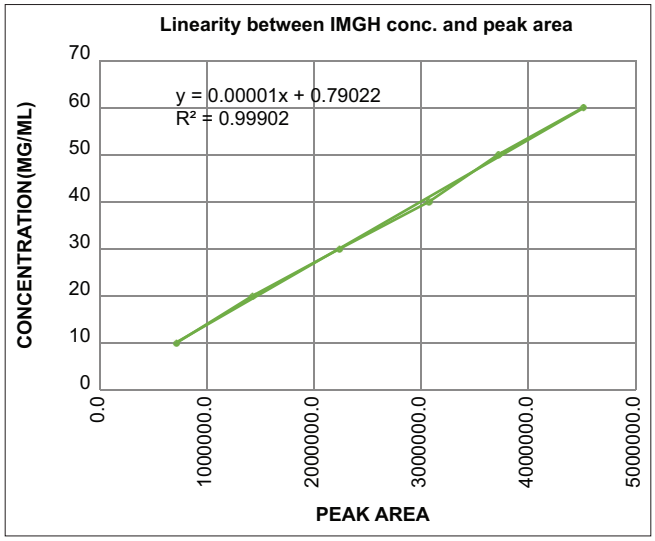


Figure 8: Calibration curve of imeglimin hydrochloride

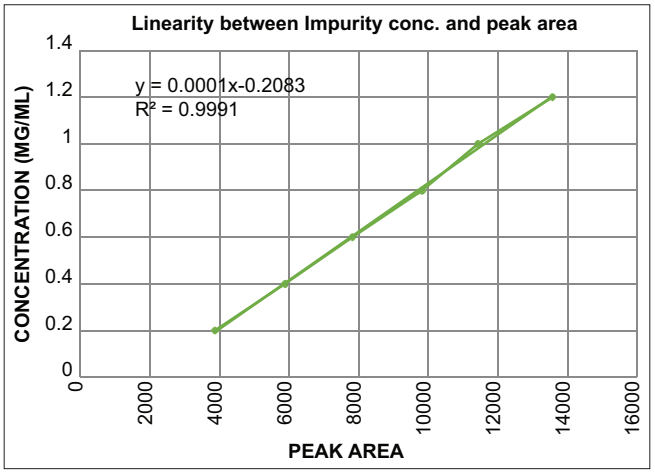


Figure 9: Calibration curve of impurity N2,N2,6-trimethyl-1,3,5-triazine-2,4-diamine

and validated method for estimating the IMGH and Impurity N2,N2,6-trimethyl-1,3,5-triazine-2,4-diaminealigned with the ICH Q2 guidelines and was determined to be simple, specific, robust, precise, and accurate.

CONCLUSION

It was discovered that the developed and optimized RP-HPLC method was reliable, precise, sensitive, and accurate for estimating IMGH and its impurities. The ICH Q2 (R1) requirements were followed for validating the newly developed approach. The LOD and LOQ for IMGH and impurity were determined. LOQ found to be 6.021 µg/mL and 0.125 µg/mL, respectively, whereas the LOD was 2.158 µg/mL and 0.04117 µg/mL, respectively,

indicating the sensitivity of the approach. The approach offers greater resolution and is cost-effective and eco-friendly. Consequently, it was determined that the RP-HPLC method's development and validation were appropriate for routine analysis in pharmaceutical laboratories to regulate product quality.

REFERENCES

1. Jain A, Soni I, Sharma RJ. Development and validation of stability indicating rp-uhplc method for the estimation of imeglimin hydrochloride used for the treatment of metabolic disorder diabetes mellitus. Int J Appl Pharm 2023;15:211-7.
2. WHO. Diabetes. Switzerland: World Health Organization; 2024.
3. Goyal R, Singhal M, Jialal IJ. Type 2 Diabetes. Treasure Island, FL: StatPearls Publishing; 2023.
4. Adhao VS, Chaudhari SP, Ambhore JP. Stability indicating RP-HPLC method development and validation for Imeglimin HCL in pharmaceutical dosage form. Chem Sci Int J 2024;33:1-10.
5. Gouda AA, Amin AS, Fahium S, Mahdy AE, Elsaify NE, Soliman NS. Spectrophotometric methods for quantitative determination of imeglimin HCl in pure and dosage forms. Bull Faculty Sci Zagazig Univ 2025;2024:190-200.
6. Giri PT, Chabukswar AR, Jagdale SC, Chindhe SA. QBD (Quality by design) approach: Development and validation of an Rp-Hplc method for estimating imeglimin hcl and its ketone impurity. Int J Appl Pharm 2025;17:241-53.
7. Mangore MM, Bhosale NR, Chavan RS, Chikane PT, Kardile MS, Khade PH. Formulation and optimization of imeglimin-loaded nanoparticles using central composite design. J Chem Healrth Risks 2025;15:2687-96.
8. Tsuno T, Li J, Nishiyama K, Kawasawa YI, Inoue R, Yajima EO, *et al*. Imeglimin suppresses glucagon secretion and induces a loss of α cell identity. Cell Rep Med 2025;6:102254.
9. Gogikar SK, Sen S, Pathinti S, Samanthula G, Dikundwar AG. Forced degradation study of an anti-diabetic drug imeglimin: Impurity profiling and structure elucidation using LC-Q-ToF-MS/MS and NMR. Rapid Commun Mass Spectrom 2025;39:e9960.
10. Mubeen G, Navali S, Lalitha N. RP-HPLC method for determination of imeglimin hydrochloride in bulk and tablet formulation. Asian J Pharm Res Dev 2024;12:92-6.
11. Pepakayala S, Ratnakaram VN, Zunjarrao Y, Lohia YK. Eco-friendly, trace-level, and multianalyte UPLC-APCI-MS/MS method for simultaneous quantification of genotoxic nitrosamines in imeglimin. Microchem J 2026;220:116283.
12. Chandarana CV, Vashi VH, Bera A, Patel J. Green bioanalytical method development and validation for estimation of Imeglimin hydrochloride using human plasma in pure form by LC-MS/MS. Future J Pharm Sci 2025;11:81.
13. Tamil Selvan R, Senthilkumar S, Elakkiya A, Gayathri M, Gokulraj M, Hajima H, *et al*. A Novel method development and Validation of imeglimin HCl by UV-visible spectroscopy. Int J Pharm Sci 2023;1:852-9.
14. Tandel JN. Development and validation of RP-Hplc method for the simultaneous determination of amiloride hydrochloride and furosemide in pure and pharmaceutical Dosage form. Eurasian J Analy Chem 2017;124:385-94.
15. Fukuda IM, Pinto CF, Moreira CD, Saviano AM, Lourenço FR. Design of experiments (DoE) applied to pharmaceutical and analytical quality by design (QbD). Brazilian J Pharm Sci 2018;54:e01006.
16. Saha P, Pandey MM. Design of experiment (DoE)-approach based RP-HPLC analytical method development and validation for estimation of efavirenz in bulk and formulations. J Chromatogr Sci 2022;60:35-44.
17. Jain AK, Dubey B, Khare S, Joshi A, Ahirwar M, Jain P. Comparison of RP-HPLC and UV spectrophotometric methods for estimation of haloperidol in pure and pharmaceutical formulation. J. Drug Deliv Ther 2018;8:277-82.
18. Tomar B, Sharma A, Kumar I, Jain S, Ahirrao PJ. Technology. Development and validation of the analytical method for the estimation of a combination of 5-fluorouracil and imiquimod by RP-HPLC. Res J Pharm Technol 2021;14:3313-8.
19. Patil S, Adhyapak A, Shetti P, Gurao R. Development of a robust and reliable reverse-phase high-performance liquid chromatography (RP-HPLC) method using analytical quality by design principles for the accurate determination of esculin in its bulk form. Future J Pharm Sci 2023;9:69.
20. Kaviani RJ. Comparison of different approaches for calculating LOD and LOQ in an HPLC-based analysis method. Pharm Sci 2024;31:106-9.
21. Devdikar S, Shetkar B, Satpute K, Lohiya G, Sonvane S, Kore S, *et al*. Implementation of QbD approach in RP-HPLC method optimization and validation for concurrent estimation of deflazacort and tamsulosin hydrochloride under ICH Q14 and Q2R2 framework. Curr Pharm Anal 2025;21:486-99.
22. Ramalingam S, Subramania MN, Basuvan B, Jaganathan R, Dhavamani AJ, Kandukuri NK, *et al*. A sensitive direct chiral liquid chromatography tandem mass spectrometry method for the enantio-selective analysis of imeglimin in formulation. J Appl Pharm Sci 2023;13:214-9.
23. Chikhale H, Ambekar A, Avhad S, Borse L. Development and Validation of RP-HPLC Method for Determination of Antidiabetic Drug (Imeglimin HCL) in Bulk and its Dosage Form. J ChemHealthRisks 2024;14.

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