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Research Article

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AQbD-Assisted Development and Validation of a Green RP-HPLC Method for Simultaneous Quantification of Telmisartan, Amlodipine Besylate, and Bisoprolol Fumarate

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ABSTRACT

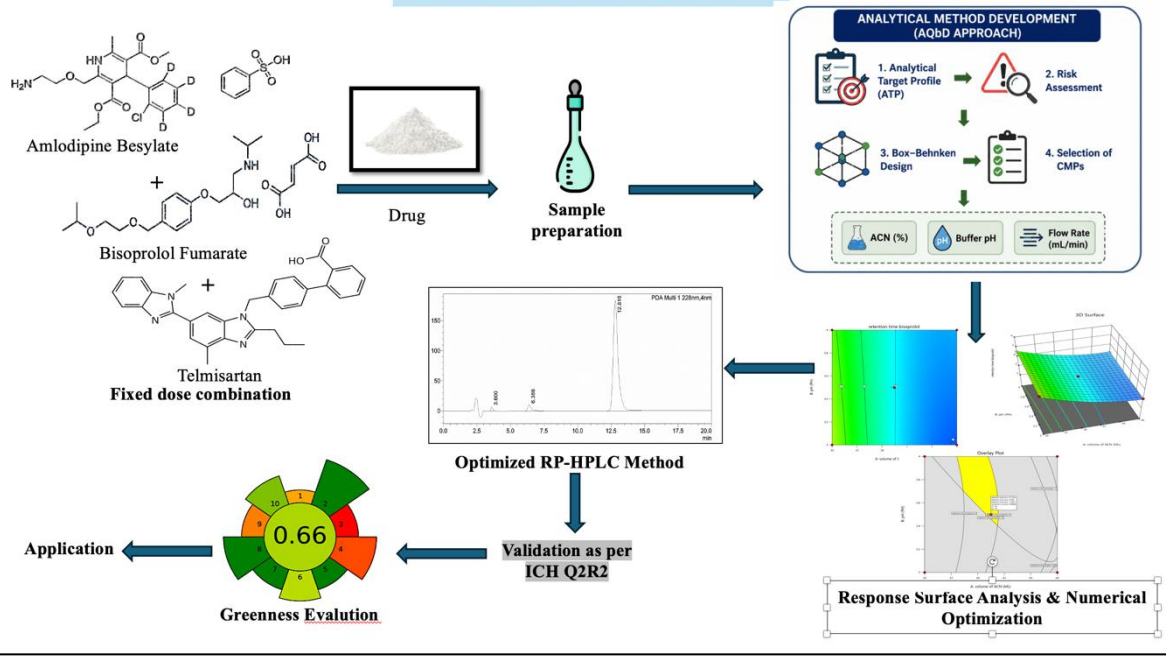
The simultaneous determination of Telmisartan (TEL), Amlodipine besylate (AML), and Bisoprolol fumarate (BIS) presents a significant analytical challenge owing to their markedly different physicochemical properties, including variations in lipophilicity and ionization behavior. In the present study, a novel Analytical Quality by Design (AQbD)-driven reverse-phase high-performance liquid chromatographic (RP-HPLC) method was developed and validated for the simultaneous quantification of TEL, AML, and BIS in bulk and synthetic mixtures. Critical chromatographic variables, including acetonitrile content, buffer pH, and flow rate, were systematically optimized using a Box–Behnken Design to establish a robust analytical design space and improve method understanding. The optimized chromatographic separation was achieved on a C18 column (250 × 4.6 mm, 5 µm) using phosphate buffer and acetonitrile (60:40, v/v) adjusted to pH 3.5, delivered at a flow rate of 1.0 mL min^{-1} and column temperature of 35 °C. Under optimized conditions, BIS, AML, and TEL were eluted at 3.56, 6.38, and 12.63 min, respectively, with satisfactory peak symmetry (tailing factor ≤1.4) and column efficiency (3754–6725 theoretical plates). Statistical analysis demonstrated excellent model adequacy, with adjusted R^2 values ranging from 0.8737 to 0.9921 and adequate precision values between 16.01 and 66.05, confirming the reliability of the developed AQbD model.

The method exhibited excellent linearity over concentration ranges of 1–4 µg mL^{-1} for BIS, 2–8 µg mL^{-1} for AML, and 16–64 µg mL^{-1} for TEL, with correlation coefficients of 0.9990, 0.9988, and 0.9988, respectively. Recovery studies demonstrated high accuracy, with recoveries ranging from 99.55–101.11% for BIS, 99.64–100.68% for AML, and 99.23–102.82% for TEL. Precision studies showed %RSD values below 2% for repeatability, intraday precision, and intermediate precision, confirming excellent method reproducibility. Deliberate variations in chromatographic parameters produced negligible effects on analytical performance, indicating robust method behavior. Furthermore, greenness assessment using the AGREE metric yielded a score of 0.66, demonstrating acceptable environmental sustainability of the developed procedure.

The proposed AQbD-assisted RP-HPLC method offers a reliable, precise, accurate, and environmentally conscious analytical platform for the simultaneous determination of TEL, AML, and BIS. The integration of statistical optimization and green analytical principles provides enhanced method robustness and supports its application in routine pharmaceutical quality control and future industrial analyses.

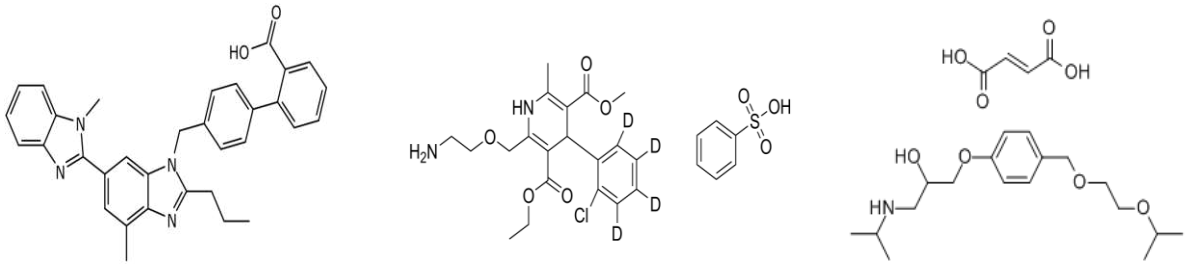
Keywords: Telmisartan; Amlodipine Besylate; Bisoprolol Fumarate; Analytical Quality by Design; Box–Behnken Design; RP-HPLC; Method Validation; Green Analytical Chemistry.

GRAPHICAL ABSTRACT



INTRODUCTION

Hypertension is one of the most prevalent cardiovascular disorders worldwide and represents a major risk factor for myocardial infarction, stroke, heart failure, chronic kidney disease, and premature mortality **[1,2]**. Although numerous antihypertensive agents are available, monotherapy often fails to achieve adequate blood pressure control, necessitating the use of combination therapy. The recently introduced triple-drug combination of telmisartan (TEL), amlodipine besylate (AML), and bisoprolol fumarate (BIS), Chemical structures of telmisartan, amlodipine besylate, and bisoprolol fumarate shown in the Figure 1, offers superior therapeutic efficacy by targeting multiple pathophysiological pathways involved in hypertension **[3,4]**. Telmisartan is a selective angiotensin II type-1 receptor blocker **[5]** that inhibits vasoconstriction and aldosterone secretion while also exhibiting partial peroxisome proliferator-activated receptor gamma (PPAR-γ) agonistic activity, providing additional metabolic benefits. Amlodipine besylate is a long-acting dihydropyridine calcium channel blocker **[6]** that reduces peripheral vascular resistance through inhibition of calcium influx into vascular smooth muscle cells, whereas bisoprolol fumarate is a highly selective β1-adrenergic receptor blocker **[7]** that lowers blood pressure by reducing heart rate, cardiac output, and renin release. The complementary pharmacological actions of these drugs make their combination highly effective for the management of hypertension and associated cardiovascular complications **[3,4]**.



Telmisartan Amlodipine Besylate Bisoprolol Fumarate

Figure 1. Chemical structures of telmisartan, amlodipine besylate, and bisoprolol fumarate.

From an analytical perspective, simultaneous determination of TEL, AML, and BIS presents considerable chromatographic challenges due to their distinct physicochemical properties. Telmisartan possesses a pKa of approximately 4.2 and a high Log P value of 7.7, indicating strong lipophilicity and prolonged retention on reversed-phase columns **[5]**. In contrast, amlodipine besylate and bisoprolol fumarate are basic compounds with pKa values of approximately 8.6 and 9.5 and Log P values of 2.6 and 2.2, respectively **[6,7]**. These differences significantly influence ionization behavior, retention characteristics, selectivity, and peak symmetry during RP-HPLC analysis. Therefore, optimization of chromatographic variables such as mobile phase composition, pH, and flow rate is essential to achieve satisfactory separation and analytical performance.

Analytical Quality by Design (AQbD) has emerged as a systematic, science-based, and risk-oriented approach for analytical method development that enhances method understanding and robustness throughout the analytical lifecycle **[8,9]**. Unlike conventional trial-and-error approaches, AQbD utilizes predefined analytical objectives, risk assessment, and design of experiments (DoE) to identify and control critical method parameters affecting analytical performance. Among various experimental designs, Box–Behnken Design (BBD) is widely employed for chromatographic optimization because it efficiently evaluates linear, interaction,

and quadratic effects of multiple variables while minimizing the number of experimental runs **[10]**. Furthermore, increasing emphasis on environmental sustainability has led to the incorporation of Green Analytical Chemistry (GAC) principles into method development **[11,12]**. Greenness assessment tools such as the National Environmental Methods Index (NEMI), Green Analytical Procedure Index (GAPI), Analytical Eco-Scale, and Analytical Greenness Metric Approach (AGREE) facilitate evaluation of the environmental impact of analytical procedures and promote the development of safer and more sustainable methods **[13,14]**.

An extensive literature survey revealed several UV spectrophotometric, HPTLC, RP-HPLC, and LC-MS methods for the individual estimation of telmisartan, amlodipine besylate, and bisoprolol fumarate, as well as for certain binary combinations **[15-17]**. However, no AQbD-assisted RP-HPLC method has been reported for the simultaneous estimation of TEL, AML, and BIS in a single chromatographic run. Therefore, the present study aimed to develop and validate a novel AQbD-based RP-HPLC method for simultaneous determination of telmisartan, amlodipine besylate, and bisoprolol fumarate in bulk and synthetic mixture. The method was optimized using Box–Behnken Design, validated according to ICH Q2(R2) guidelines, and evaluated using green analytical chemistry assessment tools to establish its suitability for routine pharmaceutical quality control applications.

MATERIALS AND METHODS

Selection of Detection Wavelength

To establish an appropriate detection wavelength for simultaneous estimation, individual standard solutions of telmisartan (40 µg/mL), amlodipine besylate (5 µg/mL), and bisoprolol fumarate (2.5 µg/mL) were prepared and scanned over the wavelength range of 200–400 nm using a UV–Visible spectrophotometer. Telmisartan exhibited maximum absorbance within the range of 210–220 nm, while bisoprolol fumarate showed appreciable absorbance around 220–230 nm. Amlodipine besylate displayed a characteristic absorption band at higher wavelengths (350–370 nm). An isosbestic point was observed at 228 nm where all three analytes exhibited comparable absorbance. Therefore, 228 nm was selected as the analytical wavelength for simultaneous RP-HPLC determination of telmisartan, amlodipine besylate, and bisoprolol fumarate due to its adequate sensitivity and response for all analytes.

Chemicals and Reagents

Telmisartan was obtained from Yarrow Chem Products (Mumbai, Maharashtra, India), amlodipine besylate from Atom Pharma (Surat, Gujarat, India), and bisoprolol fumarate from Zydus Pharmaceuticals (Vadodara, Gujarat, India) as reference standards. HPLC-grade acetonitrile was procured from Thermo Fisher Scientific Pvt. Ltd., while HPLC-grade methanol was purchased from Merck Specialties Pvt. Ltd. (Mumbai, Maharashtra, India). Potassium dihydrogen phosphate was obtained from Astron Chemicals, and orthophosphoric acid was procured from Loba Chemicals Pvt. Ltd. Ultrapure HPLC-grade water was obtained using a Milli-Q water purification system (Millipore, USA). All chemicals, reagents, and solvents used during method development, AQbD optimization, validation, and greenness assessment were of HPLC or analytical reagent grade.

Instrumentation and Chromatographic Conditions

Chromatographic analysis was performed using a Shimadzu LC-2030C high-performance liquid chromatography system equipped with a quaternary pump, autosampler, column oven, and photodiode array (PDA) detector controlled through LabSolutions software. Separation was achieved on a BDS Hypersil C18 column (250 mm × 4.6 mm, 5 µm particle size) maintained at 35°C. The mobile phase consisted of phosphate buffer (pH 3.5) and acetonitrile

(60:40, v/v) delivered under isocratic conditions at a flow rate of 1.0 mL min^{-1}. Prior to use, the mobile phase was filtered through a 0.45 µm membrane filter and degassed by sonication. The injection volume was 20 µL, and chromatographic detection was carried out at 228 nm.

Preparation of Phosphate Buffer (pH 3.5)
A phosphate buffer of pH 3.5 was prepared by dissolving 2.72 g of potassium dihydrogen phosphate in approximately 800 mL of water. The pH was adjusted to 3.5 using orthophosphoric acid, and the final volume was made up to 1000 mL with water. The prepared buffer was filtered through a 0.45 µm membrane filter and degassed before use.

AQbD-Based Method Development
Analytical Quality by Design (AQbD) principles were implemented to develop a robust and reliable RP-HPLC method. An Analytical Target Profile (ATP) was established to achieve baseline separation of telmisartan, amlodipine besylate, and bisoprolol fumarate with acceptable retention time, peak symmetry, theoretical plate count, and resolution **[8,9]**.
Critical method parameters (CMPs) identified during preliminary risk assessment included acetonitrile concentration, buffer pH, and mobile phase flow rate. Their effects on critical analytical attributes (CAAs), including retention time and chromatographic resolution, were systematically evaluated using a Box–Behnken Design (BBD) **[10]**.

Experimental Design and Optimization
A three-factor, three-level Box–Behnken Design consisting of seventeen experimental runs was employed using Design-Expert® software. The selected independent variables were volume of acetonitrile (35–45 mL), buffer pH (3.0–4.0), and flow rate (0.8–1.2 mL/min). The responses studied were retention times of telmisartan, amlodipine besylate, and bisoprolol fumarate, together with chromatographic resolution. Statistical analysis of the experimental design generated quadratic polynomial models describing the influence of critical method parameters on chromatographic responses. The optimized analytical design space was established using desirability functions, and optimum chromatographic conditions were identified as phosphate buffer: acetonitrile (60:40, v/v), pH 3.5, and flow rate of 1.0 mL/min.

Preparation of Standard Stock Solutions
Primary stock solutions of telmisartan, amlodipine besylate, and bisoprolol fumarate were prepared separately by accurately weighing appropriate quantities of each drug and dissolving them into methanol. Further dilutions were prepared to obtain working concentrations corresponding to the analytical concentration levels employed during method development and validation studies.

Preparation of Standard Mixture
A mixed standard solution containing telmisartan (40 µg/mL), amlodipine besylate (5 µg/mL), and bisoprolol fumarate (2.5 µg/mL) was prepared using the optimized mobile phase. The solution was filtered through a 0.45 µm membrane filter prior to chromatographic analysis.

Preparation of Synthetic Mixture
A laboratory-prepared synthetic mixture was formulated to simulate the pharmaceutical dosage form. Appropriate quantities of telmisartan, amlodipine besylate, and bisoprolol fumarate were blended with commonly used pharmaceutical excipients, including microcrystalline cellulose, starch, croscarmellose sodium, talc, and magnesium stearate. An accurately weighed quantity equivalent to the required drug content was transferred into a volumetric flask, extracted with

mobile phase, sonicated for 15 min, filtered through a 0.45 µm membrane filter, and appropriately diluted prior to chromatographic analysis.

Method Validation

The developed RP-HPLC method was validated according to International Council for Harmonization (ICH) guideline Q2(R2) **[18]** with respect to system suitability, specificity, linearity, accuracy, precision, robustness, and sensitivity.

System Suitability

System suitability was assessed prior to analysis **[19]** by injecting six replicate preparations of the standard mixture. Parameters including retention time, theoretical plates, peak area, tailing factor, and percentage relative standard deviation (%RSD) were evaluated to verify chromatographic system performance.

Specificity

Specificity was evaluated by analyzing blank, placebo, synthetic mixture, and standard solutions under optimized chromatographic conditions. The absence of interfering peaks at the retention times of telmisartan, amlodipine besylate, and bisoprolol fumarate confirmed the specificity of the developed method.

Linearity and Working Range

Linearity was established over concentration ranges of 16–64 µg/mL for telmisartan, 2–8 µg/mL for amlodipine besylate, and 1–4 µg/mL for bisoprolol fumarate. Calibration curves were constructed by plotting peak area against concentration, and regression analysis was performed to determine linearity characteristics.

Precision

Precision was evaluated in terms of repeatability, intraday precision, and intermediate precision. Repeatability was assessed using six replicate injections at the target concentration. Intraday precision was determined by analyzing three concentration levels within a single day, whereas intermediate precision was assessed on different days. Results were expressed as percentage relative standard deviation (%RSD).

Accuracy

Accuracy was determined using recovery studies performed at 80%, 100%, and 120% concentration levels. Known amounts of standard drugs were added to pre-analyzed samples, and percentage recoveries were calculated to assess method trueness.

Sensitivity

Method sensitivity was evaluated by calculating the limit of detection (LOD) and limit of quantification (LOQ) based on the standard deviation of the response and the slope of the calibration curve using the following equations:

$LOD = 3.3\sigma/S$

$LOQ = 10\sigma/S$

where σ represents the standard deviation of the response and S denotes the slope of the calibration curve.

Robustness

Robustness was assessed by introducing deliberate variations in critical chromatographic parameters. Flow rate (1.0 mL/min) was varied to 0.8 and 1.2 mL/min, acetonitrile content was varied between 35% and 45%, and buffer pH was adjusted between 3.3 and 3.7. The effect of

these changes on chromatographic performance was evaluated through retention time, peak area, and system suitability parameters.

Greenness Assessment

The environmental sustainability of the developed RP-HPLC method was evaluated in accordance with Green Analytical Chemistry principles. Various greenness assessment tools, including the Analytical Greenness Metric Approach (AGREE), Green Analytical Procedure Index (GAPI), National Environmental Methods Index (NEMI), and Analytical Eco-Scale, were considered for evaluating the environmental impact of the analytical procedure. Among these tools, the greenness assessment was performed using the AGREE software **[14]**, which provided a comprehensive evaluation based on the twelve principles of Green Analytical Chemistry.

RESULT & DISCUSSION

Selection of Detection Wavelength

The UV absorption spectra of telmisartan, amlodipine besylate, and bisoprolol fumarate were recorded over the wavelength range of 200–400 nm. Overlay spectral analysis revealed an isosbestic point at 228 nm where all three analytes exhibited adequate absorbance, enabling simultaneous detection with satisfactory sensitivity. Therefore, 228 nm was selected as the optimum wavelength for chromatographic analysis.

Selection of Critical Method Parameters and Responses

Analytical Quality by Design (AQbD) principles were employed to systematically develop and optimize the RP-HPLC method for simultaneous estimation of bisoprolol fumarate (BIS), amlodipine besylate (AML), and telmisartan (TEL). Preliminary investigations identified the percentage of acetonitrile (A), buffer pH (B), and flow rate (C) as critical method parameters (CMPs) affecting chromatographic performance tabulated in Table 1.

A three-factor, three-level Box–Behnken Design (BBD) comprising 17 experimental runs was selected to evaluate the influence of these variables on critical analytical attributes (CAAs), namely retention time of BIS, AML, and TEL, and resolution of AML.

Table 1. Independent variables and responses used in Box–Behnken Design

Factor	Parameter	Low (-1)	Center (0)	High (+1)
A	Acetonitrile (%)	35	40	45
B	Buffer pH	3.0	3.5	4.0
C	Flow rate (mL min^{-1})	0.8	1.0	1.2

Response	Description
Y1	Retention time of BIS
Y2	Retention time of AML
Y3	Retention time of TEL
Y4	Resolution of AML

Experimental Design and Observed Responses

Seventeen experimental runs were performed according to the Box–Behnken Design and the obtained chromatographic responses were recorded tabulated in Table 2.

Table 2. Experimental runs and observed responses obtained from Box–Behnken Design

Run	ACN (%)	pH	Flow Rate	RT BIS (min)	RT AML (min)	RT TEL (min)	Resolution AML
1	40	3.0	0.8	4.378	7.338	23.400	9.130

2	40	4.0	1.2	3.050	5.282	7.033	7.831
3	45	4.0	1.0	3.166	4.373	19.657	5.060
4	40	3.5	1.0	3.587	6.358	12.840	8.718
5	35	3.5	0.8	5.922	14.699	28.208	15.297
6	35	3.0	1.0	4.941	10.510	33.299	9.835
7	40	3.5	1.0	3.500	6.358	12.840	8.718
8	40	3.5	1.0	3.500	6.358	12.840	8.718
9	45	3.5	1.2	2.703	3.785	14.192	5.044
10	40	3.5	1.0	3.500	6.358	12.840	8.718
11	45	3.0	1.0	3.034	4.306	13.615	5.673
12	40	3.0	1.2	2.965	5.005	15.839	8.508
13	35	3.5	1.2	3.994	9.981	19.169	13.970
14	35	4.0	1.0	4.538	10.325	14.227	12.845
15	40	4.0	0.8	4.528	7.810	10.375	8.739
16	45	3.5	0.8	4.009	5.725	9.871	5.547
17	40	3.5	1.0	3.500	6.358	12.840	8.718

Analysis of Variance (ANOVA)

ANOVA was applied to evaluate the significance of the developed quadratic models and to identify the influence of critical method parameters on chromatographic responses. All developed models were statistically significant ($p < 0.0001$), confirming the suitability of the selected experimental design. Results are tabulated in Table 3.

Table 3. Summary of ANOVA results for Box–Behnken Design

Response	Model F-value	p-value	Significant Factors ($p < 0.05$)	Adjusted R^2	Predicted R^2	Adequate Precision
Retention time of BIS	334.27	<0.0001	A, C, AB, AC, A^2, C^2	0.9921	0.9792	66.045
Retention time of AML	59.80	<0.0001	A, C, AC, A^2, B^2, C^2	0.9707	0.7946	27.740
Retention time of TEL	21.57	<0.0001	A, B, C, AC, A^2	0.9271	0.7034	21.570
Resolution of AML	16.01	<0.0001	A, B^2, C^2	0.8737	0.7318	16.013

The ANOVA results indicated that acetonitrile concentration exerted the greatest influence on analyte retention times, while pH significantly affected chromatographic selectivity and resolution. High adjusted and predicted R^2 values confirmed good agreement between experimental and predicted responses.

Response Surface Analysis

Response surface methodology was employed to visualize the interaction effects among critical method parameters. Increasing acetonitrile concentration and flow rate significantly reduced retention times of all analytes. Conversely, lower acetonitrile concentrations increased retention and improved resolution. Buffer pH showed a pronounced effect on chromatographic selectivity, particularly for amlodipine and telmisartan.

The generated contour plots and three-dimensional response surface plots facilitated identification of a robust chromatographic region and demonstrated the suitability of

multivariate optimization for method development. Contour plots and three-dimensional response surface plots illustrating the effects of acetonitrile concentration (A), buffer pH (B), and flow rate (C) on chromatographic responses including retention time of bisoprolol fumarate, amlodipine besylate, telmisartan, and resolution of amlodipine besylate shown in the Figure 2.

Polynomial equations generated by BBD

Retention time of BIS:

3.5123−0.8104A−0.7656C+0.1338AB+0.1555AC+0.4139A2+0.2244C2

Retention time of AML:

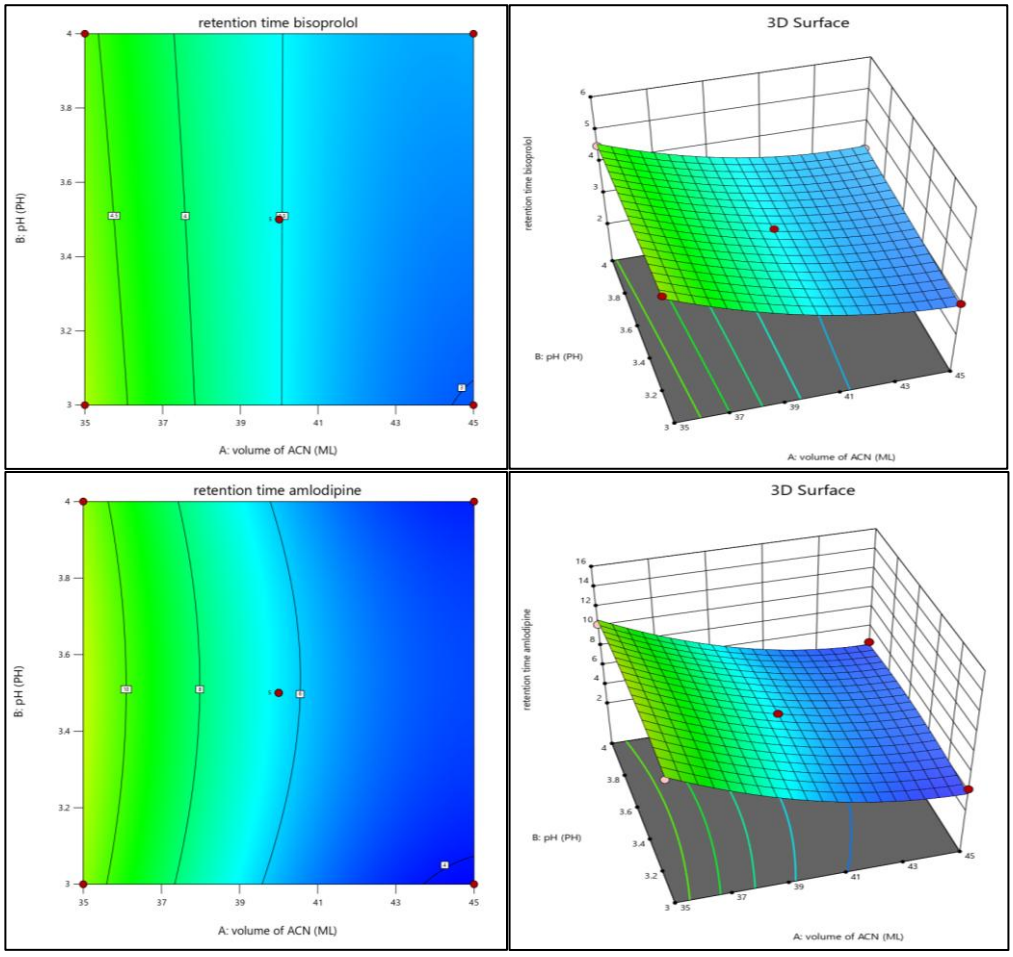
6.358−3.4158A+0.0789B−1.4399C+0.0630AB+0.6945AC−0.0488BC+1.6046A2−0.5841B2+0.5

Retention time of TEL:

6.358−3.4158A+0.0789B−1.4399C+0.0630AB+0.6945AC−0.0488BC+1.6046A2−0.5841B2+0.5

Resolution of AML

8.9386−3.8279A+0.1661B−0.4200C−0.8611B2+0.7502C2



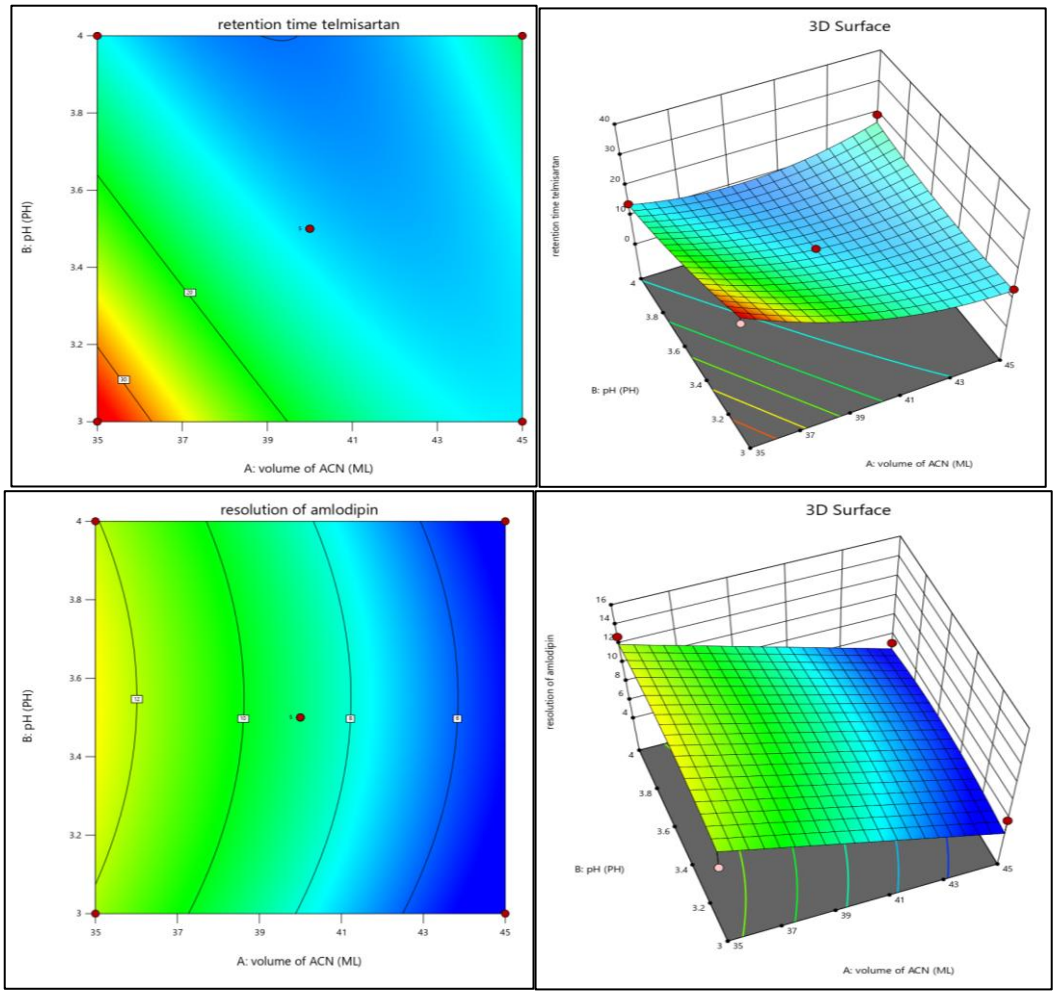


Figure 2. Contour plots and three-dimensional response surface plots illustrating the effects of acetonitrile concentration (A), buffer pH (B), and flow rate (C) on chromatographic responses including retention time of bisoprolol fumarate, amlodipine besylate, telmisartan, and resolution of amlodipine besylate.

Numerical Optimization and Design Space Establishment

Numerical optimization using desirability function analysis was performed to simultaneously minimize retention times and maximize chromatographic resolution.

The desirability function predicted optimum chromatographic conditions consisting of phosphate buffer (pH 3.5) and acetonitrile (60:40, v/v) delivered at a flow rate of 1.0 mL min^{-1}. Experimental verification demonstrated excellent agreement between predicted and observed chromatographic responses, confirming the validity of the developed AQbD model, shown in Figure 3.

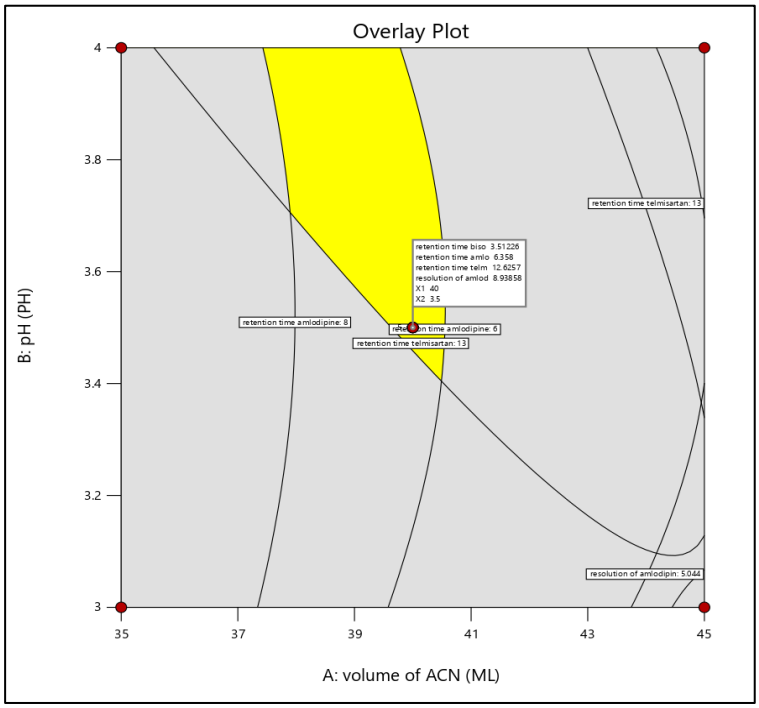


Figure 3: Desirability Plot showing optimized chromatographic conditions.

Optimized Chromatographic Performance & Method Validation

Under optimized chromatographic conditions, complete baseline separation of bisoprolol fumarate, amlodipine besylate, and telmisartan was achieved. Bisoprolol fumarate, amlodipine besylate, and telmisartan eluted at retention times of 3.56, 6.38, and 12.63 min, respectively, shown in Figure 4. The chromatographic peaks exhibited satisfactory symmetry with tailing factors below 1.4 and theoretical plate counts ranging from 3754 to 6725, indicating excellent chromatographic efficiency. Results are tabulated in Table 4.

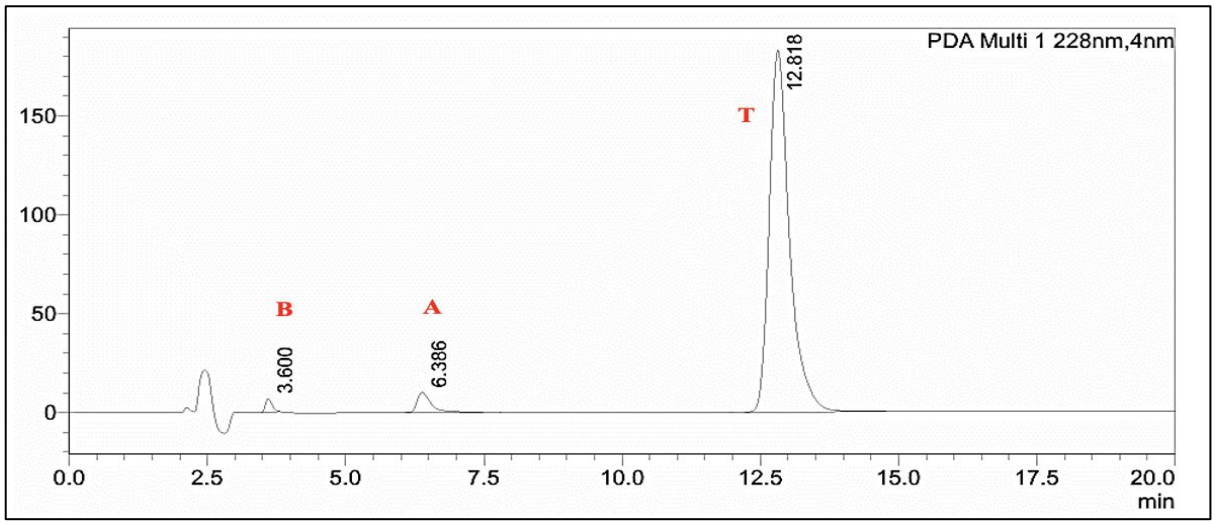
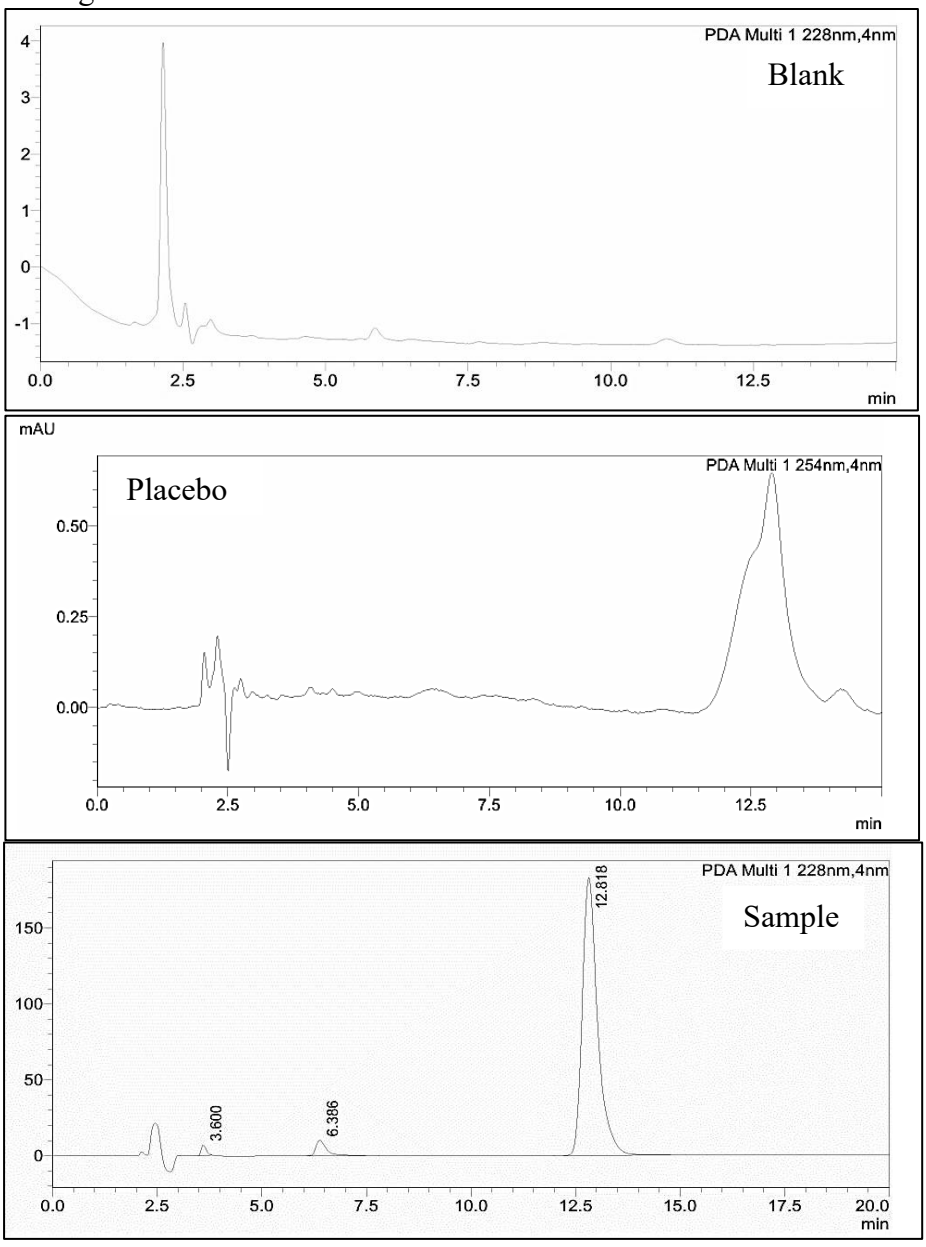


Figure 4: Optimized chromatogram showing separation of BIS, AML, and TEL.

Table 4: System Suitability Parameters of the Developed RP-HPLC.

Parameter	Telmisartan	Amlodipine Besylate	Bisoprolol Fumarate
Retention time (min)	12.63 ± 0.24	6.38 ± 0.12	3.56 ± 0.07
%RSD of retention time	1.93	1.93	1.83
Peak area	615856 ± 1265.81	127372.8 ± 1714.36	63451 ± 764.71
%RSD of peak area	0.21	1.35	1.21
Theoretical plates	6725	3754	4225
Tailing factor	1.4	1.2	1.3

Specificity was evaluated by analyzing blank, placebo, sample, and Standard preparations. No interfering peaks were observed at the retention times corresponding to bisoprolol fumarate, amlodipine besylate, and telmisartan, confirming excellent selectivity of the developed method. Refer Figure 5.



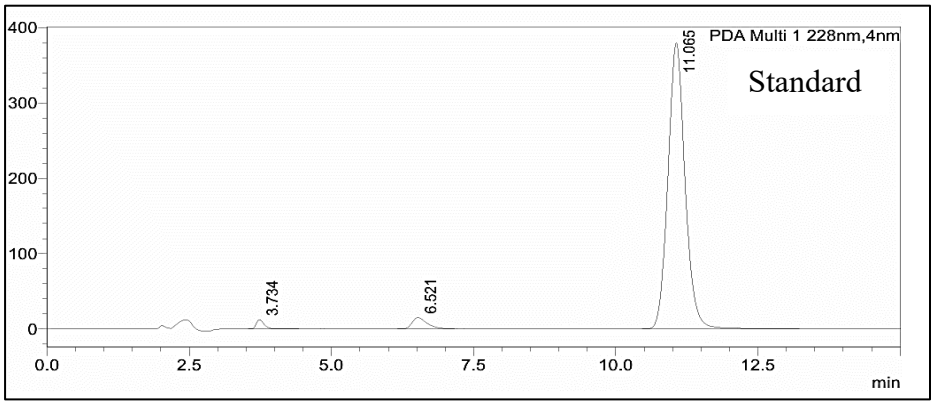


Figure 5: Chromatograms of blank, placebo, sample, and Standard mixture preparations.

Excellent linear relationships between concentration and peak area were observed over the concentration ranges of 1–4 µg mL^{-1} for bisoprolol fumarate, 2–8 µg mL^{-1} for amlodipine besylate, and 16–64 µg mL^{-1} for telmisartan presented in Figure 6.

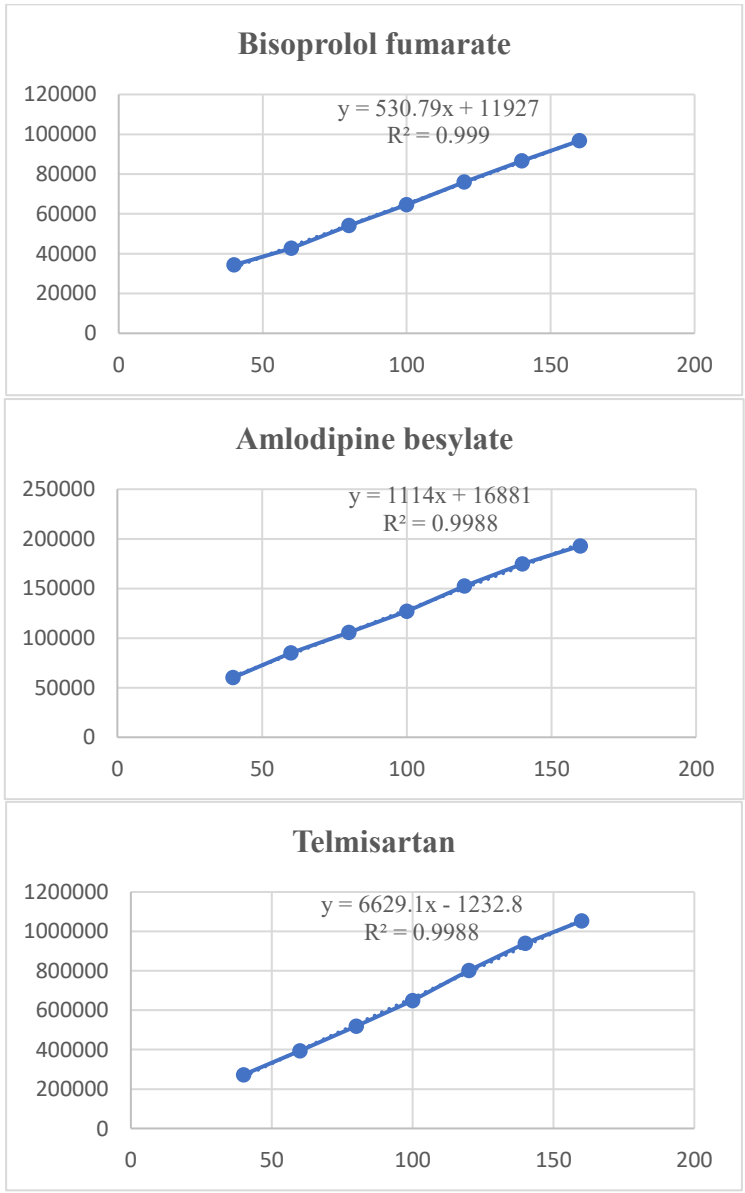


Figure 6. Calibration plots of bisoprolol fumarate, amlodipine besylate, and telmisartan.

The calibration curves exhibited good linearity with regression equations of y = 530.79x + 11927 for bisoprolol fumarate, y = 1114x + 16881 for amlodipine besylate, and y = 6629.1x − 1232.8 for telmisartan. The corresponding slope values were 530.79, 1114, and 6629.1, respectively, while the intercept values were 11927, 16881, and −1232.8. The correlation coefficients (R^2) obtained were 0.9990 for bisoprolol fumarate, 0.9988 for amlodipine besylate, and 0.9988 for telmisartan, indicating excellent linearity and proportionality between concentration and detector response throughout the studied ranges

The low LOD and LOQ values obtained for all analytes further confirmed the high sensitivity of the developed method and its suitability for quantitative analysis at low concentration levels.

Repeatability, intraday precision, and intermediate precision studies were performed to assess method reproducibility. Results are tabulated in Table 5. Percentage relative standard deviation values remained below **2%** for all analytes under all investigated conditions, demonstrating excellent repeatability and intermediate precision.

Table 5: Repeatability, intraday precision, and intermediate precision results.

Drug	Precision Parameter	Concentration (µg/mL)	Mean Peak Area ± SD	%RSD
Telmisartan	Repeatability	40	615856 ± 1265.81	0.21
	Intraday Precision	32	510234 ± 3797.21	0.74
		40	613519 ± 6428.19	1.04
		48	831505 ± 948.58	0.11
	Intermediate Precision	32	514863 ± 6451.62	1.25
		40	613520 ± 1673.47	0.27
		48	829020 ± 4797.63	0.58
Amlodipine Besylate	Repeatability	5	127373 ± 1714.36	1.35
	Intraday Precision	4	109299 ± 3440.19	0.03
		5	128018 ± 1613.00	1.26
		6	159693 ± 1716.56	1.07
	Intermediate Precision	4	110335 ± 3942.15	0.04
		5	127528 ± 2208.56	1.73
		6	154895 ± 1716.56	1.11
Bisoprolol Fumarate	Repeatability	2.5	63451 ± 764.71	1.21
	Intraday Precision	2	54327 ± 268.95	0.50
		2.5	63219 ± 222.27	0.35
		3	81596 ± 174.54	0.21
	Intermediate Precision	2	53973 ± 715.05	1.32
		2.5	62853 ± 437.51	0.70
		3	81159 ± 901.49	1.11

Accuracy was evaluated through recovery studies at 80%, 100%, and 120% concentration levels. Mean recoveries ranged from 99.55–101.11% for bisoprolol fumarate, 99.64–100.68% for amlodipine besylate, and 99.23–102.82% for telmisartan presented in Table 6. The obtained results complied with ICH acceptance criteria and confirmed the accuracy of the developed analytical procedure.

Table 6: Accuracy Study by Recovery Method

Drug	Recovery Level (%)	Initial Conc. (µg/mL)	Spiked Conc. (µg/mL)	Total Conc. (µg/mL)	Amount Found (µg/mL)	% Recovery ± SD (n=3)	%RSD
Telmisartan	80	40	32	72	32.90	102.82 ± 0.015	0.014
	100	40	40	80	39.69	99.24 ± 0.025	0.025
	120	40	48	88	47.63	99.23 ± 0.017	0.017
Amlodipine Besylate	80	5	4	9	3.98	99.73 ± 0.086	0.087
	100	5	5	10	5.03	100.68 ± 0.086	0.086
	120	5	6	11	5.97	99.64 ± 0.096	0.096
Bisoprolol Fumarate	80	2.5	2.0	4.5	1.99	99.55 ± 0.340	0.341
	100	2.5	2.5	5.0	2.52	101.11 ± 0.055	0.054
	120	2.5	3.0	5.5	2.98	99.51 ± 0.037	0.038

Robustness was investigated by introducing deliberate variations in chromatographic conditions, including flow rate, buffer pH, and mobile phase composition. No significant changes in chromatographic performance were observed, and percentage relative standard deviation values remained below **2%**, confirming the robustness and reliability of the method. Results are presented in Table 7.

Table 7: Robustness Study Results

Parameter Varied	Condition	Telmisartan (%RSD)	Amlodipine Besylate (%RSD)	Bisoprolol Fumarate (%RSD)
Flow rate	0.8 mL min^{-1}	0.18	0.41	0.24
	1.2 mL min^{-1}	0.20	0.51	0.20
ACN content	35%	0.13	0.53	0.26
	45%	0.10	0.20	0.14
Buffer pH	3.3	0.11	0.30	0.40
	3.7	0.09	0.86	0.35

Analysis of Synthetic Mixture

The developed RP-HPLC method was successfully applied to the analysis of a laboratory-prepared synthetic mixture containing telmisartan, amlodipine besylate, and bisoprolol fumarate. Assay values of 99.2%, 100.6%, and 101.1%, respectively, were obtained, demonstrating excellent agreement with the nominal concentrations. The low %RSD values (< 2.0%) confirmed the precision of the method, while the absence of interfering peaks established its selectivity. These findings highlight the suitability of the proposed method for routine quality control and quantitative analysis of the studied antihypertensive combination.

Greenness Assessment
The developed method achieved an AGREE score of 0.66 shown in Figure 7, indicating acceptable environmental performance. The favorable score was attributed to reduced solvent consumption, simple sample preparation, and efficient chromatographic conditions, demonstrating the suitability of the proposed method as an environmentally responsible analytical procedure.

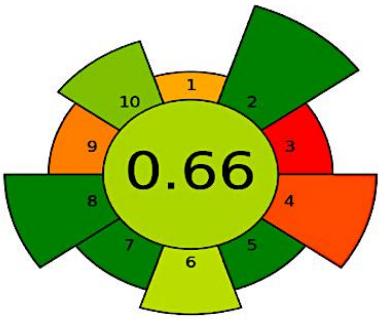


Figure 7: AGREE assessment pictogram.

CONCLUSION
A novel AQbD-assisted RP-HPLC method was successfully developed and validated for the simultaneous determination of telmisartan, amlodipine besylate, and bisoprolol fumarate. Application of the Box–Behnken Design enabled systematic optimization of critical chromatographic parameters and establishment of a robust analytical design space. The optimized method demonstrated excellent separation efficiency, linearity ($R^2 \geq 0.9988$), accuracy (99.23–102.82% recovery), precision (%RSD < 2%), sensitivity, specificity, and robustness in accordance with ICH Q2(R2) guidelines. Successful analysis of the synthetic mixture confirmed its applicability for routine pharmaceutical quality control. Furthermore, the AGREE score of 0.66 highlighted the acceptable environmental sustainability of the procedure. Overall, the integration of AQbD principles with green analytical chemistry provided a reliable, efficient, and environmentally responsible RP-HPLC method suitable for future pharmaceutical and industrial applications.

DECLARATIONS

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Availability of data and materials
The datasets generated or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests
The authors declare no competing interests.

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Author's contributions

Paresh Ghughuskar & Prexa Tandel performed the experimental work and drafted the manuscript. Ratna Musale & Dr. Hiralben Mehta supervised the study and critically revised the manuscript. All authors read and approved the final manuscript.

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REFERENCES

1. Whelton PK, Carey RM, Aronow WS, Casey DE Jr, Collins KJ, Dennison Himmelfarb C, et al. 2017 ACC/AHA guideline for the prevention, detection, evaluation, and management of high blood pressure in adults. *Hypertension*. 2018;71(6): e13–e115.
2. World Health Organization. *Hypertension*. Geneva: World Health Organization; 2023.
3. Williams B, Mancia G, Spiering W, Agabiti Rosei E, Azizi M, Burnier M, et al. 2018 ESC/ESH Guidelines for the management of arterial hypertension. *Eur Heart J*. 2018;39(33):3021–3104.
4. Unger T, Borghi C, Charchar F, Khan NA, Poulter NR, Prabhakaran D, et al. 2020 International Society of Hypertension Global Hypertension Practice Guidelines. *Hypertension*. 2020;75(6):1334–1357.
5. National Center for Biotechnology Information. PubChem Compound Summary for Telmisartan. Bethesda (MD): National Library of Medicine; 2025. Available from: https://pubchem.ncbi.nlm.nih.gov/compound/Telmisartan
6. National Center for Biotechnology Information. PubChem Compound Summary for Amlodipine. Bethesda (MD): National Library of Medicine; 2025. Available from: https://pubchem.ncbi.nlm.nih.gov/compound/Amlodipine
7. National Center for Biotechnology Information. PubChem Compound Summary for Bisoprolol. Bethesda (MD): National Library of Medicine; 2025. Available from: https://pubchem.ncbi.nlm.nih.gov/compound/Bisoprolol
8. Reid GL, Morgado J, Barnett K, Harrington B, Wang J, Harwood JW, et al. Analytical Quality by Design (AQbD) in pharmaceutical development. *Am Pharm Rev*. 2013;16(3):62–73.
9. Peraman R, Bhadraya K, Reddy YP. Analytical Quality by Design: A tool for regulatory flexibility and robust analytics. *Int J Anal Chem*. 2015;2015:868727.
10. Box GEP, Behnken DW. Some new three level designs for the study of quantitative variables. *Technometrics*. 1960;2(4):455–475.

11. Anastas PT, Warner JC. *Green Chemistry: Theory and Practice*. New York: Oxford University Press; 1998.
12. Płotka-Wasylka J. A new tool for the evaluation of analytical procedure: Green Analytical Procedure Index (GAPI). *Talanta*. 2018;181:204–209.
13. Gałuszka A, Migaszewski ZM, Konieczka P, Namieśnik J. Analytical Eco-Scale for assessing the greenness of analytical procedures. *TrAC Trends Anal Chem*. 2012;37:61–72.
14. Pena-Pereira F, Wojnowski W, Tobiszewski M. AGREE—Analytical GREEnness metric approach and software. *Anal Chem*. 2020;92(14):10076–10082.
15. Sultana N, Arayne MS, Ali SN. Simultaneous determination of amlodipine and telmisartan by RP-HPLC. *J Chromatogr Sci*. 2013;51(5):463–470.
16. Chitlange SS, Imran M, Sakarkar DM. Development and validation of RP-HPLC method for estimation of telmisartan in pharmaceutical dosage forms. *J Pharm Biomed Anal*. 2008;48(2):525–528.
17. Patel A, Patel M, Shah D. Development and validation of RP-HPLC method for bisoprolol fumarate and related substances. *Int J Pharm Sci Res*. 2021;12(5):2654–2661.
18. International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH). *ICH Harmonised Guideline Q2(R2): Validation of Analytical Procedures*. Geneva: ICH; 2023.
19. United States Pharmacopeial Convention. *United States Pharmacopeia and National Formulary (USP–NF)*. General Chapter <621> Chromatography. Rockville (MD): United States Pharmacopeial Convention; 2024.