

AQbD-assisted Hptlc Method for Simultaneous Determination of Bisoprolol Fumarate and Perindopril Arginine: Optimization, Validation and Comprehensive Sustainability Assessment

Ratna Musale

Gujarat Technological University

Dewoo Patil

Parul University Limda

Hiralben Mehta

`hiralben.mehta29533@paruluniversity.ac.in`

Parul University

Prexa Tandel

Parul University Limda

Research Article

Keywords: Analytical Quality by Design, High-performance thin-layer chromatography, Bisoprolol fumarate, Perindopril arginine, Box–Behnken design, Green Method, White analytical chemistry, Analytical sustainability

Posted Date: September 1st, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-10675369/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Additional Declarations: No competing interests reported.

AQbD-ASSISTED HPTLC METHOD FOR SIMULTANEOUS DETERMINATION OF BISOPROLOL FUMARATE AND PERINDOPRIL ARGININE: OPTIMIZATION, VALIDATION AND COMPREHENSIVE SUSTAINABILITY ASSESSMENT

Ratna Musale¹, Dewoo Patil², Hiralben Mehta^{3*}, Prexa Tandel²

¹Research Scholar, Gujarat Technological University, Chankheda, Ahmedabad, 382424, Gujarat, India

²Research Scholar, Department of Quality Assurance, Parul institute of Pharmacy and Research, Parul University Limda, Vadodara 391760, Gujarat, India

^{3*}Assistant Professor, Department of Quality Assurance, Parul institute of Pharmacy and Research, Parul University Limda, Vadodara 391760, Gujarat, India

Address for correspondence:

Dr. Hiralben Mehta
HOD Pharmaceutical Analysis,
Department of Quality Assurance,
Parul Institute of Pharmacy and Research,
Parul University, Vadodara, Gujarat, India
E-mail: hiralben.mehta29533@paruluniversity.ac.in
Tel: +919033388478

ABSTRACT

Background: The development of robust analytical methods is essential to ensure the quality, safety, and therapeutic efficacy of fixed-dose combination (FDC) pharmaceutical products.

Objective: In the present study, an Analytical Quality-by-Design (AQbD)-assisted high-performance thin-layer chromatographic (HPTLC) method was developed and validated for the simultaneous determination of bisoprolol fumarate (BIS) and perindopril arginine (PER) in tablet formulations.

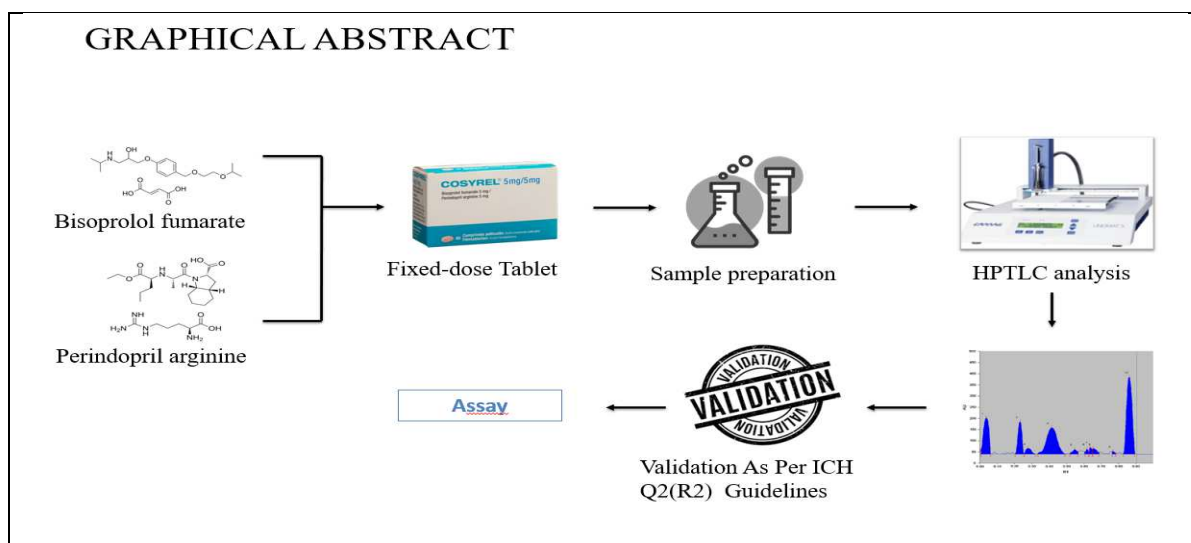
Method: Critical method parameters affecting chromatographic performance were systematically optimized using a three-factor, three-level Box–Behnken experimental design to establish a scientifically justified analytical design space. The developed method was validated according to the International Council for Harmonisation (ICH Q2(R2)) guideline for specificity, linearity, accuracy, precision, robustness, detection capability, quantitation capability, and assay.

Results: Under the optimized chromatographic conditions, BIS and PER were well resolved with compact and symmetrical peaks, demonstrating excellent chromatographic performance. The method exhibited satisfactory linearity over the selected concentration ranges with high correlation coefficients, while recovery, precision, and robustness studies confirmed its reliability and reproducibility. Application of the optimized method to a marketed tablet formulation demonstrated accurate quantification of both analytes without interference from excipients. The developed method also demonstrated excellent analytical sustainability, achieving an AGREE score of 0.71, an Analytical Eco-Scale score of 81, and favourable GAPI and BAGI assessments. Furthermore, the RGB 12 evaluation yielded Redness, Greenness, and Blueness scores of 98.8%, 82.5%, and 89.6%, respectively, with an overall Method Brilliance score of 90.3%.

Conclusion: The integration of AQbD principles with HPTLC provided enhanced method understanding, improved robustness, and reduced experimental variability, demonstrating the suitability of the proposed method for routine quality control and regulatory applications in pharmaceutical analysis, confirming an excellent balance between analytical performance, environmental sustainability, and practical applicability.

Keywords: Analytical Quality by Design; High-performance thin-layer chromatography; Bisoprolol fumarate; Perindopril arginine; Box–Behnken design; Green Method, White analytical chemistry, Analytical sustainability

GRAPHICAL ABSTRACT



Abbreviations:

ACE	Angiotensin-Converting Enzyme
AGREE	Analytical GREENness Metric
ANOVA	Analysis of Variance
AQbD	Analytical Quality-by-Design
AR	Analytical Reagent
ATP	Analytical Target Profile
BAGI	Blue Applicability Grade Index
BBD	Box–Behnken Design
BIS	Bisoprolol Fumarate
CAA	Critical Analytical Attribute
CMP	Critical Method Parameter
FDC	Fixed-Dose Combination
FMEA	Failure Mode and Effects Analysis
GAC	Green Analytical Chemistry
GAPI	Green Analytical Procedure Index
HPLC	High-Performance Liquid Chromatography
HPTLC	High-Performance Thin-Layer Chromatography
ICH	International Council for Harmonisation
LC–MS	Liquid Chromatography–Mass Spectrometry
LOD	Limit of Detection

LOQ	Limit of Quantitation
MB	Method Brilliance
MODR	Method Operable Design Region
PER	Perindopril Arginine
R _f	Retention Factor
RGB	Red–Green–Blue (model)
RSD	Relative Standard Deviation
SD	Standard Deviation
TLC	Thin-Layer Chromatography
UPLC	Ultra-Performance Liquid Chromatography
UV	Ultraviolet
WAC	White Analytical Chemistry

INTRODUCTION

Hypertension is one of the most prevalent chronic non-communicable diseases and remains a major contributor to cardiovascular morbidity and mortality worldwide. Persistent elevation of arterial blood pressure significantly increases the risk of myocardial infarction, stroke, heart failure, chronic kidney disease, and other cardiovascular complications.^[1-3] Despite the availability of several antihypertensive agents, achieving optimal blood pressure control often requires combination therapy involving drugs with complementary mechanisms of action.^[1-3] Fixed-dose combination (FDC) products have therefore become an integral component of modern hypertension management because they improve therapeutic efficacy, reduce pill burden, enhance patient adherence, and minimize dose-related adverse effects.^[4,5]

Among the available antihypertensive combinations, bisoprolol fumarate (BIS) and perindopril arginine (PER) represent a clinically important therapeutic option. Bisoprolol fumarate is a highly selective β adrenergic receptor blocker that reduces heart rate, myocardial contractility, and cardiac output, thereby lowering blood pressure and myocardial oxygen demand. Perindopril arginine is a long-acting angiotensin-converting enzyme (ACE) inhibitor that is hydrolyzed to the active metabolite perindoprilat, producing vasodilation through inhibition of the renin–angiotensin–aldosterone system.^[6,7] The complementary pharmacological actions of these drugs provide superior cardiovascular protection compared with monotherapy and are widely employed in the long-term management of hypertension and associated cardiovascular disorders. Chemical structures of (A) bisoprolol fumarate and (B) perindopril arginine shown in Figure 1.^[8,9]

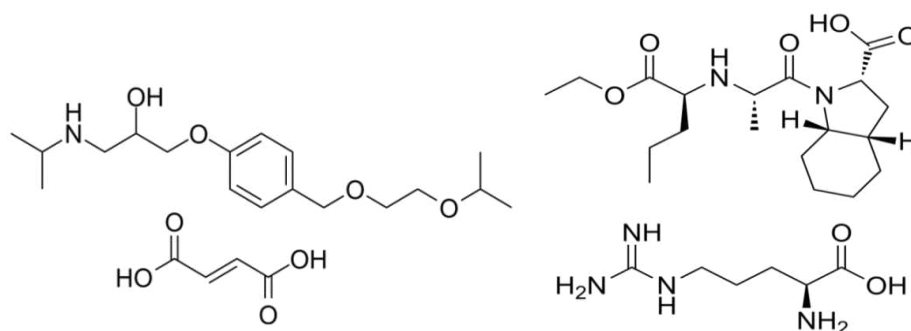


Figure 1. Chemical structures of (A) bisoprolol fumarate and (B) perindopril arginine.

Reliable analytical methods are essential throughout the pharmaceutical product lifecycle to ensure dosage accuracy, batch-to-batch consistency, regulatory compliance, and patient safety. Various analytical techniques, including UV spectrophotometry, high-performance liquid chromatography (HPLC), ultra-performance liquid chromatography (UPLC), liquid chromatography mass spectrometry (LC–MS), and high-performance thin-layer chromatography (HPTLC), have been reported for the estimation of bisoprolol fumarate, perindopril arginine, or their combinations.^[10-14] Although liquid chromatographic techniques provide excellent sensitivity and selectivity, they are frequently associated with higher solvent consumption, increased operational costs, longer analysis times, and lower sample throughput. Conversely, HPTLC offers several advantages, including simultaneous analysis of multiple samples, minimal solvent usage, shorter analytical time, cost-effectiveness, and suitability for routine pharmaceutical quality control.

Recent regulatory expectations have encouraged the implementation of Analytical Quality-by-Design (AQbD) principles for analytical method development.^[15,16] AQbD adopts a systematic, science-based, and risk-oriented strategy that emphasizes predefined analytical objectives, identification of critical method parameters (CMPs), understanding of critical analytical attributes (CAAs), experimental design, and lifecycle management.^[17,18] Compared

with conventional one-factor-at-a-time optimization, AQbD enables comprehensive evaluation of factor interactions and establishes a robust analytical design space that enhances method reliability, reproducibility, and regulatory flexibility.

Response surface methodologies, particularly the Box–Behnken Design (BBD), have become valuable statistical tools for chromatographic optimization because they efficiently evaluate the individual and interaction effects of experimental variables while minimizing the number of experimental trials. Such approaches improve analytical robustness and provide a deeper understanding of method performance, thereby facilitating the development of reproducible and reliable chromatographic methods.^[19]

In recent years, Green Analytical Chemistry (GAC) has gained considerable attention by promoting analytical methods that minimize the use of hazardous chemicals, solvent consumption, waste generation, and energy requirements without compromising analytical performance. To evaluate the environmental sustainability of analytical procedures, established greenness assessment tools such as the Analytical Eco-Scale, and Analytical GREENness Metric (AGREE) are widely employed. These complementary metrics provide a comprehensive assessment of the environmental profile of analytical methods. White Analytical Chemistry (WAC) is a holistic approach that aims to achieve an optimal balance between analytical performance, environmental sustainability, and practical applicability. In the present study, the RGB 12 model was employed to assess the developed AQbD-assisted HPTLC method according to the principles of WAC.

Although several analytical procedures have been reported for the determination of bisoprolol fumarate and perindopril arginine, systematic application of AQbD principles to the development and optimization of an HPTLC method for their simultaneous estimation remains limited. Furthermore, many reported methods rely on empirical optimization strategies without establishing a statistically validated analytical design space or comprehensively evaluating method robustness in accordance with current regulatory expectations.

Therefore, the present study aimed to develop a simple, rapid, economical, and robust AQbD-assisted HPTLC method for the simultaneous determination of bisoprolol fumarate and perindopril arginine in pharmaceutical tablet formulations. A three-factor, three-level Box–Behnken experimental design was employed to optimize the chromatographic conditions by investigating the influence of critical method parameters on chromatographic responses. The optimized method was subsequently validated according to the International Council for Harmonisation (ICH Q2(R2)) guideline^[20] with respect to specificity, linearity, accuracy, precision, robustness, detection capability, quantitation capability, and assay. The developed method is intended to provide a reliable, reproducible, and cost-effective analytical platform for routine pharmaceutical quality control and regulatory applications.

MATERIALS AND METHODS

Chemicals and reagents

Bisoprolol fumarate (BIS) and perindopril arginine (PER) reference standards were obtained from Zydus Pharmaceuticals (Ahmedabad, India) and Globela Pharma (Surat, India), respectively. A commercially available fixed-dose combination tablet containing 5 mg of BIS and 5 mg of PER (Cosyrel®, Servier India Pvt. Ltd., India) was purchased from the local market and used for assay studies. Analytical reagent (AR)-grade methanol was procured from Rankem Chemicals (India), whereas chloroform and ethanol were purchased from Merck India Pvt. Ltd. (Mumbai, India). Glacial acetic acid and ammonia solution were obtained from Prakash Chemicals (India). All chemicals and solvents employed throughout the study were of analytical reagent grade and used without further purification.

Instrumentation

Chromatographic analysis was carried out using a CAMAG high-performance thin-layer chromatography (HPTLC) system (Muttentz, Switzerland) comprising a Linomat V automatic sample applicator, twin-trough glass development chamber, TLC Scanner IV, and VisionCATS software for instrument control, data acquisition, and densitometric analysis. Chromatographic separation was performed on precoated aluminium-backed silica gel 60 F254 HPTLC plates (20 cm × 10 cm; Merck, Darmstadt, Germany). Samples were applied as bands using a nitrogen-assisted spray system to ensure uniform sample deposition. Ultraviolet spectral measurements were performed using a double-beam UV–Visible spectrophotometer equipped with matched 1 cm quartz cells for wavelength selection. Routine sample preparation involved an ultrasonic bath to facilitate complete dissolution of the analytes, while all weighing operations were carried out using a calibrated analytical balance with a readability of 0.1 mg.

Selection of detection wavelength

Selection of an appropriate detection wavelength is essential for obtaining adequate sensitivity during densitometric analysis. Individual working solutions of BIS and PER were prepared in methanol and scanned over the wavelength range of 200–400 nm using the UV–Visible spectrophotometer. The absorption spectra of both analytes were subsequently superimposed to identify a wavelength that provided sufficient absorbance for simultaneous determination without compromising analytical sensitivity. Based on the spectral overlap and signal intensity, 219 nm was selected as the optimum wavelength for densitometric scanning throughout the study.

Preparation of standard and sample solutions

Primary stock solutions of BIS and PER were prepared separately by accurately weighing 10 mg of each reference standard into individual 10 mL volumetric flasks. The contents were dissolved in methanol with intermittent sonication to ensure complete dissolution and diluted to volume with the same solvent to obtain stock solutions containing 1000 µg mL⁻¹ of each analyte. Working standard solutions required for chromatographic optimization, calibration, and validation studies were prepared by appropriate dilution of the stock solutions with methanol immediately before analysis.

For analysis of the pharmaceutical formulation, twenty tablets were accurately weighed and finely powdered to obtain a homogeneous mixture. An amount of powder equivalent to the labelled content of one tablet was transferred into a volumetric flask containing methanol and sonicated for 20 min to achieve complete extraction of both active pharmaceutical ingredients. The extract was cooled to room temperature and diluted to volume with methanol. The resulting solution was filtered through Whatman No. 41 filter paper to remove insoluble excipients, and suitable dilutions were prepared with methanol to obtain the required analytical concentration prior to HPTLC analysis.

Analytical Target Profile (ATP)

An Analytical Target Profile (ATP) was established at the beginning of method development following the principles of Analytical Quality-by-Design (AQbD). The ATP defined the desired analytical performance and guided the systematic development of an HPTLC method for the simultaneous estimation of bisoprolol fumarate and perindopril arginine in pharmaceutical tablets. The method was designed to provide reliable chromatographic separation with acceptable retention factors, adequate resolution (>1.5), and compliance with ICH Q2(R2) requirements. In addition, emphasis was placed on minimizing solvent consumption and analysis time to develop a cost-effective method suitable for routine quality control (Table 1).

Table 1. Analytical Target Profile (ATP)

ATP Element	Target Requirement	Justification
Analytical technique	HPTLC	Rapid, economical and suitable for routine quality control
Analytes	Bisoprolol fumarate and Perindopril arginine	Simultaneous estimation in fixed-dose combination tablets
Sample matrix	Pharmaceutical tablet dosage form	Routine quality control application
Analytical objective	Simultaneous quantitative estimation	Accurate determination of both analytes
Specificity	No interference from excipients or solvent	Reliable identification and quantification
Chromatographic separation	Resolution (R_s) > 1.5	Complete separation of analytes
Retention factor (R_f)	BIS: 0.30–0.40; PER: 0.60–0.70	Appropriate migration and reproducibility
Accuracy	98–102% recovery	Compliance with ICH Q2(R2)
Precision	%RSD < 2.0	Repeatability and intermediate precision
Linearity	$R^2 \geq 0.99$	Reliable quantitative performance
Robustness	No significant effect after deliberate variation	Consistent analytical performance
Greenness objective	Reduced solvent consumption and analysis time	Sustainable analytical procedure

Knowledge Management

Prior knowledge from published literature, the physicochemical properties of the analytes, and preliminary laboratory experiments was used to support method development. Parameters such as analyte polarity, solubility, and chromatographic behaviour on silica gel were considered during the selection of the mobile phase, stationary phase, and detection wavelength. Initial trial experiments helped identify the chromatographic variables that significantly influenced method performance and provided the basis for selecting the critical method parameters (CMPs) for subsequent optimization using the Box–Behnken design (Table 2).

Table 2. Knowledge Management Summary

Knowledge Source	Observation	Impact on Method Development
Literature review	Limited HPTLC methods reported for BIS–PER combination	Necessitated development of a new method
Physicochemical properties	Both drugs exhibit moderate polarity	Guided mobile phase selection

Preliminary chromatographic trials	Different solvent combinations evaluated	Chloroform–ethanol–glacial acetic acid selected
Stationary phase	Silica gel 60 F254	Provided satisfactory separation
Detection wavelength	UV spectra overlapped at 219 nm	Selected for simultaneous densitometric analysis
Mobile phase optimization	Chloroform ratio strongly affected migration	Identified as a Critical Method Parameter
Chamber saturation	Influenced chromatographic resolution	Included in optimization study
Experimental design	Response Surface Methodology	Box–Behnken Design selected for optimization

Analytical Quality-by-Design (AQbD)-Assisted Method Development

The chromatographic method was developed using the Analytical Quality-by-Design (AQbD) approach, which provides a systematic and risk-based strategy for analytical method development. Based on the predefined Analytical Target Profile (ATP) and quality risk assessment, three Critical Method Parameters (CMPs)—chloroform volume (Factor A), glacial acetic acid volume (Factor B), and chamber saturation time (Factor C)—were selected for optimization, while the ethanol content was kept constant. The Critical Analytical Attributes (CAAs), namely the retention factors (R_f) of bisoprolol fumarate and perindopril arginine and the chromatographic resolution between the analytes, were chosen as the analytical responses because they are essential for achieving reliable separation and accurate quantification (Table 3) (Figure 2).

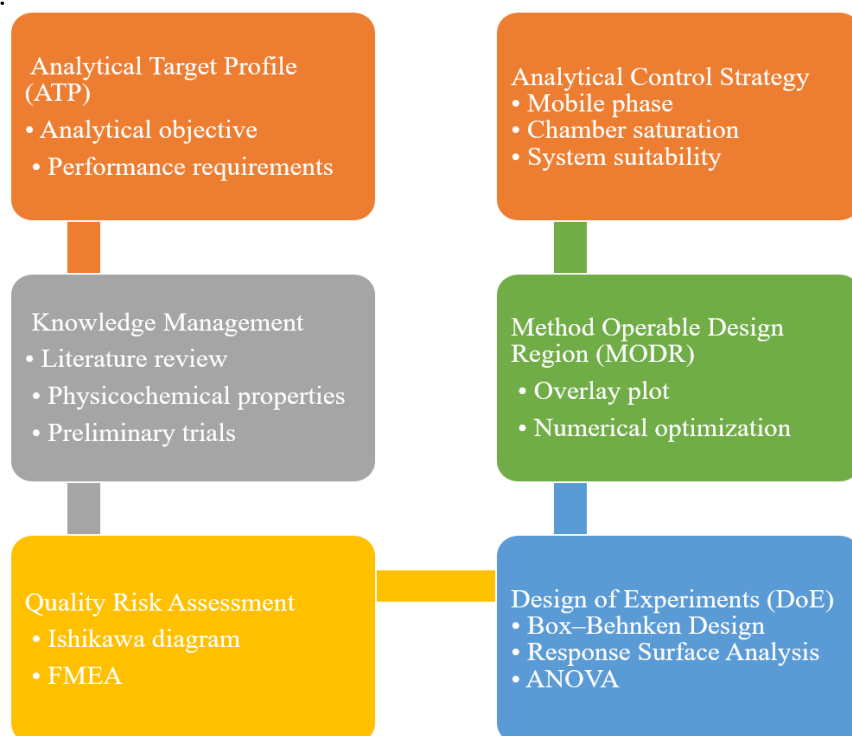


Figure 2. Analytical Quality-by-Design (AQbD) workflow adopted for HPTLC method development.

Table 3. Critical Method Parameters (CMPs) and Critical Analytical Attributes (CAAs)

Category	Parameter	Role in AQbD	Outcome
Critical Method Parameter (CMP)	Volume of chloroform (A)	Controls analyte migration and separation	Optimized using BBD
	Volume of glacial acetic acid (B)	Improves peak symmetry and selectivity	Optimized using BBD
	Chamber saturation time (C)	Controls solvent equilibration and reproducibility	Optimized using BBD
Constant Parameter	Ethanol volume	Maintained constant	Fixed during optimization
	Stationary phase	Silica gel 60 F254	Constant
	Development distance	80 mm	Constant
	Detection wavelength	219 nm	Constant
Critical Analytical Attribute (CAA)	R_f of Bisoprolol	Peak identification	Response (Y_1)
	R_f of Perindopril	Peak identification	Response (Y_2)
	Chromatographic Resolution	Peak separation	Response (Y_3)

Quality Risk Assessment

A systematic quality risk assessment was performed in accordance with ICH Q9(R1) to identify the factors that could influence chromatographic performance. An Ishikawa (fishbone) diagram was first used to identify potential sources of analytical variability, followed by Failure Mode and Effects Analysis (FMEA) to prioritize the risks based on severity, occurrence, and detectability (Figure 3). The assessment identified the volume of chloroform, glacial acetic acid, and chamber saturation time as the most critical method parameters. These variables were therefore selected for optimization using the Box–Behnken design, while the remaining parameters were kept constant throughout the study (Table 4).

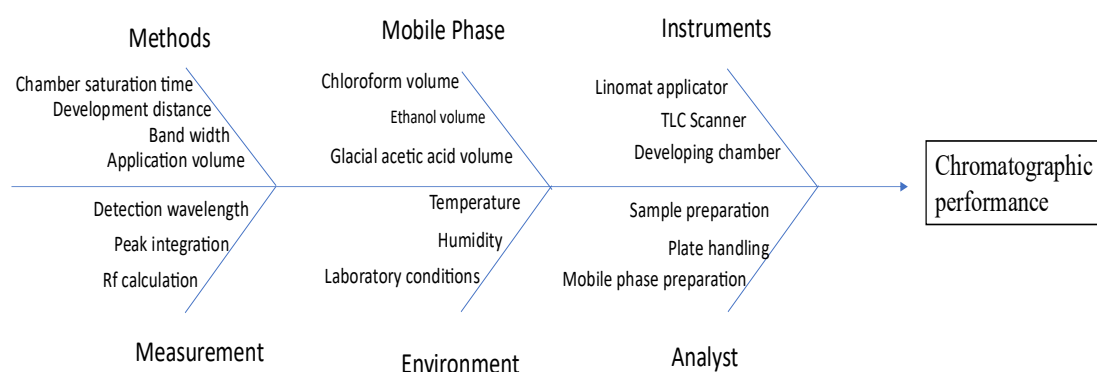
**Figure 3: Ishikawa Diagram**

Table 4. Failure Mode and Effects Analysis (FMEA) for Risk Assessment

Method Parameter	Possible Effect	Severity (S)	Occurrence (O)	Detectability (D)	RPN (S × O × D)	Risk Level
Volume of chloroform	Poor separation, shifted R_f	5	5	3	75	High
Volume of glacial acetic acid	Peak tailing, reduced selectivity	4	4	3	48	High
Chamber saturation time	Variation in migration and resolution	5	4	2	40	High
Ethanol volume	Minor change in migration	3	2	3	18	Medium
Development distance	Small variation in R_f	3	2	2	12	Low
Detection wavelength	Variation in sensitivity	3	2	2	12	Low
Band width	Minor effect on peak shape	2	2	2	8	Low
Sample application volume	Minor effect on peak intensity	2	2	2	8	Low

Risk Classification: RPN ≥ 40: High Risk, RPN 20–39: Medium Risk, RPN < 20: Low Risk

Experimental design and statistical optimization

Optimization of the chromatographic conditions was carried out using a three-factor, three-level Box–Behnken Design (BBD) generated with Design-Expert® software (Stat-Ease Inc., Minneapolis, MN, USA). The three independent variables were evaluated at three coded levels (−1, 0, and +1), resulting in 17 experimental runs, including replicated center points for estimation of experimental error. The experimental responses were fitted to second-order polynomial models, and analysis of variance (ANOVA) was used to evaluate model significance and adequacy. Response surface and contour plots were generated to investigate the effects of individual variables and their interactions on chromatographic performance. Numerical optimization based on the desirability function approach was subsequently performed to identify the optimum chromatographic conditions that provided suitable R_f values and maximum chromatographic resolution.

Optimized chromatographic conditions

Chromatographic separation was performed on precoated silica gel 60 F₂₅₄ aluminium plates using a mobile phase consisting of chloroform: ethanol: glacial acetic acid (7.3:2.4:0.29, v/v/v). The development chamber was saturated with the mobile phase for 22 min before plate development. Standard and sample solutions were applied as 8 mm bands with an application volume of 10 µL, followed by densitometric scanning at 219 nm. Under the optimized conditions, bisoprolol fumarate and perindopril arginine were well resolved with mean R_f values of 0.339 and 0.650, respectively, demonstrating satisfactory chromatographic performance for subsequent validation studies.

Method validation

The optimized HPTLC method was validated in accordance with the recommendations of the International Council for Harmonisation (ICH) Q2(R2) ^[20] guideline to establish its suitability for the simultaneous quantitative determination of bisoprolol fumarate (BIS) and perindopril arginine (PER) in pharmaceutical formulations. Validation parameters included specificity, system suitability, linearity, accuracy, precision, robustness, detection capability, quantitation capability, and assay.

Specificity

Method specificity was evaluated by comparing chromatograms obtained from blank solvent, standard solutions, and sample preparations developed under identical chromatographic conditions. The chromatograms were examined to verify complete separation of BIS and PER and to confirm the absence of interfering peaks arising from excipients, solvents, or other matrix components at the corresponding retention factors.

System suitability

System suitability was assessed before sample analysis to verify the performance of the chromatographic system. A mixed standard solution containing 20 µg band⁻¹ of each analyte was applied in five replicate bands under the optimized chromatographic conditions. The retention factor (R_f) and peak area of each replicate were recorded, and the mean, standard deviation (SD), and percentage relative standard deviation (%RSD) were calculated to evaluate system repeatability ^[23].

Linearity

The linearity of the method was established by analysing five concentration levels ranging from 10 to 30 µg band⁻¹ for both BIS and PER. Each concentration level was analysed in triplicate. Calibration curves were constructed by plotting peak area against analyte concentration, and linear regression analysis was performed to determine the regression equation and correlation coefficient (R^2).

Accuracy

Method accuracy was determined using the standard addition technique at 80%, 100%, and 120% of the nominal assay concentration. Known amounts of BIS and PER reference standards were added to the pre-analysed sample solution, and each concentration level was analysed in triplicate. Accuracy was expressed as percentage recovery together with the corresponding %RSD.

Precision

Method precision was evaluated in terms of repeatability and intermediate precision.

Repeatability was determined by analysing five independently prepared replicate applications containing 20 µg band⁻¹ of each analyte under identical experimental conditions. Intermediate precision was assessed by determining intra-day and inter-day precision at three concentration levels (10, 20, and 30 µg band⁻¹). Intra-day precision was evaluated by analysing the samples at three different time intervals within the same day, whereas inter-day precision was determined by analysing the samples on three consecutive days. Precision was expressed as %RSD of the measured peak areas.

Robustness

The robustness of the developed method was investigated by introducing small deliberate variations in selected chromatographic parameters while maintaining all other conditions constant. The influence of changes in chamber saturation time (±2 min) and detection wavelength (±2 nm) on chromatographic performance was evaluated using standard solutions containing 20 µg band⁻¹ of each analyte. The robustness of the method was assessed by calculating the corresponding %RSD values.

Detection capability and quantitation capability

The detection capability (limit of detection, LOD) and quantitation capability (limit of quantitation, LOQ) of the proposed method were estimated from the calibration data according to the recommendations of ICH Q2(R2). The values were calculated using the standard deviation of the response (σ) and the slope (S) of the calibration curve according to the following equations^[20]:

$$\text{LOD} = 3.3 \times (\sigma/S)$$

$$\text{LOQ} = 10 \times (\sigma/S)$$

Assay of marketed formulation

The validated method was applied to the quantitative estimation of BIS and PER in the marketed tablet formulation. For analysis of the pharmaceutical formulation, twenty tablets were accurately weighed and finely powdered to obtain a homogeneous mixture. An amount of powder equivalent to the labelled content of one tablet was transferred into a volumetric flask containing methanol and sonicated for 20 min to achieve complete extraction of both active pharmaceutical ingredients. The extract was cooled to room temperature and diluted to volume with methanol. The resulting solution was filtered through Whatman No. 41 filter paper to remove insoluble excipients, and suitable dilutions were prepared with methanol to obtain the required analytical concentration prior to HPTLC analysis.

Statistical analysis

The experimental design and numerical optimization were performed using Design-Expert® software (Stat-Ease Inc., Minneapolis, MN, USA). Response surface methodology was employed to evaluate the effects of the selected critical method parameters on the chromatographic responses. Analysis of variance (ANOVA) was used to assess the statistical significance and adequacy of the developed polynomial models. Validation data were analysed using Microsoft Excel 2021 (Microsoft Corporation, Redmond, WA, USA) and expressed as the mean ± standard deviation (SD). Precision was evaluated using the percentage relative standard deviation (%RSD), while regression analysis was used to determine calibration equations and correlation coefficients. Statistical significance was considered at $p < 0.05$.

Assessment of Analytical Sustainability and Practical Applicability

The environmental sustainability of the developed AQbD-assisted HPTLC method was evaluated using the Analytical Eco-Scale^[24], Analytical GREENness (AGREE) metrics^[25],

GAPI^[26], BAGI^[27] and the RGB model^[28,29]. The Analytical Eco-Scale was calculated by assigning penalty points based on the chemicals used, solvent consumption, energy requirements, waste generation, and occupational hazards. The AGREE metric was used to evaluate the method according to the twelve principles of Green Analytical Chemistry. These complementary metrics enabled the method to be assessed from multiple perspectives, including analytical performance, environmental friendliness, and practical applicability, providing a balanced evaluation of its overall sustainability.

RESULT & DISCUSSION

Selection of Detection Wavelength

The detection wavelength was optimized by recording the UV absorption spectra of bisoprolol fumarate (BIS) and perindopril arginine (PER) over the wavelength range of 200–400 nm. Superimposition of the spectra showed that both analytes exhibited adequate absorbance at 219 nm, providing maximum analytical sensitivity with minimal baseline interference. Consequently, 219 nm was selected as the optimum densitometric scanning wavelength for simultaneous estimation of both drugs throughout the study.

Analytical Target Profile and Risk Assessment

The Analytical Target Profile established before experimentation served as the predefined analytical objective throughout method development. The ATP emphasized simultaneous determination of both analytes with acceptable chromatographic separation, high analytical precision, robustness, and suitability for routine pharmaceutical quality control. Knowledge management involving literature review and preliminary chromatographic experiments identified several chromatographic variables that could potentially influence analytical performance. Subsequent quality risk assessment using Ishikawa analysis and Failure Mode and Effects Analysis ranked mobile phase composition and chamber saturation time as the highest-risk variables affecting chromatographic performance. Consequently, these parameters were selected as Critical Method Parameters for further optimization using response surface methodology.

AQbD-Based Method Development

An Analytical Quality by Design (AQbD) strategy was adopted to systematically develop and optimize a robust HPTLC method for the simultaneous estimation of bisoprolol fumarate and perindopril arginine. Preliminary chromatographic investigations identified three critical method parameters (CMPs), namely chloroform volume in the mobile phase (A), glacial acetic acid (GAA) volume (B), and chamber saturation time (C), as the variables having the greatest influence on chromatographic performance. The investigated experimental factors and their corresponding levels are summarized in Table 5.

Table 5. Independent variables and analytical responses employed in the Box–Behnken experimental design

Factor	Independent variable	Symbol	Unit	Low (−1)	Centre (0)	High (+1)
A	Chloroform in mobile phase	A	mL	7.30	7.50	7.70
B	Glacial acetic acid	B	mL	0.20	0.25	0.30
C	Chamber saturation time	C	min	20	25	30

Analytical responses

Response	Analytical attribute
R1	R _f value of bisoprolol fumarate
R2	R _f value of perindopril arginine

A three-factor, three-level Box–Behnken design (BBD) consisting of fifteen experimental runs was employed to investigate the individual and interaction effects of the selected chromatographic variables on the critical analytical attributes (CAAs). The experimental design enabled systematic evaluation of chromatographic performance while minimizing the number of experimental trials required. Representative chromatograms obtained during the optimization study are shown in Figure 4.

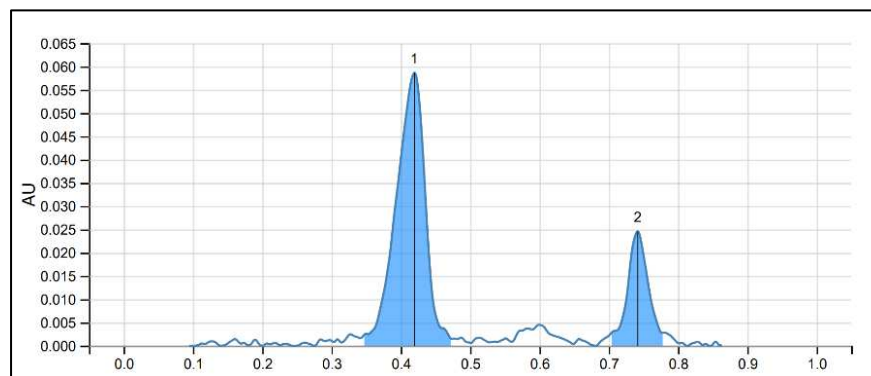


Figure 4. Representative HPTLC chromatograms obtained during the Box–Behnken experimental runs.

Experimental Design and Chromatographic Responses

The chromatographic responses obtained from the fifteen experimental runs demonstrated that changes in mobile phase composition and chamber saturation time markedly influenced analyte migration and chromatographic separation. The observed retention factors of both analytes and the corresponding chromatographic resolution varied considerably across the investigated design space, confirming that the selected experimental region was appropriate for optimization. The complete experimental design together with the observed responses is presented in Table 6.

Table 6. Experimental design matrix and observed chromatographic responses

Run	Chloroform (mL)	GAA (mL)	Saturation time (min)	R_f (BIS)	R_f (PER)	Resolution
1	7.30	0.30	25	0.399	0.685	2.00
2	7.70	0.30	25	0.419	0.774	2.49
3	7.50	0.20	30	0.665	0.754	0.62
4	7.30	0.25	20	0.411	0.665	1.78
5	7.70	0.20	25	0.339	0.650	2.18
6	7.70	0.25	20	0.493	0.678	1.30
7	7.70	0.25	30	0.678	0.695	0.12
8	7.50	0.30	30	0.658	0.745	0.61
9	7.30	0.20	25	0.563	0.731	1.18
10	7.30	0.25	30	0.686	0.672	0.10
11	7.50	0.20	20	0.425	0.744	2.23
12	7.50	0.30	20	0.421	0.685	1.85
13	7.50	0.25	25	0.415	0.775	2.50
14	7.50	0.25	25	0.400	0.790	2.75
15	7.50	0.25	25	0.385	0.805	3.00

The experimental responses indicated that increasing the proportion of chloroform generally enhanced analyte migration, whereas chamber saturation time played a crucial role in establishing solvent equilibrium and achieving improved chromatographic resolution. These findings confirmed that simultaneous optimization of the selected chromatographic variables was essential for obtaining reproducible and well-resolved chromatographic peaks suitable for quantitative analysis.

Statistical Modelling and ANOVA

The experimental responses generated from the Box–Behnken design were fitted to quadratic polynomial models using Design-Expert® software. The adequacy of the developed models was evaluated by analysis of variance (ANOVA), and the statistical parameters obtained are summarized in Table 7. All three responses exhibited statistically significant quadratic models ($p < 0.05$), confirming that the selected chromatographic variables adequately explained the observed variation in chromatographic performance.

Table 7. Statistical summary of the quadratic models generated by the Box–Behnken design

Response	Model F-value	Model p-value	Lack-of-fit p-value	R ²	Adjusted R ²	Predicted R ²	Adeq. Precision	Interpretation
R _f of BIS	10.44	0.0094	0.0618	0.9495	0.8586	0.2206	9.435	Significant model
R _f of PER	5.11	0.0436	0.1877	0.9014	0.7244	−0.2743	5.910	Significant model
Resolution	7.25	0.0210	0.2033	0.9019	0.7254	−0.3944	5.910	Significant model

Table 8. Verification of numerical optimization under optimized chromatographic conditions

Response	Predicted value	Experimental value	Relative error (%)
R _f of Bisoprolol	0.350	0.339	3.143
R _f of Perindopril	0.648	0.650	0.309
Resolution	2.080	2.200	5.769

For the R_f value of bisoprolol fumarate, the quadratic model produced an F-value of 10.44 with a model p-value of 0.0094, while the lack-of-fit was non-significant ($p = 0.0618$), indicating satisfactory model adequacy. Similarly, the model developed for the R_f value of perindopril arginine was statistically significant ($F = 5.11$, $p = 0.0436$) with a non-significant lack-of-fit ($p = 0.1877$). The quadratic model describing chromatographic resolution also demonstrated statistical significance ($F = 7.25$, $p = 0.0210$) and an acceptable lack-of-fit ($p = 0.2033$). The coefficients of determination (R²) ranged from 0.9014 to 0.9495, indicating good agreement between experimental and predicted responses. Furthermore, Adeq Precision values exceeding four confirmed an adequate signal-to-noise ratio and satisfactory predictive capability of the developed mathematical models.

Model Adequacy

The adequacy of the developed quadratic models was confirmed through multiple statistical parameters. The significant model F-values together with non-significant lack-of-fit indicated

that the developed polynomial equations adequately described the relationship between chromatographic variables and analytical responses. High coefficients of determination (R^2), satisfactory adjusted R^2 values, and Adeq Precision values exceeding the recommended threshold of four confirmed good predictive capability of the developed models. These observations demonstrate that the selected experimental design efficiently characterized the chromatographic system and was suitable for optimization.

Response Surface Analysis

The interaction between the selected chromatographic variables was investigated using contour plots and three-dimensional response surface plots generated from the fitted quadratic models (Figures 5 and 6). These graphical representations facilitated visualization of the combined influence of the independent variables on chromatographic performance.

The interaction between chloroform volume and chamber saturation time exerted the greatest influence on all three analytical responses. Increasing the proportion of chloroform enhanced analyte migration, whereas appropriate chamber saturation promoted solvent equilibrium, resulting in improved peak symmetry and reproducible retention behavior. In comparison, the influence of glacial acetic acid was less pronounced but remained important for minimizing peak tailing and improving chromatographic selectivity.

For chromatographic resolution, the response surface plots demonstrated that maximum separation was consistently achieved near the center of the experimental design space, confirming the presence of an optimum operating region rather than a simple linear relationship between the investigated variables. These observations demonstrate the advantage of the AQBd approach in defining a robust chromatographic design space capable of consistently producing satisfactory analytical performance.

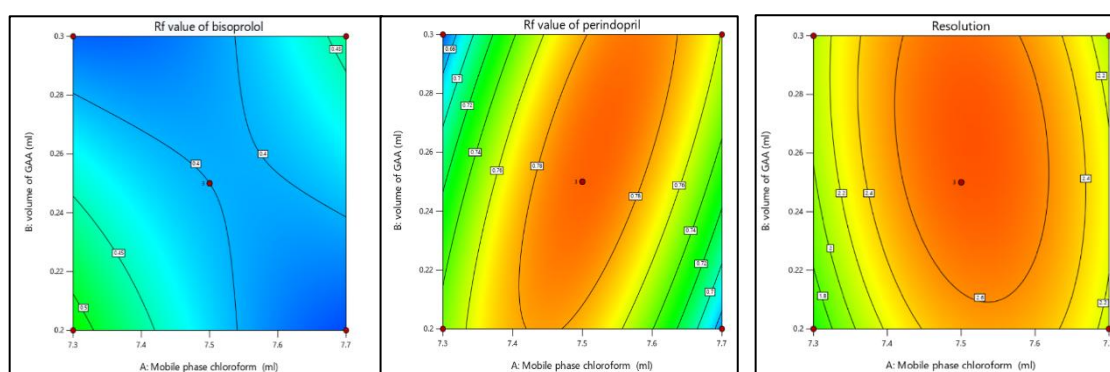


Figure 5. Contour plots illustrating the effects of chromatographic variables on (A) R_f of bisoprolol fumarate, (B) R_f of perindopril arginine, and (C) chromatographic resolution.

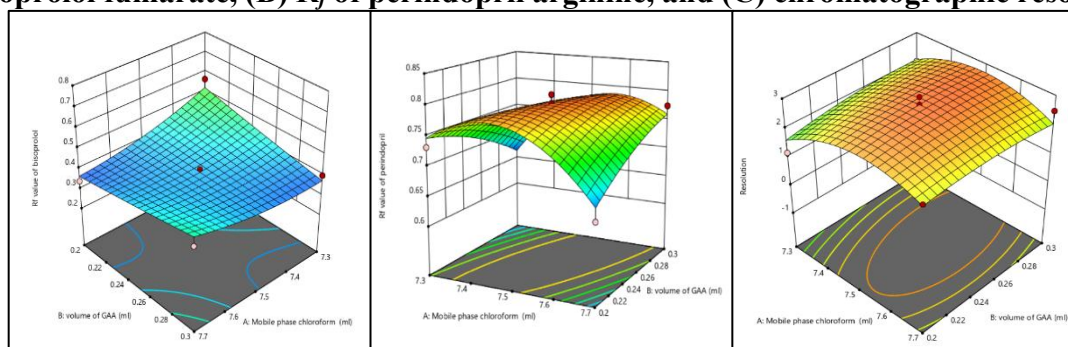


Figure 6. Three-dimensional response surface plots showing the interaction effects of chromatographic variables on (A) R_f of bisoprolol fumarate, (B) R_f of perindopril arginine, and (C) chromatographic resolution.

Method Operable Design Region (MODR)

Response surface methodology enabled identification of a multidimensional Method Operable Design Region (MODR) within which all predefined Critical Analytical Attributes simultaneously satisfied the desired acceptance criteria. The overlay plot generated from numerical optimization visually defined this region and demonstrated that minor variations in chromatographic conditions within the MODR are unlikely to compromise analytical performance. Establishment of the MODR represents an important outcome of the AQbD strategy because it provides enhanced process understanding, improved robustness, and greater regulatory flexibility throughout the analytical lifecycle (Figure 6 and 7).

Number	Mobile phase chloroform	volume of GAA	chamber saturation time	Rf value of bisoprolol	Rf value of perindopril	Resolution	Desirability	
32	7.302	0.292	22.100	0.351	0.649	2.084	1.000	Selected

Figure 7. Desirability plot generated by Design-Expert® software showing simultaneous optimization of chromatographic responses.

Numerical Optimization and Optimized Chromatographic Conditions

Numerical optimization was performed using the desirability function approach to simultaneously optimize all chromatographic responses. The optimization process identified 7.30 mL chloroform, 2.40 mL ethanol, 0.29 mL glacial acetic acid, and a chamber saturation time of 22 min as the optimum chromatographic conditions, yielding an overall desirability value of 1.000. The desirability function and overlay plot defining the optimized design space are presented in Figures 7 and 8, respectively.

Experimental verification of the optimized conditions demonstrated excellent agreement between the predicted and observed chromatographic responses. Relative prediction errors ranged from 0.31% to 5.77%, confirming the reliability and predictive capability of the developed mathematical models (Table 7).

The optimized chromatographic conditions provided compact, symmetrical, and well-resolved chromatographic bands for both analytes, confirming the suitability of the AQbD-assisted optimization strategy for routine pharmaceutical analysis.

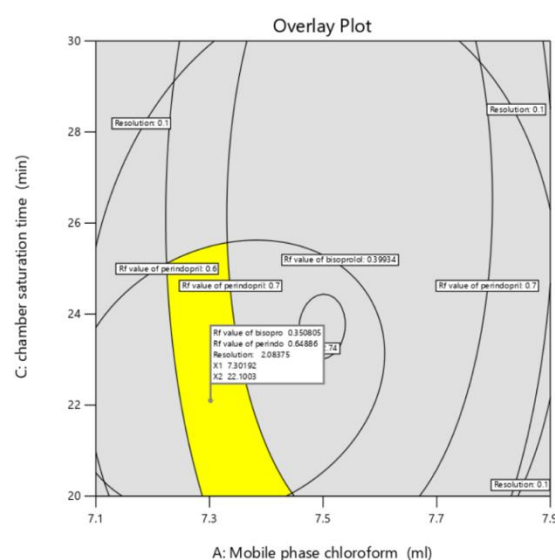


Figure 8. Overlay plot illustrating the optimized chromatographic design space

Design Space Verification

The optimized chromatographic conditions predicted by the mathematical models were experimentally verified to confirm the reliability of the established design space. Excellent agreement between predicted and experimental responses, with relative prediction errors below 6%, confirmed the validity of the developed models and demonstrated the predictive capability of the AQbD approach. These findings indicate that the optimized operating conditions are robust and reproducible for routine pharmaceutical analysis.

Analytical Control Strategy

Based on the optimized Method Operable Design Region, an analytical control strategy was established to ensure consistent chromatographic performance during routine application. Critical Method Parameters, including mobile phase composition, chamber saturation time, development distance, and detection wavelength, were maintained within predefined operating ranges throughout validation studies. Continuous monitoring of system suitability parameters such as retention factor, chromatographic resolution, and peak symmetry ensured that the analytical procedure remained under statistical control during routine analysis.

Table 9. Optimized chromatographic conditions

Parameter	Optimized condition
Stationary phase	Silica gel 60 F254 aluminium TLC plate
Mobile phase	Chloroform: Ethanol: GAA (7.3:2.4:0.29, v/v/v)
Chamber saturation time	22 min
Band width	8 mm
Application volume	10 μ L
Development distance	80 mm
Detection wavelength	219 nm
R_f value of Bisoprolol	0.339
R_f value of Perindopril	0.650
Resolution	2.20

Method Validation

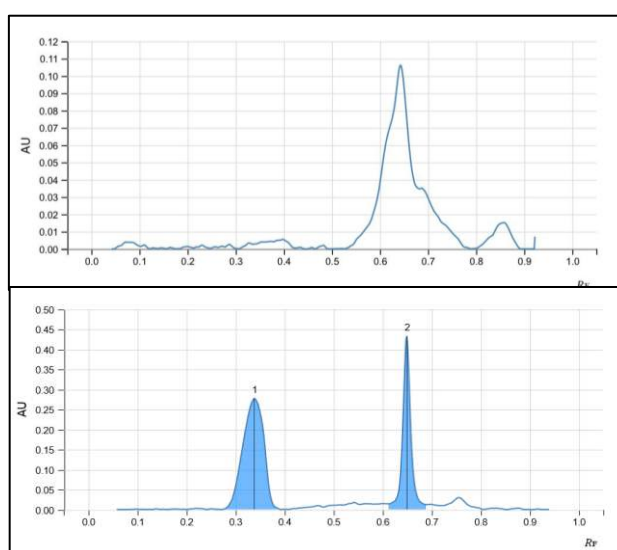
The optimized AQbD-based HPTLC method was validated according to the recommendations of ICH Q2(R2) to establish its suitability for the simultaneous determination of bisoprolol fumarate and perindopril arginine in pharmaceutical dosage forms. Representative densitograms of blank, standard, and sample solutions demonstrated the specificity of the method by confirming the absence of interfering peaks at the retention factors corresponding to either analyte (Figure 7). Peak purity assessment using overlay UV spectra further confirmed the spectral homogeneity of both chromatographic bands (Figure 10).

The developed method exhibited excellent linearity over the concentration range of 10–30 μ g band⁻¹ for both analytes, producing regression equations of $y = 0.0008x + 0.0096$ ($R^2 = 0.9963$) for bisoprolol fumarate and $y = 0.0006x + 0.0138$ ($R^2 = 0.9972$) for perindopril arginine (Figure 11). System suitability testing produced consistent retention factors and peak area responses with %RSD values below 2%, confirming satisfactory chromatographic repeatability. Accuracy studies performed using the standard addition method demonstrated mean recoveries of 98.75–102.26% for bisoprolol fumarate and 98.40–102.00% for perindopril arginine. Repeatability, intraday precision, and interday precision studies also produced %RSD values below 2%, indicating excellent precision and reproducibility of the proposed method. Robustness evaluation, performed by deliberate variation of the detection wavelength and

chamber saturation time, showed no significant influence on chromatographic performance, confirming the robustness of the developed analytical procedure. The calculated limits of detection (LOD) and quantification (LOQ) were 0.92 and 2.80 $\mu\text{g band}^{-1}$, respectively, for bisoprolol fumarate, and 1.24 and 3.75 $\mu\text{g band}^{-1}$, respectively, for perindopril arginine, demonstrating satisfactory analytical sensitivity. Application of the validated method to the marketed fixed-dose combination tablet yielded assay values of 100.62% for bisoprolol fumarate and 99.16% for perindopril arginine, which complied with the pharmacopeial acceptance criteria for finished pharmaceutical products. A comprehensive summary of all validation characteristics is presented in Table 10.

Table 10. Summary of validation characteristics of the proposed AQbD-based HPTLC method according to ICH Q2(R2)

Validation parameter	Acceptance criterion	Bisoprolol fumarate	Perindopril arginine
Specificity	No interfering peaks	Passed	Passed
System suitability (%RSD)	<2%	1.17	1.82
Linearity range ($\mu\text{g band}^{-1}$)	As intended	10–30	10–30
Regression equation	—	$y = 0.0008x + 0.0096$	$y = 0.0006x + 0.0138$
Correlation coefficient (R^2)	>0.99	0.9963	0.9972
Accuracy (% recovery)	98–102%	98.75–102.26	98.40–102.00
Repeatability (%RSD)	<2%	1.17	1.76
Intraday precision (%RSD)	<2%	0.25–0.29	0.84–1.50
Interday precision (%RSD)	<2%	1.46–1.98	1.61–1.84
Robustness (%RSD)	<2%	0.69–0.78	0.74–0.84
LOD ($\mu\text{g band}^{-1}$)	—	0.92	1.24
LOQ ($\mu\text{g band}^{-1}$)	—	2.80	3.75
Assay (% label claim)	98–102%	100.62	99.16



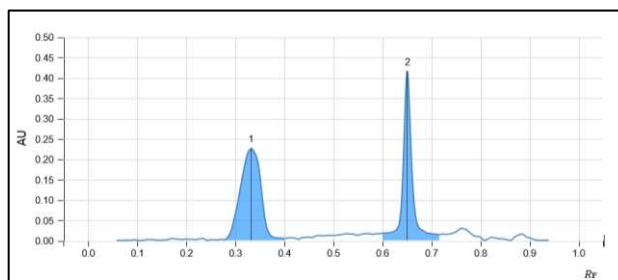


Figure 9. Representative HPTLC densitograms of blank, standard, and sample solutions demonstrating method specificity.

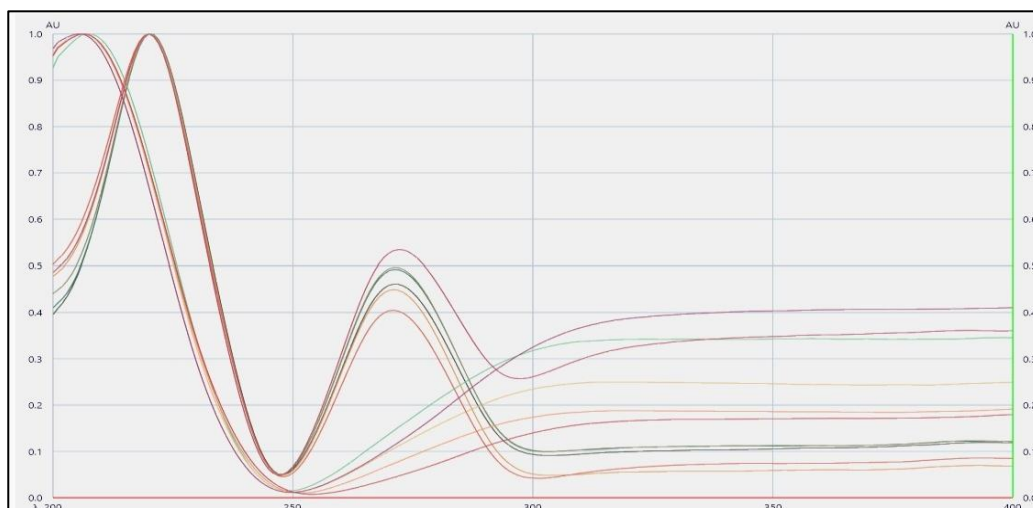


Figure 10. Overlay UV spectra confirming peak purity of bisoprolol fumarate and perindopril arginine.

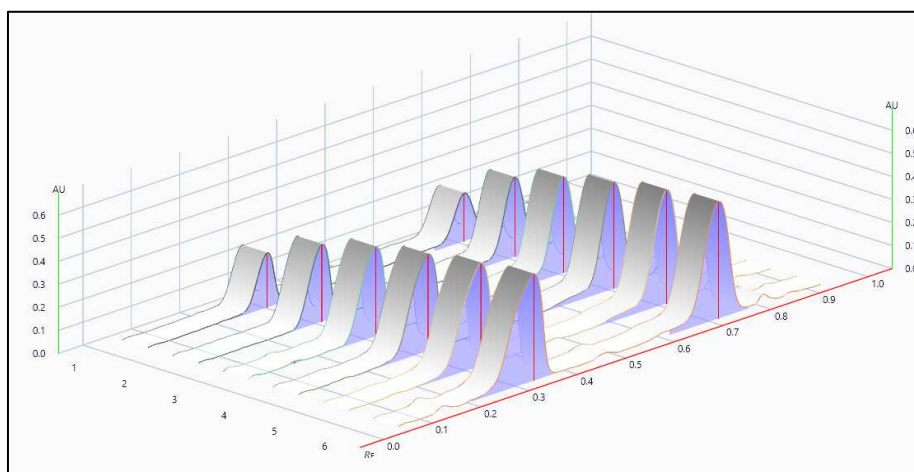


Figure 11. Overlay 3D Densitogram of linearity

Assessment of Analytical Sustainability and Practical Applicability

The environmental sustainability of the developed AQbD-assisted HPTLC method was assessed using the Analytical Eco-Scale, Analytical Greenness (AGREE) metrics. The method achieved an Analytical Eco-Scale score of 81 (Table 11), indicating excellent environmental performance. This high score was primarily attributed to low solvent consumption, the absence of derivatization, simple sample preparation, and minimal waste generation. The AGREE score of 0.71 demonstrated good compliance with the principles of Green Analytical Chemistry, confirming the sustainability of the proposed method (Figure 12). The environmental

performance of the proposed method was further evaluated using the Green Analytical Procedure Index (GAPI). The GAPI pictogram showed predominantly green and yellow zones, indicating that most analytical steps are environmentally favourable (Figure 12). The few red zones were mainly associated with the use of chloroform during chromatographic development. Nevertheless, simple sample preparation, low solvent consumption, minimal waste generation, and the absence of derivatization or energy-intensive processes contributed to an overall favourable GAPI profile, confirming the environmental suitability of the developed method.

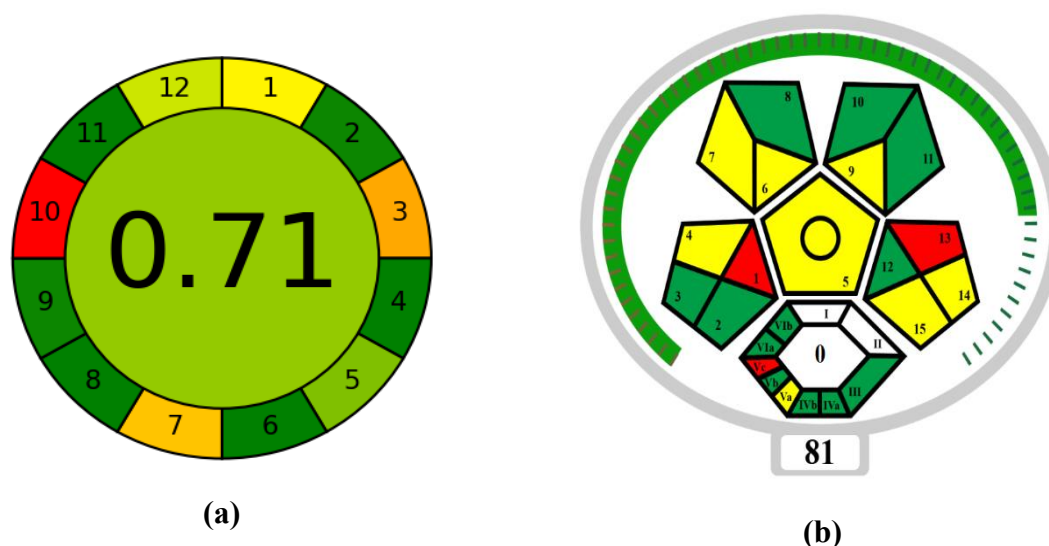


Figure 12: Greenness evaluation by (a) AGREE and (b) ComplexMoGAPI

Table 11. Analytical Eco-Scale Penalty Point Calculation

Parameter	Experimental Condition	Penalty Points (PP)
Reagents	Chloroform, ethanol, glacial acetic acid and methanol	17
Energy consumption	HPTLC sample applicator, chamber development and densitometric scanning	1
Occupational hazard	Handling of organic solvents under laboratory conditions	1
Waste generation	Approximately 10 mL organic solvent waste per plate	0
Waste treatment	Proper collection and disposal of organic solvent waste	0
Total Penalty Points (ΣPP)		19
Analytical Eco-Scale Score	$100 - \Sigma$ PP	81
Interpretation		Excellent green analytical method

Note: Analytical Eco-Scale score = $100 - \Sigma$ Penalty Points (PP). According to Gałuszka et al., Eco-Scale scores >75 indicate an excellent green analytical method, scores between 50 and 75 indicate an acceptable green method, and scores <50 indicate an inadequate green analytical procedure.

The practical applicability of the developed method was evaluated using the Blue Applicability Grade Index (BAGI) (Figure 13). The method demonstrated excellent practical performance owing to its low cost, rapid analysis, simple sample preparation, and ability to analyze multiple

samples simultaneously. Moreover, the robustness achieved through the AQbD approach supports reliable implementation in routine pharmaceutical quality-control laboratories. Overall, the BAGI assessment confirmed that the proposed HPTLC method combines operational simplicity with excellent practicality for routine analysis.



Figure 13: Blue Applicability Grade Index (BAGI) score

The RGB 12 model based on the principles of White Analytical Chemistry (WAC) was used to evaluate the analytical performance (Redness), environmental sustainability (Greenness), and practical applicability (Blueness) of the proposed AQbD-assisted HPTLC method. The method achieved Redness, Greenness, and Blueness scores of 98.8%, 82.5%, and 89.6%, respectively, with an overall Method Brilliance (MB) score of 90.3% (Figure 14).

Method: QbD Assisted HPTLC Method					
R1: Scope of application	100.0	G1: Toxicity of reagents	70.0	B1: Cost-efficiency	95.0
R2: LOD and LOQ	95.0	G2: Amount of reagents and waste	80.0	B2: Time-efficiency	95.0
R3: Precision	100.0	G3: Energy and other media	90.0	B3: Requirements	90.0
R4: Accuracy	100.0	G4: Direct impacts	90.0	B4: Operational simplicity	78.3
R (%) = 98.8		G (%) = 82.5		B (%) = 89.6	
Whiteness (Method Brilliance): 90.3%					

Figure 14: Method Brilliance score by RGB assessment

The excellent Redness score reflects the method's outstanding analytical performance, supported by high accuracy, excellent precision, satisfactory sensitivity, and reliable validation results. The Greenness score demonstrates good environmental sustainability, mainly due to low solvent consumption, minimal waste generation, and low energy requirements, although the use of chloroform in the mobile phase slightly reduced the overall green rating. The high Blueness score highlights the method's practical advantages, including low cost, rapid analysis, operational simplicity, and suitability for routine quality control. Overall, the high Method Brilliance score confirms that the proposed method successfully balances analytical

performance, environmental sustainability, and practical applicability, making it well suited for routine pharmaceutical analysis (Table 12).

Table 12. Summary of Analytical Sustainability Assessment Using AGREE, GAPI, BAGI, RGB 12, and Analytical Eco-Scale

Assessment tool	Result	Interpretation
Analytical Eco-Scale	81	Excellent green analytical method
AGREE	0.71	Good overall greenness
GAPI	Predominantly green and yellow zones	Environmentally acceptable analytical procedure
BAGI	High applicability	Excellent practical suitability for routine QC
RGB – Redness	98.80%	Excellent analytical performance
RGB – Greenness	82.50%	High environmental sustainability
RGB – Blueness	89.60%	Excellent practical applicability
RGB – Method Brilliance	90.30%	Outstanding balance between analytical performance, greenness, and practicality

White Analytical Chemistry (WAC) extends Green Analytical Chemistry by considering not only environmental sustainability but also analytical performance and practical applicability. Accordingly, the RGB 12 model was used to provide a holistic evaluation of the proposed AQbD-assisted HPTLC method. Together with AGREE, GAPI, BAGI, and the Analytical Eco-Scale, the results confirmed that the developed method is analytically reliable, environmentally sustainable, and suitable for routine pharmaceutical analysis.

CONCLUSION

The present study successfully developed an Analytical Quality-by-Design (AQbD)-based HPTLC method for the simultaneous estimation of bisoprolol fumarate and perindopril arginine in fixed-dose combination tablets. A systematic AQbD approach comprising predefined Analytical Target Profile (ATP), risk assessment, and Box–Behnken Design enabled identification and optimization of the critical method parameters, resulting in a robust Method Operable Design Region (MODR). The optimized method exhibited satisfactory chromatographic performance and successfully met the validation requirements of ICH Q2(R2), demonstrating excellent specificity, linearity, accuracy, precision, robustness, and suitability for routine assay of the marketed formulation.

In addition to its excellent analytical performance, the proposed AQbD-assisted HPTLC method demonstrated high analytical sustainability and practical applicability. The favorable AGREE (0.71), Analytical Eco-Scale (81), GAPI, and BAGI assessments, together with the RGB Method Brilliance score of 90.3%, confirmed that the method aligns well with the principles of White Analytical Chemistry by achieving an excellent balance between analytical performance, environmental sustainability, and practical applicability. Therefore, the proposed method can be considered a reliable, sustainable, and practical analytical tool for the routine quality control of bisoprolol fumarate and perindopril arginine in pharmaceutical formulations.

NOVELTY STATEMENT

This work presents a systematic AQbD-driven development of an HPTLC method for the simultaneous estimation of bisoprolol fumarate and perindopril arginine, which has not been previously reported in the literature. Unlike conventional optimization approaches, the present study employs a statistically justified Box–Behnken experimental design to establish a validated analytical design space for chromatographic separation.

The novelty of this research lies in:

- First AQbD-based HPTLC method for simultaneous estimation of BIS and PER
- Establishment of a statistically validated analytical design space using response surface methodology
- Simultaneous optimization of retention factor and chromatographic resolution
- Integration of ICH Q2(R2)-compliant validation with AQbD principles
- Development of a cost-effective, rapid, and environmentally sustainable alternative to LC-based methods

This approach enhances regulatory compliance readiness and provides deeper method understanding compared to previously reported empirical methods.

DECLARATIONS

Funding

Open access funding provided by Parul University. None received.

Author's contributions

Dewoo Patil and Prexa Tandel have performed the experimental work and drafting. Ratna Musale and Dr. Hiralben Mehta have supervised the study and critically revised the manuscript. All authors reviewed and approved the final version of the manuscript.

Competing interests

The authors declare no competing interests.

Data Availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics declaration and consent to participate

Not applicable. This study did not involve human participants, human tissues, animals, or clinical investigations.

Consent to Participate

Not applicable.

Consent for publication

Not applicable.

Acknowledgement

The authors are thankful to Zydus Pharmaceuticals (Ahmedabad, India) and Globela Pharma (Surat, India) for providing gift samples of bisoprolol fumarate and perindopril arginine, respectively. We express our gratitude to Parul University, Parul Institute of Pharmacy and Research, for offering laboratory facilities, encouragement, and continuous support throughout the research process. Finally, we appreciate all individuals who contributed to this work for their cooperation and valuable assistance.

REFERENCES

1. World Health Organization. Hypertension fact sheet. <https://www.who.int> (2023).
2. Whelton, P. K. et al. 2017 ACC/AHA guideline for hypertension. *Hypertension* **71**, e13–e115 (2018).
3. Williams, B. et al. 2018 ESC/ESH hypertension guidelines. *Eur. Heart J.* **39**, 3021–3104 (2018).
4. Burnier, M. & Egan, B. M. Adherence in hypertension. *Curr. Hypertens. Rep.* **21**, 65 (2019).
5. Bangalore, S. et al. Fixed-dose combinations in hypertension. *Am. J. Med.* **120**, 713–719 (2007).
6. Brunton, L. L. et al. *Goodman & Gilman's Pharmacological Basis of Therapeutics*. (McGraw-Hill, 2018).
7. Katzung, B. G. *Basic & Clinical Pharmacology*. (McGraw-Hill, 2021).
8. National Center for Biotechnology Information. Bisoprolol fumarate. PubChem <https://pubchem.ncbi.nlm.nih.gov/compound/Bisoprolol> (2026).
9. National Center for Biotechnology Information. Perindopril arginine. PubChem <https://pubchem.ncbi.nlm.nih.gov/compound/Perindopril> (2026).
10. British Pharmacopoeia Commission. *British Pharmacopoeia* (TSO, 2023).
11. Sahu, P. K. et al. An overview of experimental designs in HPLC method development and validation. *J. Pharm. Biomed. Anal.* **147**, 590–611 (2018). <https://doi.org/10.1016/j.jpba.2017.07.006>
12. Sherma, J. HPTLC in pharmaceutical analysis. *J. AOAC Int.* **93**, 850–863 (2010).
13. Swartz, M. E. UPLC: an introduction and review. *J. Liq. Chromatogr. Relat. Technol.* **28**, 1253–1263 (2005). <https://doi.org/10.1081/JLC-200053469>
14. Niessen, W. M. A. Liquid chromatography–mass spectrometry. *J. Chromatogr. A* **856**, 179–197 (1999). [https://doi.org/10.1016/S0021-9673\(99\)00881-1](https://doi.org/10.1016/S0021-9673(99)00881-1)
15. ICH Q14: Analytical Procedure Development. <https://www.ich.org> (2023).
16. ICH Q8(R2): Pharmaceutical Development. <https://www.ich.org> (2009).
17. ICH Q9(R1): Quality Risk Management. <https://www.ich.org> (2023).
18. ICH Q10: Pharmaceutical Quality System. <https://www.ich.org> (2008).
19. Lewis, G. A., Mathieu, D. & Phan-Tan-Luu, R. *Pharmaceutical Experimental Design*. (1999).
20. International Council for Harmonisation. ICH Q2(R2): Validation of Analytical Procedures. <https://www.ich.org> (2023).
21. Beg, S. et al. AQbD applications. *J. Pharm. Biomed. Anal.* **147**, 643–658 (2019).
22. Ermer, J. & Nethercote, P. Quality by Design in analytical chemistry. *Pharm. Technol.* **41**, 32–40 (2017).
23. United States Pharmacopeial Convention. USP–NF General Chapter <621> Chromatography. <https://www.usp.org> (2024).
24. Gałuszka A, Migaszewski ZM, Konieczka P, Namieśnik J. *Analytical Eco-Scale for assessing the greenness of analytical procedures*. Trends Anal Chem. 2012;37:61–72.
25. Pena-Pereira F, Wojnowski W, Tobiszewski M. AGREE—Analytical GREENness Metric Approach and Software. *Analytical Chemistry*. 2020;92:10076–10082.

26. Mansour FR, Omer KM, Płotka-Wasyłka J. A total scoring system and software for complex modified GAPI (ComplexMoGAPI) application in the assessment of method greenness. *Green Anal Chem.* 2024;10:100126.
27. Manousi N, Wojnowski W, Płotka-Wasyłka J, Samanidou V. Blue applicability grade index (BAGI) and software: a new tool for the evaluation of method practicality. *Green Chem.* 2023;25(19):7598–7604.
28. Nowak PM, Kościelniak P. What color is your method? Adaptation of the RGB additive color model to analytical method evaluation. *Anal Chem.* 2019;91(16):10343–52.
29. Nowak PM, Wietecha-Posłuszny R, Pawliszyn J. White analytical chemistry: an approach to reconcile the principles of green analytical chemistry and functionality. *Trends Anal Chem.* 2021;138:116223.