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ORIGINAL RESEARCH PAPER

DoE-empowered development and validation of an environmentally sustainable RP-HPLC method for simultaneous estimation of antihypertensive drugs: AQbD perspective

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ABSTRACT

The primary objective of the present inquiry is to formulate a sustainable method employing Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) for determination of Amlodipine (AM) and Irbesartan (IRB) simultaneously, compounds commonly prescribed for hypertension treatment. Existing literature underscores the absence of a comprehensive method in this regard. This research endeavors to align with the tenets of green chemistry by seamlessly integrating Analytical Quality by Design (AQbD) with RP-HPLC, replacing environmentally hazardous chemical modifiers with eco-friendly solvents. Identifying the critical variables as the 70% ethanol level and flow rate, a central composite design is applied for optimization. The separation is achieved utilizing a Phenomenex Luna column *(C_{18}, 250 mm × 4.6 mm i.d, 5 µm)* with a mobile phase comprising ethanol and 0.1 % o-phosphoric acid in a 70:30 v/v ratio, flowing at 0.8 mL min^{-1}, and detection wavelength of 242 nm. Green assessment methodologies are implemented to gauge the adherence of the proposed RP-HPLC method to eco-friendly principles while ensuring efficiency in chromatographic performance. The current developed method is rapid with retention time of 2.3 and 3.3 min for AM and IRB respectively and having a wide linear range from 55 to 130 µg mL^{-1}, which makes the suitable for the accurate quantification of AM and IRB simultaneously in bulk and tablet dosage form, there by minimize environmental impact by providing a conscientious choice for the routine analysis which is achieved through the amalgamation of AQbD with a sustainable approach.

KEYWORDS

analytical quality by design, amlodipine, irbesartan, eco-friendly RP-HPLC, hypertension

1. INTRODUCTION

Hypertension represents a significant contributory factor in the advancement of cardiovascular, cerebrovascular, and mortality-related pathologies. Effective management of blood pressure (BP) emerges as a crucial factor in augmenting prognostic outcomes for individuals suffering with hypertension [1]. The European Society for Hypertension/European Society of Cardiology (ESH/ESC) has recommended the use of combination therapy as the main approach to improving blood pressure (BP) reduction in most patients [2]. A frequently advocated therapeutic regimen involves the co-administration of irbesartan and amlodipine in a unified single-tablet format. Their concurrent usage precipitates an additive antihypertensive effect, underscoring a synergistic impact [3]. Irbesartan, a prevalent pharmaceutical agent, exerts its therapeutic effects through the non-peptidic blockade of angiotensin II receptors and operates by selectively inhibiting the binding of angiotensin II to AT1 receptors (Fig. 1a) [4]. This action leads to vasodilation and improves the effects caused by aldosterone

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by decreasing sodium excretion and increasing potassium excretion [5]. Amlodipine is a dihydropyridine derivative that has calcium antagonist characteristics (Fig. 1b). Amlodipine works by blocking the entry of calcium ions across the cell membrane in both vascular smooth muscle and cardiac muscle tissues muscle [6].

HPLC is the dominant analytical technique in pharmaceutical analysis, playing a crucial role in the quality check procedures for both bulk pharmaceuticals and dosage form. The importance of the technique extends to the examination of bulk drugs, impurity profiling, evaluation of stability of drugs, and the evaluation of enantiomers. AQbD approach utilizes statistical Design of Experiments (DoE) to create a 'design space' that defines a robust analytical procedure [7]. The Food and Drug Administration (FDA) has actively promoted the principles of QbD, which involve systematically integrating quality into both the product and the process in a science-driven and risk-based manner during development. This approach differs from the retrospective attempt to determine quality detailed testing. The utilization of QbD and its principles in the formulation of analytical techniques has become increasingly popular, with the goal of attaining improved method effectiveness and makes method robust [8]. The optimization of HPLC methods often involves the common use of the Central Composite Design (CCD) [9]. As a result of increased concerns from scientists and the public about environmental pollution, different sectors, including both society and research, have adopted practices that are environmentally sustainable. Green analytical chemistry places significant importance on sample preparation and liquid chromatography (LC) analysis within its scope, primarily due to the widespread use of dangerous solvents. Green life cycle strategies encompass minimizing solvent usage, switching to less harmful solvents, and, when possible, eliminating organic solvents entirely [10]. In spite of their commendable attributes, acetonitrile (ACN) and methanol (MeOH) give rise to apprehensions pertaining to environmental safety. Acetonitrile, with its highly flammable and easily vaporizable nature, coupled with its toxicity, raises concerns. Conversely, although MeOH exhibits lower toxicity and enhanced biodegradability relative to acetonitrile, it is nonetheless categorized as a dangerous solvent due to its intrinsic poisonous nature and the imperative for proper waste disposal. Careful consideration is required due to the substantial amount of waste produced during analyses using RP-HPLC [11]. Acetonitrile has been incorporated into the mobile phase or extraction processes in the previously mentioned liquid chromatography (LC) methods. These solvents are classified as harmful by the US Environmental Protection Agency (EPA) [10]. Given their inherent toxicity [11] it is crucial to safely detoxify waste solvents and to reduce the amounts, which may result in significant or extremely high disposal expenses. The use of environmentally friendly liquid chromatographic methods has become a central focus in pharmaceutical analysis, with the goal of protecting the health of operators and maintaining ecological integrity. The extensive use of several chromatographic instruments worldwide for pharmaceutical quality control highlights the significant consumption of organic solvents and the resulting production of waste [12]. Originating in the 2000s as a branch of green chemistry, Green Analytical Chemistry (GAC) has gradually gained

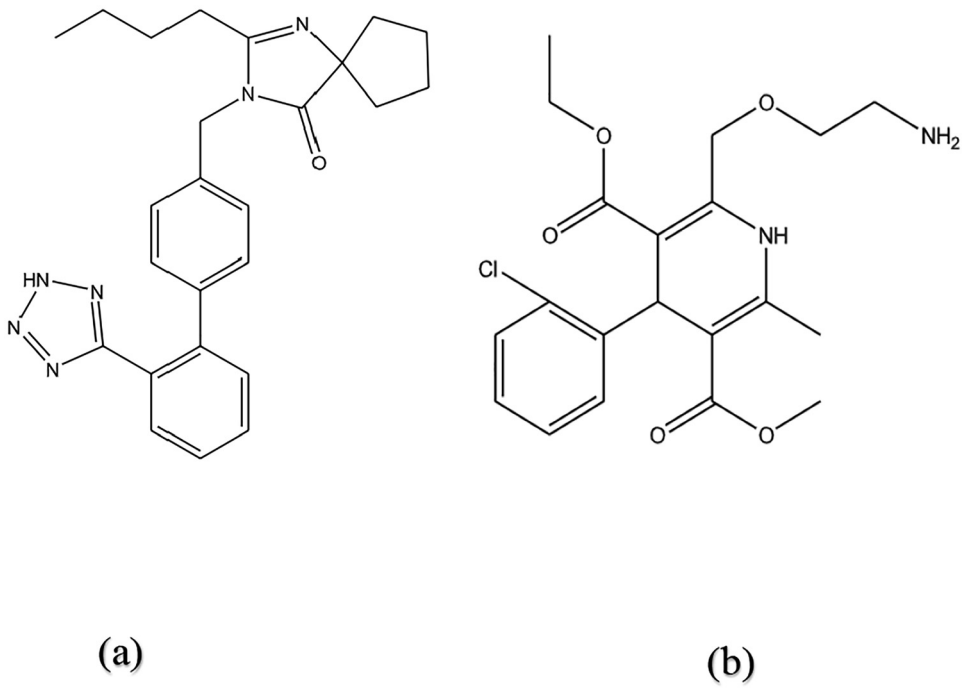


Fig. 1. Structural formula for Irbesartan (a) and Amlodipine (b)

recognition and endorsement in the scientific community [10]. GAC focuses on removing or minimizing dangerous compounds from analytical procedures in order to improve environmental and health compatibility while maintaining analytical performance [13]. While developing a RP-HPLC method without using organic solvents is difficult, a strategic approach for improving its ecological sustainability is to replace acetonitrile and methanol with alternative biodegradable solvents that are less harmful. The evaluation of the greenness of a solvent depends on its compliance with environmental, health, and safety standards, as well as a life-cycle assessment (LCA) [14]. Ethanol, propylene carbonate, ethyl lactate, isopropanol, are considered environmentally sustainable and fit to use in Reversed-Phase RP-HPLC [15].

After an in-depth review of published research work for the simultaneous estimation of irbesartan and amlodipine we concluded that these techniques take long time to elute the standard peaks hence, the analysis time was extended. Furthermore, no method is supported by QbD, even minor changes to the method's criteria will necessitate a revalidation. Toxic solvents like acetonitrile that are harmful to both the environment and to the analyst have been used in the HPLC procedures developed so far, which is not in line with the principles of green chemistry [16, 17].

The current study produced a reliable and environmentally safe HPLC method by using the AQbD methodology. The study proposes a unique chromatographic method that uses ethanol as a solvent, which is less toxic. The fundamental goal of this research is to present concepts in a pragmatic way. The method has been developed to reduce any dangers to the environment and the analyst. Thus, our goal was to develop and validate a novel, environmentally friendly HPLC technique that supports AQbD and allows for the rapid quantification of amlodipine and irbesartan simultaneously. Method shows its superiority in terms of lesser retention time, wider linearity range, accuracy, high sensitivity and repeatability. The method's eco-friendly results were then calculated by the utilisation of GAPI, AES, and AGREE tools. The suggested method has been assessed in compliance with the ICH Q2 (R2) guidelines.

2. EXPERIMENTAL

2.1. Chemicals and reagents

The Irbesartan (98%) and Amlodipine (98%) standards, which have been certified, were obtained from Yarrow Chem Products, a company located in Mumbai, India. Ethanol, the organic phase HPLC was obtained from Hayman Group Ltd., a supplier based in East Ways Park, Witham, UK. Sodium Hydroxide (NaOH) was purchased from Sisco Research Laboratories Pvt. Ltd, Maharashtra, India. analytical-grade o-phosphoric acid (OPA), 30% hydrogen peroxide (H_2O_2), and hydrochloric acid (HCl) were sourced from Rankem, New Delhi, India. HPLC water was internally obtained by ELGA Lab Water in Lane End, High Wycombe, UK. The pharmaceutical product Irbepex – AM (consisting of Irbesartan 150 mg and Amlodipine 2.5 mg) was obtained from a nearby pharmacy. It is manufactured by Shilpex Pharmysis.

2.2. Equipment and optimised HPLC parameters

Utilizing an HPLC Agilent 1220 Infinity II system (Santa Clara, California, USA), the technique was developed, consisting of a binary pump, a diode array detector and an autosampler injector. Chromatographic separation was carried out using a Phenomenex Luna column *(C_{18}, 250 mm × 4.6 mm i.d, 5 µm)*. The filtration process involved 0.45 µm nylon membranes, while solvent degassing was achieved through ultrasonication. The analytical processes were performed at a column oven temperature of 35 ± 1 °C using isocratic conditions. Conducting the analysis with a PDA detector set at 242 nm, the most effective mobile phase, identified through research-oriented methods, consisted of ethanol and o-phosphoric acid (0.1%) at a volumetric ratio of 70:30 v/v. The rate of flow was adjusted to 0.80 mL min^{-1}, and the volume of the injection was 5.0 µL.

2.3. Software

The experimental design, focused on optimizing various chromatographic parameters and establishing the design space, was executed using the trial version 12 of Design-Expert® software by Stat-Ease Inc., headquartered in Minneapolis, USA. Data collection and processing were carried out through the utilization of Agilent ChemStation (Version B) software.

2.4. Preparation

2.4.1. Preparation of aqueous phase. Exactly 1 mL of o-phosphoric acid was accurately measured and then transferred to a standard flask with a volume of 100 mL of HPLC grade water. Then the volume was subsequently modified to 1,000 mL with HPLC quality water. The solution was then filtered and degassed through the utilization of a sonicator in accordance with research-oriented practices.

2.4.2. Formulation of standardized stock solution. Stock solutions of Irbesartan and Amlodipine at 1,000 µg mL^{-1} concentration were prepared by dissolving 10.0 mg of Irbesartan and Amlodipine in 10.0 mL of pure ethanol (100%) respectively, further working standard solutions are prepared from the previously prepared stock solutions of Irbesartan and Amlodipine.

2.4.3. Sample analysis procedure. 20 Irbepex-AM tablets each containing 150 mg of Irbesartan and 2.5 mg Amlodipine were weighed, coarsely pulverized. Subsequently, 10.0 mg each of IRB and AM were precisely weighed and placed into a 10.0 mL volumetric flask. The volume is completed to the mark using 100% ethanol, resulting in a theoretical concentration of 1,000 µg mL^{-1} for both IRB and AM. The solution obtained was subjected to sonication for approximately 20 min and then filtered through Whatman

filter paper. Later the filtrate is diluted to attain the concentrations of 15.0 and 30.0 µg mL^{-1} of IRB and AM respectively. The above prepared 15.0 and 30.0 µg mL^{-1} sample solutions of IRB and AM are subsequently spiked with 85.0 and 100.0 µg mL^{-1} of standard solutions of IRB and AM and % recovery study is performed using standard addition method.

3. HPLC METHOD DEVELOPMENT ENABLED BY QUALITY BY DESIGN (QBD)

3.1. Quality target method profile (QTMP) and critical analytical attributes (CAAs)

In Analytical Quality by Design (A-QbD) methodology, the initiation phase involves utilizing the Quality Target Methodology Protocol (Table 1). This protocol constitutes a comprehensive compilation of all conceivable components that establish precise criteria for the designated analytical technique. The primary aim of QTMP is to furnish a reliable and precise HPLC method. Furthermore, this methodology seeks to endorse environmentally conscious practices by incorporating sustainable chemicals. To meet the outlined goals in QTMP, a subset of Critical Analytical Attributes (CAAs) and Critical Method Parameters (CMPs) have been selected.

3.2. Risk assessment – evaluation of potential dangers

The mobile phase and column design are two of the many HPLC variables that might affect the quality of the separation. Other variables, such as the sample composition, injection volume, temperature of the column, and detection conditions, could affect a method's results. As a result, the fishbone diagram in Fig. 3 was designed to identify the most important method attributes (CAAs), which is a systematic risk assessment. All the factors influencing the analytical quality are shown in this pictorial depiction, and those that are most important were chosen for further investigation.

3.3. Conducting experimental design

Preliminary method development trials were conducted to ascertain the appropriate ranges of method variables after performing the risk analysis. To optimise these technique variables, a DOE approach was used, and Central Composite Study design was used for this objective. In addition to a full factorial design, this design has center points and axial points, which help with sequential experimentation and estimating of quadratic or higher order trends. Validity and reproducibility are enhanced by Central Composite Design in experimental designs. The proportion of organic phase, mobile phase flow rate are selected as CMPS and the selected CAAS are resolution, tailing factor and number of theoretical plates of the HPLC process. The experimental variables are listed in Table 2.

Table 2. Levels of selected CMPS

Variables	Level	
Selected variables	Low	High
A: % Ethanol (V/V)	65	75
B: Flow rate (mL min^{-1})	0.6	1.0

Table 1. Quality target method profile (QTMP) postulated for the HPLC analysis of Irbesartan and Amlodipine

Method parameters	Target	Justification
Analyte	Irbesartan, Amlodipine	Development of an HPLC for the simultaneous quantification of active analyte for routine analysis.
Sample	Liquid	Drugs should be completely soluble in the suitable liquid phase ensuring complete solubility.
Chromatographic Technique	Reverse Phase – High Performance Liquid Chromatography	Highly hydrophobic drugs are better separated and eluted in the non-polar C_{18} stationary phase due to increased retention. Not only the increased resolution, RP-HPLC also provides a rapid analysis with minute sample size and with less mobile phase usage.
Instrument	HPLC coupled with PDA detector, binary pump, and an autosampler.	the PDA detector detects the compounds at their λmax, thereby increasing the sensitivity. A binary pump facilitates mixing of the solvents used in the method.
Method	Environment friendly, linear, precise, accurate, specific, robust, reproducible, simple and economical.	The method should use environmentally friendly solvents as mobile phase for the separation of analytes and agree with validation parameters, facilitating a rapid analysis and minimized usage of mobile phase.
Application	Assay	Method developed should be able to quantify active pharmaceutical ingredient IRB and AM in solution and in tablet dosage form for routine and stability analysis
CAAS	Retention time, number of theoretical plates, resolution	Quality of the methods is achieved with the aid of these attributes: lower retention time, higher theoretical plate and resolution
CMPS	Mobile phase ratio, flow rate	Risk assessment is done based on prior knowledge, to shortlist the CMPs.

*CAAs: critical analytical attributes.
*CMPs: critical method parameters.

3.4. Data analysis optimization and model validation

For the examination of data, and model validation, Design-Expert® version 12 by Stat-Ease Inc., based in Minneapolis, USA, has been employed. A quadratic model, indicating a second-order polynomial model, was utilized to know main and combined effects. In attempting the data fitting process, analyses such as analysis of variance (ANOVA), assessment of lack of fit, calculation of the adjusted R^2 (R^2-adj), predictive R^2 (R^2-pred) and coefficient of correlation (R^2) were utilized to establish a link between the factor and the response. The model is considered inadequately aligned when the lack of fit considerably exceeds the random pure error, whereas it is deemed satisfactory if the lack of fit is considerably less than the random pure error. The relationships between factors and responses were meticulously scrutinized and interpreted using 3D-Response surface plots and associated 2D contour plots. For validation, statistical optimization techniques were employed to identify optimal HPLC solutions for different Critical Analytical Attributes. These attributes encompass the minimization of retention time, maximization of resolution, and optimization of both resolution and theoretical plate count. Subsequently, a graphical method was employed in the optimization process to confirm the most favourable chromatographic solution within the designated design space.

3.5. Method development

To develop a HPLC method for assessing IRB and AM in pharmaceutical preparations, an investigation was conducted. Various mobile phases were methodically examined during the process. In accordance with the principles of green analytical chemistry, the selection of the optimal mobile phase prioritized the assay's selectivity and sensitivity. Additionally, considerations were given to the effective separation of IRB and AM from the degradation products generated during forced degradation tests. A meticulous examination of multiple wavelengths was also incorporated into the analytical method.

3.6. System suitability

Six replicated injections of standard solutions, each containing 50.0 µg mL^{-1} of IRB and 50.0 µg mL^{-1} of AM, were employed to evaluate the suitability parameters of the system. The standard IRB and AM chromatogram is illustrated in Fig. 2.

3.7. Stability of the solution

Exposed to ambient conditions for a duration of 72 h, the standard solutions of IRB and AM underwent evaluation to assess their stability. The assay results of the stored standard samples were compared to those of freshly prepared standard solutions and % ratio was calculated.

3.8. Forced degradation studies

The method's capacity to estimate stability was assessed by exposing standard solutions (1000 µg mL^{-1}) to degradation conditions, encompassing acid, alkali, oxidation, and thermal conditions. The presence of new peaks or a decrease in peak area was interpreted as evidence of degradation, with the extent assessed by percentage recovery. Simulating acidic and basic hydrolysis, sample solutions were generated in hydrochloric acid (0.01 M) and sodium hydroxide (0.01 M). Subsequently, these solutions were left at room temperature for a duration of 12 h before undergoing neutralization if required. Sample solutions were prepared in a 0.3% hydrogen peroxide solution and subjected to ambient temperature for 12 h under light protection in order to carry out the oxidative stress study. Thermal deterioration was measured by exposing the reference solution to heat for 12 h at 80 °C. The resulting solutions were subsequently diluted 10.0 µg mL^{-1} with 100% ethanol before injection into HPLC under optimized chromatographic conditions.

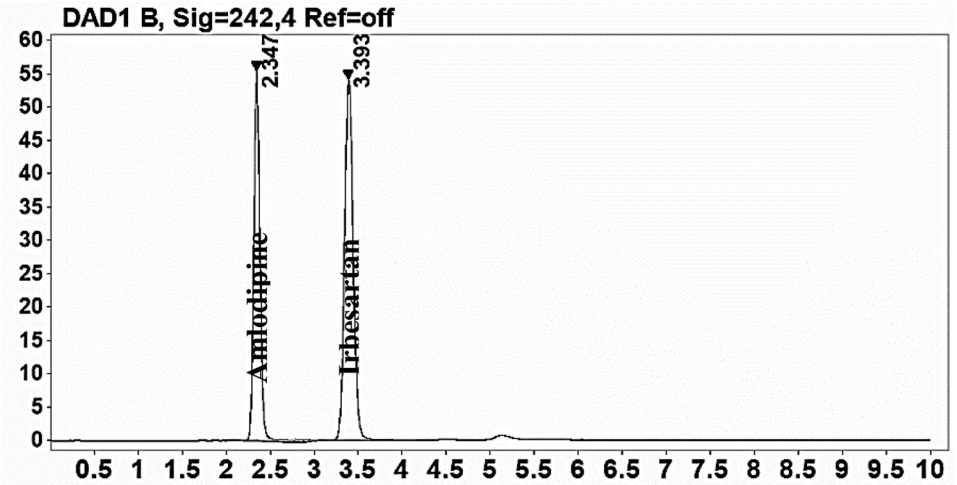


Fig. 2. Standard Amlodipine (50 µg mL^{-1}) and Irbesartan (50 µg mL^{-1}) chromatogram at Rt 2.35 min and 3.39 min

4. VALIDATION OF THE METHOD

Validation was performed in accordance with the recommendations of ICH Q2 (R2).

4.1. LOD, LOQ, and linearity

Linearity was assessed by preparing six working standard solutions, spanning concentrations from 55.0 to 130.0 μg mL^{-1}. Six distinct sets of such solutions were meticulously created and analyzed to establish individual calibration curves. The calculation of slope, intercept, and coefficient of determination for each calibration curve was undertaken to ascertain linearity. The method's sensitivity was rigorously examined by determining the limit of quantification (LOQ) and the limit of detection (LOD) by using the formula LOD = 3.3 × σ/S and LOQ = 10 × σ/S. In both cases, σ stands for the standard deviation of the responses, and S is the slope of the calibration curve.

4.2. Accuracy

The accuracy of the method has been determined by application of the analytical procedure to recovery studies using the standard addition method. Working standard solutions were subjected to HPLC analysis under optimal chromatographic conditions at of 50, 100, and 150%. The samples were prepared in triplicate for each level, and the peak area of the chromatogram was used to calculate the drug's percentage recovery.

$$\%\ \text{recovery} = \frac{\text{Cm} - \text{Cr}}{\text{Cr}} \text{X100}$$

where *Cm* and *Cr* are the measured and real concentration, respectively.

4.3. Precision

The %RSD values of within-days and between-days were calculated to evaluate the precision of the developed method. Samples were prepared in triplicate to determine both on the same day and on three distinct days. Both the peak area and the %RSD of the two medications were calculated.

4.4. Statistical data analysis

The obtained results were expressed as mean ± standard deviation (SD), and all the experiments were independently repeated at least three times.

5. RESULTS AND DISCUSSION

5.1. HPLC method development and optimization using analytical quality by design (AQbD)

The prospect of developing environmentally friendly and non-hazardous analytical methodologies that yield optimal results with minimal experimentation is a viable goal within the realm of analysis. Nevertheless, crafting a greener HPLC method without adhering to the tenets of application of Analytical Quality by Design (AQbD) may compromise method performance, necessitating periodic revalidation. This paper outlines the optimization of HPLC as a systematic process that concurrently modifying various parameters to achieve the required resolution. Consequently, the strategic integration of Green Analytical Chemistry and Analytical Quality by Design principles in a unified procedure emerges as a prudent approach to boost the method's resilience and sustainability. The study elucidates the application of AQbD principles in conjunction with Green Analytical Chemistry (GAC) concepts to systematically construct an analytical method. The emphasis on Central Composite Design (CCD) in the optimization process is underscored, given its manifold advantages as a design that elucidates the impact of factors on variables at diverse levels.

5.2. Initial investigation into method development

A comprehensive assessment of multiple factors, including the liquid phase's composition and ratio, column selection, and flow rate, was necessitated in the employment of the RP-HPLC methodology. The goal was to attain an optimal peak shape characterized by a shortened analysis duration, minimal distortion, heightened theoretical efficiency, and enhanced differentiation. Due to their adverse environmental impact and associated health risks, traditional organic solvents in HPLC methods, notably methanol and acetonitrile, were excluded. The challenges posed by existing constraints and compatibility issues were encountered in the selection of an environmentally friendly liquid chromatography mobile phase in line with Green Analytical Chemistry (GAC) principles. Solvent selection guides provided by regulatory bodies identify ethanol as a viable substitute for methanol. Thorough experimentation was conducted, trying diverse ratios of ethanol. Simultaneously, a systematic exploration was undertaken for various buffers, including ammonium formate at varying pH, acetate buffers, potassium dihydrogen orthophosphate buffer, disodium hydrogen phosphate, 0.1% o-phosphoric acid. The flow rate was systematically varied from 0.6 to 1 mL min^{-1}. The results revealed that the most suitable mobile phase was the combination of 0.1% o-phosphoric acid with ethanol as the aqueous phase. Characterized by peak symmetry, minimal tailing factor, optimal capacity factor, enhanced resolution, and a commendable theoretical plate count, this specific combination demonstrated superior chromatographic separation.

5.3. Studies on risk assessment

The establishment of correlations between various suggested method parameters and analytical parameters, as visualized in the Ishikawa fishbone diagram (Fig. 3), involved utilizing a matrix for risk assessment. Facilitating the identification of

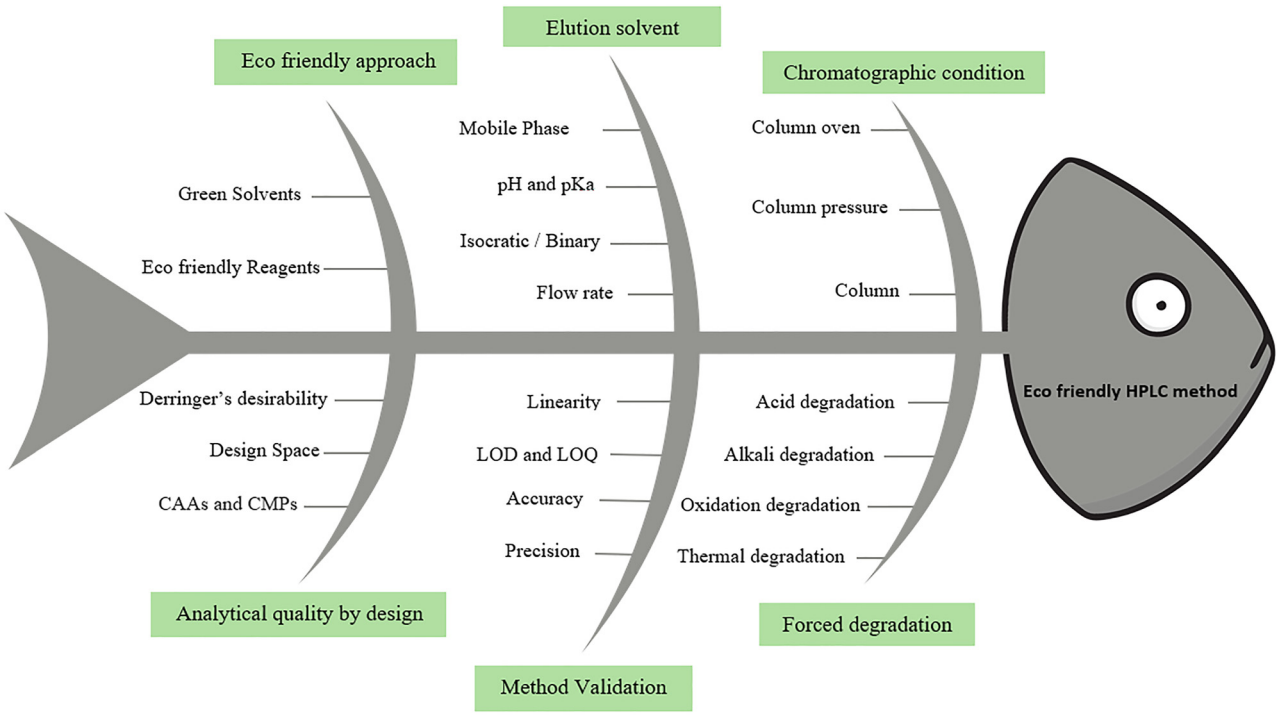


Fig. 3. Fishbone diagram

potential risk factors and enhancing the scrutiny of quality issues for subsequent evaluation during the screening phase is a key feature of this method. Utilizing risk management techniques, critical variables were determined based on the associated risks with each parameter. These assessed parameters elucidate the interplay between different analytical characteristics and method parameters. Through analysis of pertinent literature and consideration of varying levels of risk associated with each technique parameter, the study evaluated several input factors and gauged their criticality and probability. Selected for further factor screening investigations are critical factors including the organic modifiers, column temperature, injection volume, mobile phase ratio, flow rate, and pH of the aqueous phase.

5.4. Optimizing method using Central Composite Design (CCD)

Pursuing maximal efficiency or effectiveness optimization, a rotatable CCD was employed to formulate a model through the employment of response surface methods. Driven by its effectiveness in orchestrating investigations, the identified critical variables influencing the qualification of IRB and AM were the mobile phase ratio and the flow rate. Encompassing thirteen runs, the experimental design integrated five center points with the midpoint of individual variable at the zero level, repeated five times to evaluate experimental errors, the factors and their responses are given in Table 3.

The importance of each coefficient in the model was underscored by Fischer ratios (F values). Indicating a well-fitted model, higher R^2 and lesser lack-of-fit values were observed, with a high F ratio establishing the significance of the method equation. Exploring the effects of variables on each response, graphical interpretation of data through 3D surface graphs, contour and perturbation graphs was conducted and the results are demonstrated in Table 4.

Delineating the effect of ethanol volume and rate of flow on resolution, the perturbation plot revealed that increased ethanol levels and flow rates resulted in decreased resolution between the two drugs (Fig. 4a). Highlighted by the perturbation plot (Fig. 4b) was the increased retention time of the AM peak with an elevated ethanol percentage, while the flow rate had a negligible effect. The perturbation plot (Fig. 4c) for retention time of irbesartan revealed the increased retention time with decreased ethanol percentage. Increased ethanol percentage was illustrated by a perturbation plot (Fig. 4d) to negatively influence the amlodipine peak's theoretical plate number, and increased rate of flow resulted in a decreased number of theoretical plates. Furthermore, perturbation plot (Fig. 4e) for theoretical plate number of irbesartan illustrated that decreased plate number with increased ethanol percentage. Derived contour plots (Fig. 4(f–j)) shown consistent findings, while interactions were visually represented by 3D surface plots (Fig. 4(k–o)). Statistically represented polynomial equations obtained are as follows:

Table 3. Two-factor, thirteen run Central Composite experimental design and their measured responses

Runs	Factors		Responses				
	Ethanol (%v/v)	Flow rate (mL min^{-1})	Resolution (RS)	Retention time of AM	Retention time of IRB	Theoretical plate numbers of AM	Theoretical plate numbers of IRB
1	70	0.8	6.12	2.35	3.39	5,108	6,423
2	75	1	4.21	1.78	2.62	5,180	7,837
3	77	0.8	6.21	2.25	3.37	4,358	6,738
4	70	0.8	6.25	2.35	3.35	5,280	6,221
5	70	0.8	6.71	2.32	3.33	5,320	6,902
6	63	0.8	4.47	2.43	3.53	4,482	7,248
7	75	0.6	6.22	3.14	4.60	5,150	6,442
8	70	1.08	6.14	1.74	2.59	5,200	6,518
9	65	1	7.12	1.92	2.83	5,284	6,442
10	70	0.8	7.27	2.35	3.39	5,280	6,518
11	70	0.8	6.36	2.35	3.37	4,982	6,758
12	65	0.6	4.11	3.11	4.46	4,251	7,112
13	70	0.5	7.12	3.55	5.22	5,462	6,542

Table 4. ANOVA and regression summary of models

Responses	*F* value	*P* value	R^2	Adjusted R^2	Predicted R^2	Adequate precision	SD	CV (%)
Resolution	40.15	<0.0001	0.9986	0.9982	0.9979	90.48	0.71	11.8
Retention time of amlodipine	1486.51	<0.0001	0.9991	0.9984	0.9947	123.60	0.02	0.80
Retention time of irbesartan	837.62	<0.0001	0.9983	0.9971	0.9912	91.97	0.04	1.17
Theoretical plate number of amlodipine	40.19	<0.0001	0.8832	0.8746	0.8149	18.47	260.23	5.10
Theoretical plate number of irbesartan	7.32	<0.0001	0.8394	0.8946	0.8125	11.54	235.39	3.47

$$\text{Resolution} = -213.75332 + 5.20912 \times \text{mobile phase} + 91.54879 \times \text{flow rate} - 1.25500 \times \text{flow rate} - 0.029740 \times \text{mobile phase}^2 - 2.46250 \times \text{flow rate}^2$$

$$\text{Retention time of amlodipine} = +4.58827 + 0.052022 \times \text{mobile phase} - 6.16724 \times \text{flow rate} - 0.041250 \times \text{mobile phase} \times \text{flow rate} - 0.000201 \times \text{mobile phase}^2$$

$$\text{Retention time of irbesartan} = +12.60050 - 0.105104 \times \text{mobile phase} - 8.73983 \times \text{flow rate} - 0.086000 \times \text{mobile phase} \times \text{flow rate} + 0.001196 \times \text{mobile phase}^2 + 6.36667 \times \text{flow rate}^2$$

$$\text{Theoretical Plate number of amlodipine} = -80224.85368 + 2256.24094 \times \text{mobile phase} + 14337.79753 \times \text{flow rate} - 250.75000 \times \text{mobile phase} \times \text{Flow rate} - 14.57250 \times \text{mobile phase}^2 + 2279.68750 \times \text{mobile phase}^2$$

$$\text{Theoretical Plate number of irbesartan} = +29035.49731 - 736.54782 \times \text{mobile phase} + 2696.02001 \times \text{flow rate} - 185.50000 \times \text{mobile phase} \times \text{flow rate} + 6.85057 \times \text{mobile phase}^2 + 5824.40865 \times \text{flow rate}^2$$

The optimization of obtained responses, guided by Derringer's proposed desirability function, was performed determining favorable system suitability criteria. These criteria encompassed achieving optimal separation between peaks by enhancing resolution, minimizing analysis time through a brief retention time for the second peak, and improving column efficiency with a high number of theoretical plates. The overlay plot depicted in Fig. 5 illustrates the optimal region within the design space. The chosen expected solution, with 69.8% v/v ethanol, a resolution of 6.5, a flow rate of 0.8, a retention time of 2.3 and 3.3 min and theoretical plates of 5,194 and 6,423 for the amlodipine and irbesartan respectively, which resulted in a desirability value of 1.000. The control method was determined based on the anticipated experimental conditions.

5.5. Validation of analytical procedure

5.5.1. Linearity, LOD, and LOQ. Under the modified chromatographic conditions, a robust linear correlation was

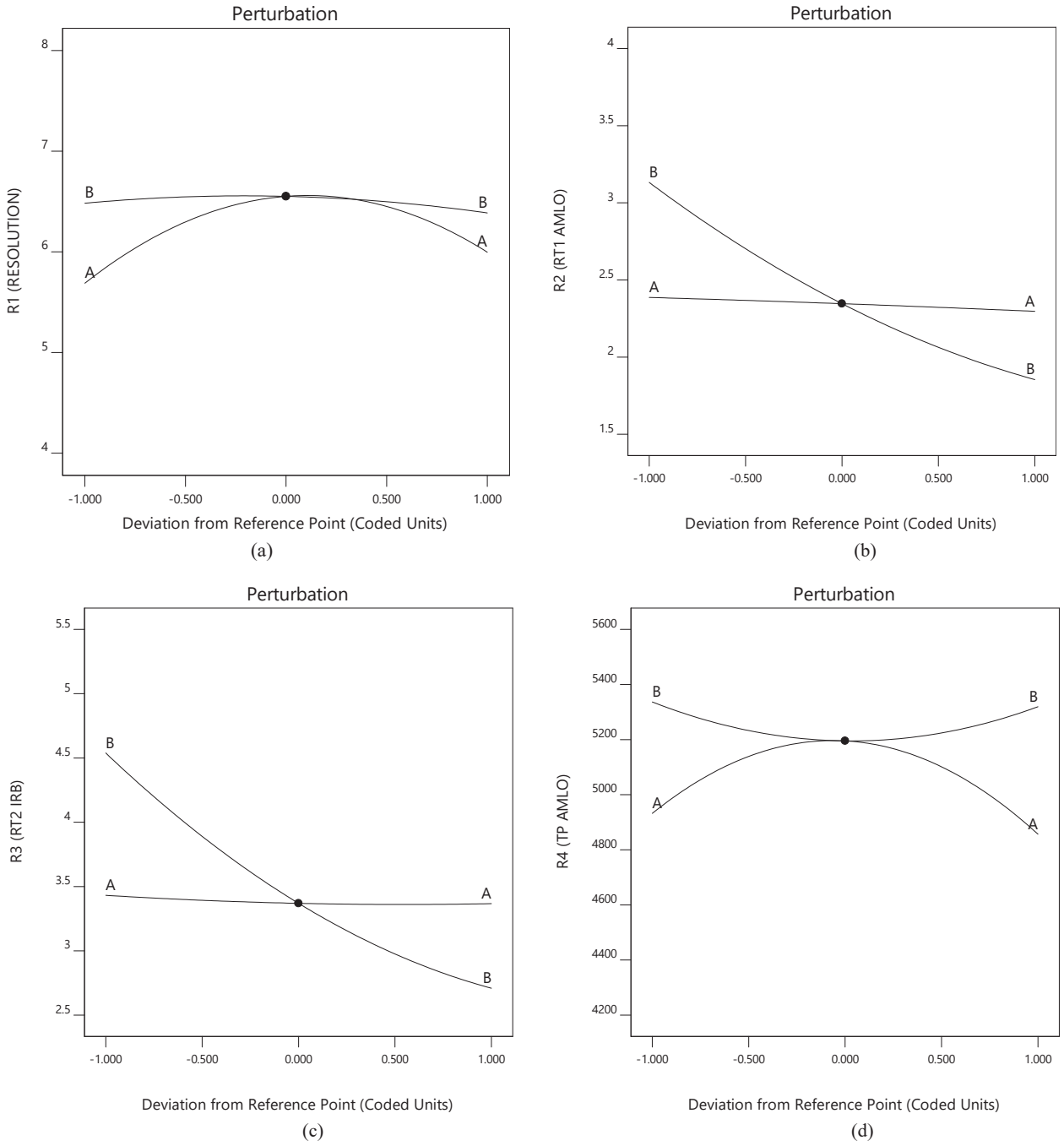
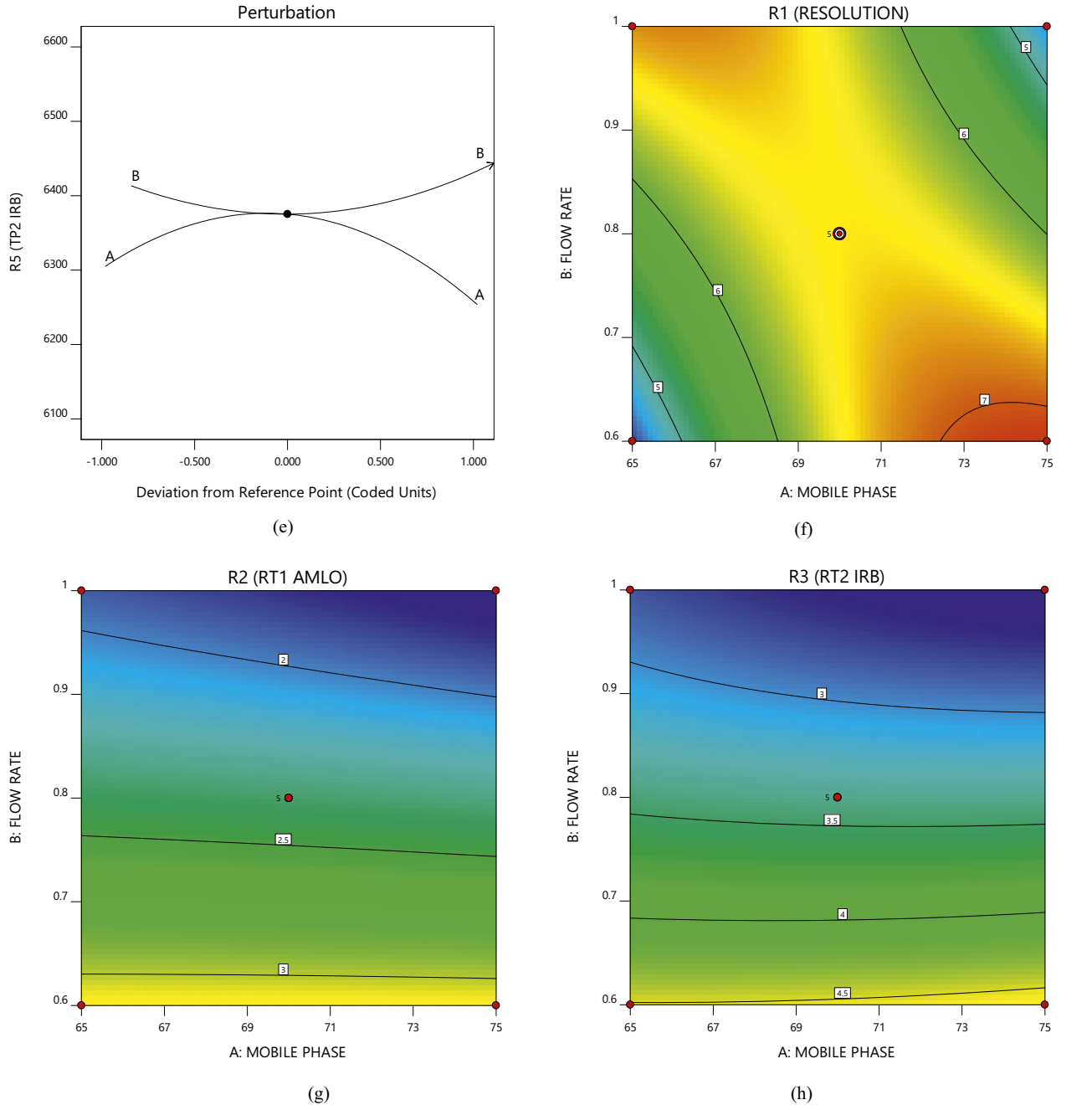


Fig. 4. Perturbation plot (a–e), contour plot (f–j), and 3-D response plot (k–o) for resolution, retention time, theoretical plate number of amlodipine and irbesartan respectively

(Figure 4 continued on next page)

identified. Spanning a range of concentration from 55.0 to 130.0 μg mL^{-1} for both drugs IRB and AM, this correlation was established. The regression coefficient (R^2) for IRB was 0.9943, and for AM, the R^2 was 0.9904, indicating an exceptionally high level of correlation in both instances. Detailed in Table 5 are the analytical results for linearity, encompassing slope and intercept. The calculation of the LOD and LOQ values utilized the SD of the response and the slope of the regression line, emphasizing the method's high sensitivity. For IRB, the determined values for the LOD and LOQ were 0.23 and 0.68 μg mL^{-1}, respectively. Conversely, for AM, the values were 0.33 and 1.00 μg mL^{-1}, respectively.

5.5.2. Accuracy and precision. The accuracy results of the method underscored the exceptional accuracy of the proposed methodology. Accuracy results are demonstrated in Table 6. Intraday and Interday precision experiments



(e) (f) (g) (h)

Fig. 4. Continued

(Figure 4 continued on next page)

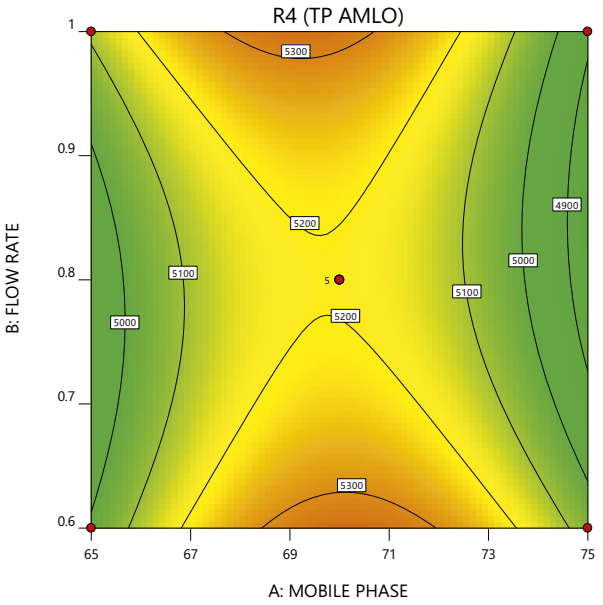
were conducted and the results are outlined in Table 6. The %RSD (below 2%) signifying a notable degree of precision.

5.5.3. System suitability. The obtained system suitability results are presented in Table 7. For all parameters, the %RSD obtained was not more than 2 which indicates the obtained results are within the limit.

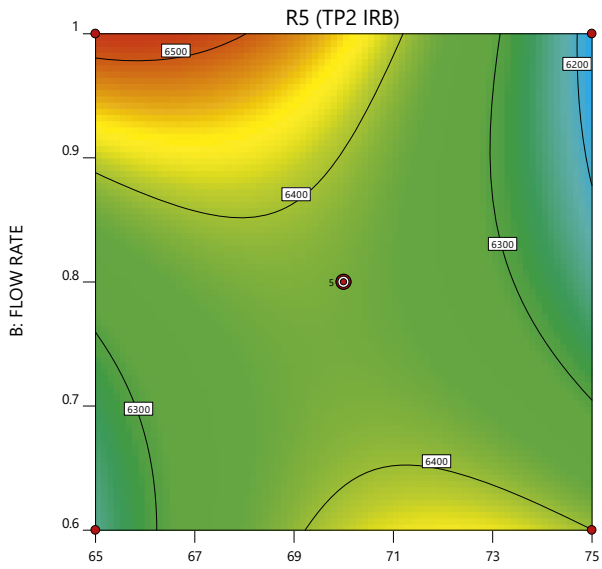
5.5.4. Solution stability. The chromatograms derived from the investigation of solution stability revealed the absence of degradation peaks and negligible changes in peak area over the 72-h duration. The test results demonstrated a deviation of less than 2% from the initial solution.

5.6. Results of forced degradation investigations

Results for degradation experiments performed on the combination of AM and IRB medicines are discussed below. The chosen primary degradation conditions included NaOH (0.01 M), HCl (0.01 M), 0.3% H_2O_2, and thermal exposure at 60°C. The drug AM was more sensitive to deterioration in acidic and alkaline circumstances, with an extra peak at Rt 2.92 and 2.93 in both acidic and



(i)



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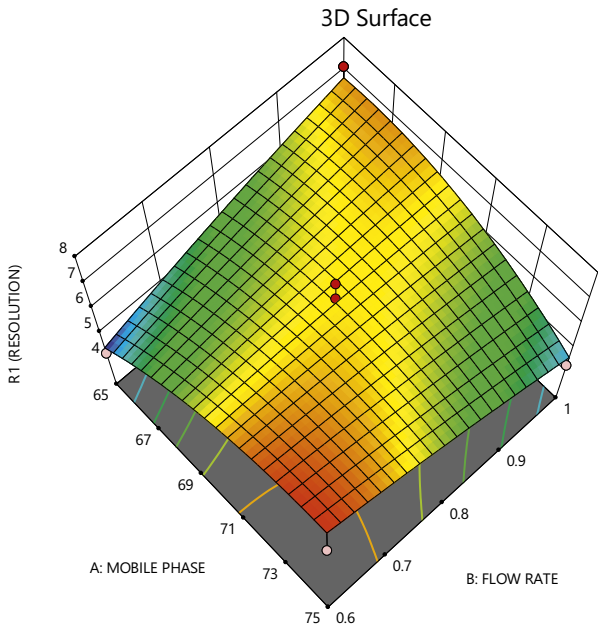

(k)

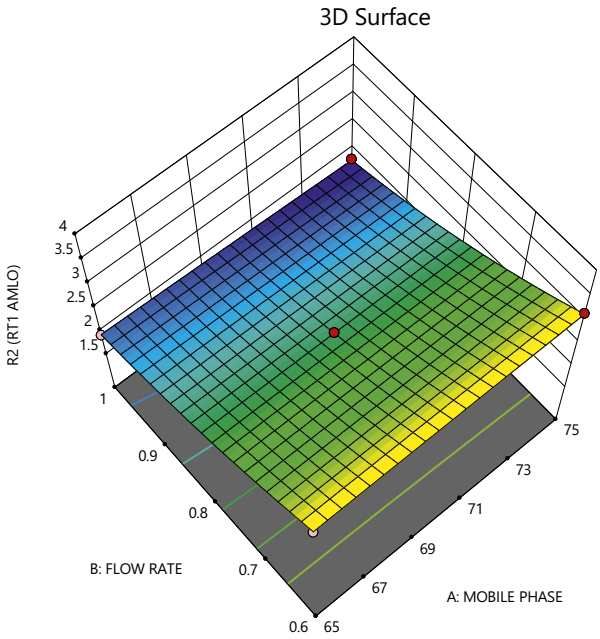

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Fig. 4. Continued

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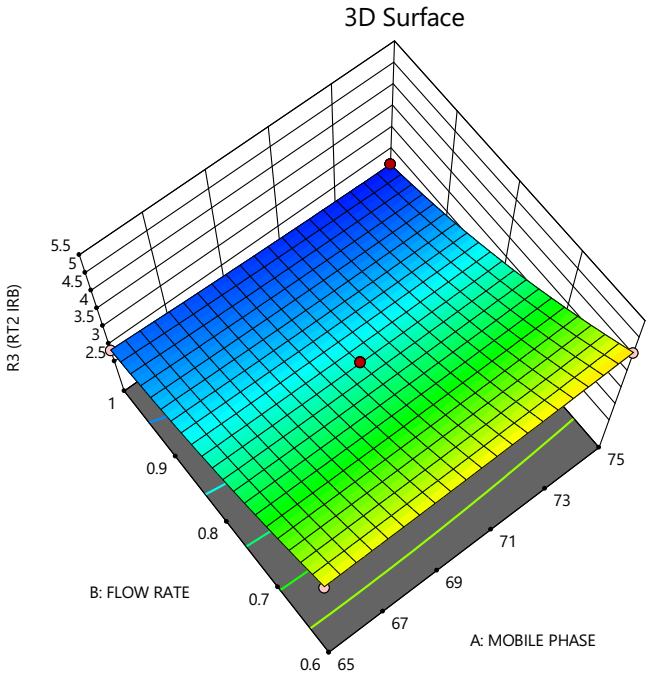
alkaline stress conditions (Fig. 6a and b). In the presence of 0.3% hydrogen peroxide, AM degraded slowly, whereas IRB was susceptible to hydrogen peroxide, with two additional peaks at 2.92 and 5.38 min and both medicines were observed to undergo heat deterioration with minimum degradation confirmed with reduction in peak area. Figure 6 and Table 8 present the results of the degradation investigation along with the accompanying chromatograms.

5.7. Assay of pharmaceutical dosage form

Assessing the effectiveness of the newly developed environmentally friendly HPLC method involved quantifying the concentrations of IRB and AM in the commercial product Irbepex AM. The suggested method exhibited robustness against the impact of tablet additives, ensuring precise quantification of drug ratios across three consecutive experiments utilizing the standard addition approach within



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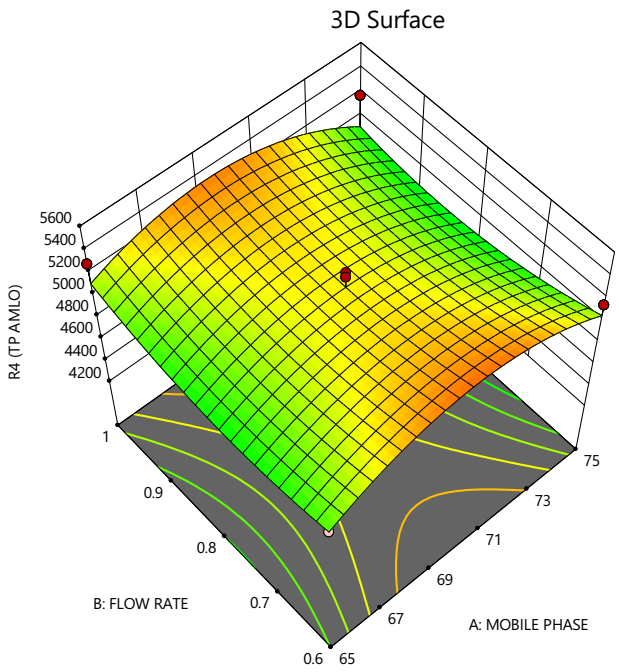

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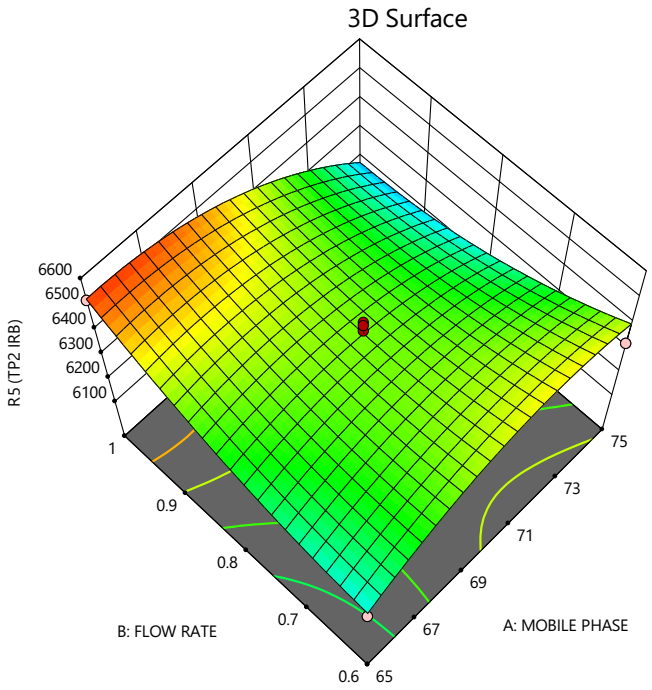


(o)

Fig. 4. Continued

permissible limits. Elaborated in Table 6 are the results of the assay.

In conclusion, the investigation showed that developing an RP-HPLC technique for AM and IRB can be successfully done using AQbD ideas. The technique of characterising the method objectives in the form of TMQP and identifying significant method attributes in the form of CAAs was used to establish the optimal chromatographic settings. It was easy to identify key method parameters as CMPs with the use of efficient risk assessment studies and proper application of response surface methodology. These CMPs were then optimised to produce the optimum chromatographic

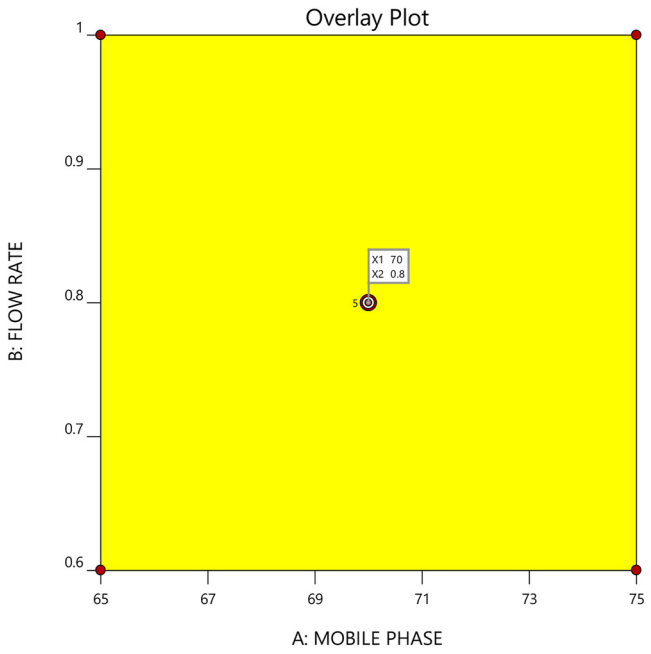


Fig. 5. Overlay plot for the interaction of ethanol and flow rate of the method

Table 5. Results of system suitability test parameters of developed HPLC method

Parameters	Mean ± SD	% RSD
Resolution	7.20 ± 0.05	0.74
Peak area of IRB	377.48 ± 4.73	1.25
Peak area of AM	293.67 ± 1.88	0.64
Retention time of IRB	3.37 ± 0.02	0.72
Retention time of AM	2.34 ± 0.01	0.58
Theoretical plates of IRB	6110.2 ± 7.1	0.12
Theoretical plates of AM	5406.5 ± 13.1	0.24
Tailing factor for IRB	1.12 ± 0.01	0.67
Tailing factor for AM	1.07 ± 0.0	0.50

*$n = 6$.

Table 6. Validation parameters for IRB and AM

Parameters	IRB	AM
Concentration (μg mL^{-1})	50.0–130.0	50.0–130.0
Regression equation	y = 6.6353x + 48.817	y = 8.8772x − 253.89
Correlation coefficient (R^2)	R^2 = 0.9943	R^2 = 0.9904
LOD (μg mL^{-1})	0.23	0.33
LOQ (μg mL^{-1})	0.68	1.00

*$n = 6$.

solutions to achieve the goals of the method. Additionally, a validation of the established method confirmed its efficiency and made it suitable for use in estimating AM and IRB in studies including both bulk and tablet dosage forms. There isn't yet a working RP-HPLC technique for AM and IRB that was created utilising the AQbD methodology. Since every established approach has used non green solvents [16–18] which are toxic to the environment. As a result, the designed and verified RP-HPLC provides a robust, cost effective and a rapid method to estimate AM and IRB in bulk and tablet form with shorter retention times (2.3 and 3.3 min).

5.8. Evaluation of environmental impact for the developed method

Modern analysts strive to create and develop environmentally friendly analytical techniques, promoting the use of cleaner solvents as substitutes for dangerous alternatives. The Green Analytical Chemistry (GAC) framework advocates for sustainable methods by using the concepts of the 3 R's - Replace, Reduce, and Re-use. This involves replacing harmful solvents with environmentally friendly alternatives or minimizing their use where necessary. A thorough study of acceptable criteria is necessary to determine the environmental sustainability of a given solution. In this study, Green Analytical Technique Index (GAPI), Analytical Eco Scale (AES), and software-driven Analytical Greenness Metric (AGREE) were employed to assess the performance of the technique. The widely used GAPI provides a quick, seamless, and dependable assessment of the environmental sustainability of analytical procedures. The application provides a fairly quantitative assessment of the environmental impact of the research process, utilizing color-coded pictograms for easy visual comparison. The GAPI diagram is shown in Fig. 7a. The Analytical Eco Scale (AES) is extra numerical assessment system that assigns penalty points (PP) based on reagents, instrumental energy, occupational hazards, and method-generated waste. In AES, the penalty points given are deducted from the maximum score of 100. High score is desirable in AES. The results of the computation using the Analytical Eco Scale are presented in Table 9.

5.8.1. AGREE metrics. A recently developed software-based tool, the Analytical Greenness Metric (AGREE), serves as a comprehensive instrument for evaluating the greenness profile by encompassing all 12 Green Analytical Chemistry (GAC) principles. Numerical value between 0 and 1 is given to each principle, showing the degree of its adherence to environmentally sound ideals, where a higher score suggests a greater dedication to environmental awareness. Visually representing greenness in a clock-like manner, AGREE assigns numbers 1–12, which correspond to the 12 GAC principles. As depicted in Fig. 7b, the technique achieved a commendable total score of 0.85, signifying a dedicated commitment to environmental considerations across all green principles. Utilizing the AGREE, AES and GAPI tools, Table 9 compares the greenness of the published and proposed methods.

Table 7. Recovery and precision results for IRB and AM

Drug	Amount of standard taken (μg mL^{-1})	Amount of sample added (μg mL^{-1})	Average concentration in the spiked sample (μg mL^{-1})	% Recovery	Mean ± SD	Intraday %RSD	Interday %RSD
IRB	70	0	69.2	98.9	100.2 ± 1.2	0.08	0.14
	70	0	70.2	100.4			
	70	0	71.0	101.4			
	85	15	100.3	100.3	100.0 ± 0.3	0.01	0.07
	85	15	99.7	99.7			
	85	15	100.3	100.3			
	100	30	130.5	100.1	100.4 ± 0.4	0.03	0.05
	100	30	131.3	101.0			
	100	30	130.5	100.4			
AM	70	0	70.1	100.2	100.0 ± 0.2	0.05	0.23
	70	0	69.8	99.8			
	70	0	70.1	100.1			
	85	15	99.9	99.9	99.8 ± 0.3	0.04	0.08
	85	15	100.1	100.1			
	85	15	99.54	99.54			
	100	30	129.9	100.0	99.9 ± 0.1	0.06	0.04
	100	30	130.1	100.1			
	100	30	129.8	99.9			

*$n = 3$.

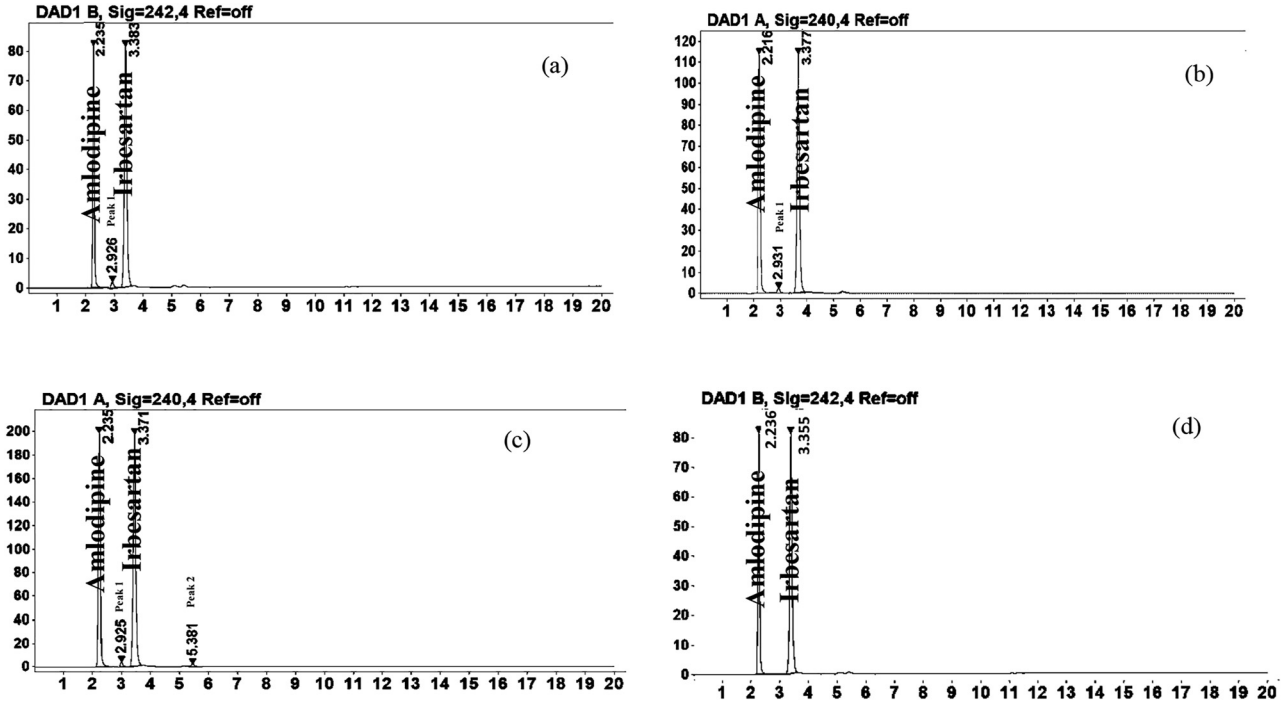


Fig. 6. The chromatograms showing degradation peaks for IRB and AM in (a) acid (b) alkali (c) peroxide and (d) thermal degradation conditions

Table 8. Forced degradation of AM and IRB

Type of degradation	Degradation condition	% Drug recovery		% Drug degradation	
		AM	IRB	AM	IRB
Acid degradation	0.01 M HCL/12 h	91.1	92.3	8.8	7.6
Alkali degradation	0.01 M NaOH/12 h	89.3	90.0	10.6	9.9
Peroxide degradation	0.3% H_2O_2/12 h	91.2	90.6	8.7	9.3
Thermal degradation	80 °C/12 h	98.7	96.5	1.2	3.4

*$n = 3$.

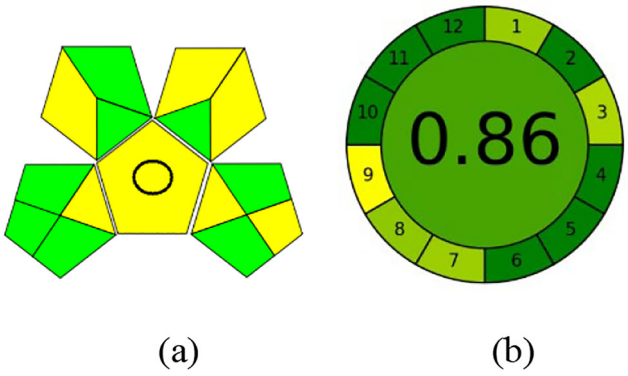
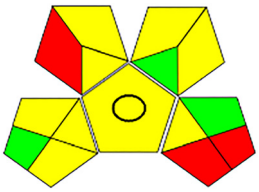
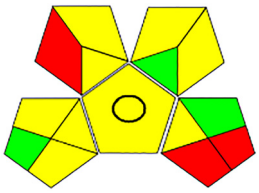
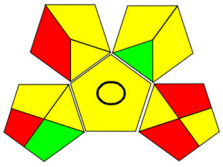
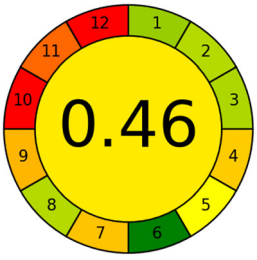
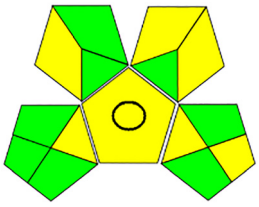


Fig. 7. a) GAPI, b) AGREE metrics. Pictograms depicting the green assessment results for the developed method

Table 9. Comparison of green assessment between developed and previously reported HPLC methods

S.No	Reported by	Optimized Chromatographic Conditions	GAPI	AES	AGREE
1.	Kamala bodapati et al.	RP-HPLC, 1 mM potassium dihydrogen phosphate (pH 3.0): acetonitrile (70:30 v/v)		14+1+1+3+3=21 AES= 79	
2.	T Hemanth Kumar et al.	RP-HPLC, Acetonitrile: Sodium acetate (pH 4.0), ratio (30:70 v/v)		14+1+1+3+3=21 AES= 79	
3.	Majdi M. Bkhaitan et al.	RP-HPLC, Acetonitrile: o-phosphoric acid buffer (pH 2.2), Gradient elution		14+4+1+3+3 AES= 75	
4.	Current developed method	RP-HPLC, Ethanol: 0.1% o-phosphoric acid (70:30 v/v)		4+4+1+0+0 =9 AES= 91	

6. CONCLUSION

The study demonstrates the collaborative integration of GAC and AQbD to formulate a precise HPLC method for quantifying IRB and AM in bulk and pharmaceutical formulations. Method shows superiority in terms of lesser retention time, wider linearity range, accuracy, and repeatability. The application of AQbD facilitates the systematic exploration of method variables, concluding the establishment of robust and reliable methodologies suitable for deployment in quality control laboratories. This eliminates the requirement for regular revalidation. Experimental investigations were conducted using statistical optimization, specifically adopting a central composite design. The obtained results unequivocally identified the ideal operating conditions within the designated design area, which practical validation experiments then confirmed. Following the principles of the GAC, ethanol was selected as the organic solvent, substituting potentially dangerous alternatives. Ultimately, assessments conducted with green evaluation tools confirmed the method's exceptional environmental compatibility, making it extremely appropriate for industrial applications and regular quality control.

Author contributions: Sinchana B Gopalaiah: Investigation, formal analysis, data curation, conceptualization, validation, writing-original draft, writing-review, and editing. Dr. Kavitha Jayaseelan: Supervision.

Conflict of interest: Authors declare no conflict of interest.

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SUPPLEMENTARY MATERIALS

Supplementary data to this article can be found online at https://doi.org/10.1556/1326.2024.01205.

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