DEVELOPMENT AND AQBD-GUIDED OPTIMIZATION OF A ROBUST RP-UPLC METHOD FOR CONCURRENT ESTIMATION OF EMPAGLIFLOZIN AND ALOGLIPTIN IN DRUG SUBSTANCES AND FORMULATIONS

Rishi Kumar [1]*, Rihana Syed [2,3],Venkata Rao Vutla[4]

[1]AssociateProfessor,School of Pharmacy, Raffles University,Neemrana-301705.

[2]Research Scholar, School of Pharmacy, Raffles University, Neemrana – 301705.

[3]Assistant Professor, Department of Pharmaceutical Analysis, Chebrolu Hanumaiah Institute of Pharmaceutical Sciences, Chowdavaram, Guntur,A.P. India.

[4]Professor,Department of Pharmaceutical Analysis, Chebrolu Hanumaiah Institute of Pharmaceutical Sciences, Chowdavaram, Guntur, A.P., India - 522019.

* Corresponding Author: Rishi Kumar, Associate Professor, SchoolofPharmacy,RafflesUniversity,Neemrana-301705, India.

Email: rishi.kumar@raffles university.edu.in Contact: +91-9911246653

ABSTRACT

Analytical Quality by Design (AQbD) extends the principles of Quality by Design (QbD) across the entire lifecycle of analytical methods, encompassing systematic development, multivariate optimization, validation, and continuous improvement. This risk-based approach enables the development of robust, reproducible, and cost-effective analytical methods with enhanced method understanding and control a fixed-dose combination of empagliflozin (EGN), a sodium-glucose co-transporter-2 (SGLT2) inhibitor, and alogliptin (AGN), a dipeptidyl peptidase-4 (DPP-4) inhibitor, is widely used in the management of type 2 diabetes mellitus due to their complementary antihyperglycemic mechanisms. In the present study, a stability-indicating reversed-phase ultra-performance liquid chromatography (RP-UPLC) method was developed and validated for the simultaneous quantification of EGN and AGN in pharmaceutical dosage forms using an AQbD-based framework. Method development was guided by risk assessment using an Ishikawa fishbone diagram and optimized through Design-Expert® software employing a design of experiments (DoE) approach, wherein the effects of critical method parameters including flow rate, organic phase composition, and column temperature were evaluated on critical quality attributes such as retention time, resolution, theoretical plates, and peak tailing. The optimized chromatographic conditions consisted of a mobile phase of 0.1 N potassium dihydrogen phosphate buffer (pH 4.7):acetonitrile (63:37, v/v), a column temperature of 30 °C, a flow rate of 0.3 mL/min, and UV detection at 252 nm, resulting in retention times of 0.977 min for EGN and 1.320 min for AGN. The method exhibited linearity over the concentration ranges of 5–30 µg/mL for EGN and 1–6 µg/mL for AGN, with LODs of 0.04 and 0.07 µg/mL and LOQs of 0.12 and 0.21 µg/mL, respectively. Precision studies showed %RSD values below 1%, while recovery values of 99.9% for EGN and 100.3% for AGN confirmed accuracy and repeatability. Forced degradation studies under acidic, alkaline, oxidative, neutral, photolytic, and thermal conditions confirmed the stability-indicating nature of the method. Overall, the developed RP-UPLC method is simple, rapid, sensitive, precise, and robust, making it suitable for routine quality control and regulatory analysis of drug formulations.

CONCLUSION

A stability-indicating RP-UPLC method was successfully developed and validated for the simultaneous determination of empagliflozin and alogliptin in pharmaceutical formulations using an AQbD-driven approach. The application of systematic risk assessment (Ishikawa fishbone diagram) and multivariate experimental design enabled effective identification and control of critical method parameters, ensuring method robustness and consistency.

The validated method demonstrated excellent linearity, sensitivity, precision, and accuracy, along with strong stability-indicating capability under various forced degradation conditions. The AQbD framework ensured comprehensive method understanding and lifecycle control, confirming the suitability of the proposed method for routine quality control and regulatory applications.

KEYWORDS

RP-UPLC; Empagliflozin; Alogliptin; Analytical Quality by Design (AQbD); Design of Experiments; Ishikawa diagram; Stability-indicating assay; Method validation; Forced degradation.

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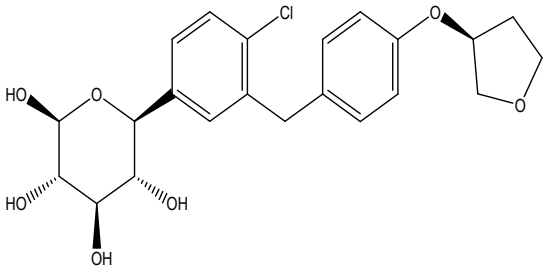
INTRODUCTION

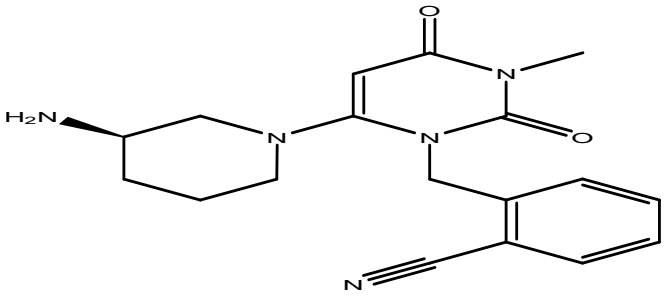
Type 2 diabetes mellitus (T2DM) is one of the most prevalent endocrine disorders worldwide and continues to increase at an alarming rate, representing a major global public health challenge. Current epidemiological projections suggest that the number of individuals living with diabetes will exceed 500 million by 2040 and surpass 800 million by 2055 [1,2]. Diabetes mellitus encompasses a heterogeneous group of metabolic disorders characterized by chronic hyperglycemia resulting from impaired insulin secretion, defective insulin action, or a combination of both, leading to disturbances in carbohydrate, lipid, and protein metabolism [3]. The disease and its associated complications impose a substantial burden on healthcare systems due to their significant impact on morbidity, mortality, and overall quality of life [2,4]. Poor glycemic control is strongly associated with the development of severe long-term complications, including cardiovascular diseases, nephropathy, neuropathy, retinopathy, and increased risk of mortality [4,5].Diabetes mellitus is broadly classified into type I and type II forms [3]. Type I diabetes arises from autoimmune-mediated destruction of pancreatic β-cells, resulting in absolute insulin deficiency, whereas T2DM is primarily characterized by insulin resistance in conjunction with relative insulin deficiency [6]. T2DM accounts for the majority of cases and is closely associated with genetic susceptibility, obesity, sedentary lifestyle, and unhealthy dietary habits [1,3]. The primary goal of diabetes management is to achieve and maintain optimal glycemic control in order to prevent or delay the onset and progression of complications [3]. Management strategies encompass both non-pharmacological approaches, including lifestyle modifications such as dietary regulation and regular physical activity, and pharmacological interventions involving oral hypoglycemic agents and insulin therapy [3,7]. In this context, combination therapy has emerged as a preferred approach over monotherapy, as the use of agents with complementary mechanisms of action enhances therapeutic efficacy, improves metabolic outcomes, and overcomes the limitations associated with single-drug regimens [7,38]. Continuous advancements in antidiabetic drug development remain essential to ensure improved efficacy, safety, and long-term disease management [7]. Empagliflozin (EMPA) is a recently developed oral antidiabetic drug primarily used in the treatment of type 2 diabetes mellitus. It produces its therapeutic effect through the selective inhibition of sodium–glucose cotransporter-2 (SGLT-2) [8]. This class of SGLT-2 inhibitors reduces blood glucose levels by acting on renal pathways, specifically by decreasing glucose reabsorption in the kidneys and enhancing urinary glucose excretion [9].

A variety of analytical methods have been reported for the determination of EMPA in bulk drug substances, pharmaceutical formulations, and human plasma. These include spectrophotometric techniques [17,18] and liquid chromatographic methods [19-31]. Related substances associated with EMPA may originate from the manufacturing process, degradation under improper storage conditions, or metabolic pathways in vivo. Such substances may be pharmacologically active, inactive, or potentially toxic [29].Chemically, empagliflozin is designated as n-glucitol, 1,5-anhydro-1-C-[4-chloro-3-[[4-[[(3S)-tetrahydro-3-furanyl]oxy]phenyl]methyl]phenyl]-, (1S). SGLT-2 is highly expressed in the kidneys and plays a crucial role in glucose transport and reabsorption from the glomerular filtrate into systemic circulation. Inhibition of this transporter by empagliflozin leads to reduced renal glucose reabsorption and increased urinary glucose elimination [30,31]. Alogliptin is an orally administered antidiabetic drug belonging to the dipeptidyl peptidase-4 (DPP-4) inhibitor class. It has a molecular formula of $C_{18}H_{21}N_5O_2$ and an IUPAC name of 2-({6-[(3R)-3-aminopiperidin-1-yl]-3-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl}methyl)benzonitrile. Alogliptin is indicated for the improvement of glycemic control in patients with type II diabetes mellitus, either as monotherapy or in combination with metformin or a peroxisome proliferator-activated receptor gamma (PPAR-γ) agonist, such as thiazolidinediones, when monotherapy fails to provide adequate glycemic control [32,33].A limited number of analytical methods have been reported for the determination of alogliptin, including spectrophotometric and high-performance liquid chromatographic (HPLC) techniques [34–37]. However, no published reports describe a rapid UPLC method with a shorter retention time for the simultaneous estimation Empagliflozin and Alogliptin in bulk drug. The present study therefore aims to develop and validate a simple, rapid, and reliable UPLC method for the quantification of Empagliflozin and alogliptin in bulk dosage form in accordance with International Council for Harmonisation (ICH) guidelines Combination therapy involving Empagliflozin and Alogliptin has gained considerable attention due to their complementary mechanisms of action, resulting in enhanced glycemic

control [38,39]. Empagliflozin, a sodium–glucose co-transporter-2 (SGLT-2) inhibitor, lowers plasma glucose levels by inhibiting renal glucose reabsorption and promoting urinary glucose excretion [39]. In contrast, alogliptin, a dipeptidyl peptidase-4 (DPP-4) inhibitor, enhances incretin activity, thereby stimulating insulin secretion and suppressing glucagon release [40]. Quality by Design (QbD) is a systematic, science-based approach to pharmaceutical development that emphasizes predefined objectives and process understanding to ensure consistent product quality [41]. The application of QbD principles to analytical method development has led to the emergence of Analytical Quality by Design (AQbD), which focuses on developing robust, reliable, and reproducible analytical methods [42]. This approach utilizes risk assessment and systematic experimentation to enhance method performance, reduce variability, and provide regulatory flexibility[42,43]. The AQbD framework aligns with the principles outlined in International Council for Harmonisation guidelines, particularly ICH Q8 (1). A key outcome of AQbD is the establishment of the Method Operable Design Region (MODR), a multidimensional space within which method parameters consistently yield acceptable performance [43]. The process begins with defining the Analytical Target Profile (ATP), which outlines the intended purpose and performance requirements of the method, analogous to the Quality Target Product Profile (QTPP) in QbD [42,44]. The ATP encompasses critical analytical attributes such as specificity, accuracy, precision, linearity, range, limit of detection (LOD), and limit of quantification (LOQ) [44]. Once the ATP is established, Critical Method Attributes (CMeAs) are identified along with their acceptance criteria and specifications. A quality risk assessment is then performed to determine the Critical Method Parameters (CMePs) the factors that have a significant impact on method performance. The relationships between CMeAs and CMePs are experimentally evaluated, and their influence on method performance is quantified using Design of Experiments (DoE)[45-47]. Subsequently, Critical Method Attributes (CMeAs) and Critical Method Parameters (CMePs) are identified through risk assessment. The relationships between these variables are systematically evaluated using Design of Experiments (DoE) to understand their impact on method performance. Analytical methods developed under AQbD principles demonstrate enhanced robustness and reduced incidence of out-of-specification (OOS) and out-of-trend (OOT) results. Consequently, AQbD is increasingly adopted in pharmaceutical research and development, supporting risk-based decision-making, regulatory compliance, and continuous improvement within the pharmaceuticalqualitysystem



EMPAGLIFLOZIN



ALOGLIPTIN

FIG. 1 STRUCTURE OF EMPAGLIFLOZIN (A) AND ALOGLIPTIN (B)

CHEMICALS AND REAGENTS

Empagliflozin and alogliptin reference standards were obtained from Lee Pharma Ltd for aloptin Srini Pharmaceuticals for empagliflozin. Analytical reagent (AR) grade hydrochloric acid and sodium hydroxide were procured from Rankem, India. Hydrogen peroxide was obtained from Qualigens. Acetic acid (AR grade) was purchased from Scientific, India, and S.D. Fine Chem Ltd. Potassium dihydrogen orthophosphate and orthophosphoric acid were sourced from S.D. Fine Chem Ltd. and Merck India Pvt. Ltd., respectively. UPLC-grade acetonitrile and methanol were supplied by Fischer Scientific. High-purity water was generated using a Merck Milli-Q water purification system. All other chemicals and reagents used were of analytical grade and employed without further purification.

Table:1 Variables and their levels in central composite design

Factor	Name	Units	Type	SubType	Minimum	Maximum	CodedLow	CodedHigh	Mean
A	FR	ml/min	Numeric	Continuous	0.2700	0.3300	-1↔27.0	+1↔33.0	30.00

							0	0	
B	MP(Organicphase)	%	Numeric	Continuous	35.00	45.00	-1↔35.00	+1↔45.00	40.00
C	Temp	0C	Numeric	Continuous	27.00	33.00	-1↔27.00	+1↔33.00	30.00

INSTRUMENTATION AND CHROMATOGRAPHIC CONDITIONS

Chromatographic analysis was performed using a Waters Acquity UPLC H-Class system equipped with a binary solvent manager, autosampler, and tunable UV detector. Data acquisition and processing were carried out using Empower 2 software. Separation was achieved on an Acquity UPLC HSS column (100 mm × 2.1 mm, 1.7 µm). The mobile phase consisted of 0.1 N potassium dihydrogen phosphate buffer and acetonitrile in the ratio of 63:37 (v/v), delivered at a flow rate of 0.3 mL/min. The column temperature was maintained at 30 °C, and detection was performed at 252 nm. The injection volume was 1 µL, with a total run time of 3 min. The mobile phase was used as the diluent throughout the analysis.

PREPARATION OF STANDARD STOCK SOLUTIONS

Accurately weighed quantities of 2 mg of alogliptin and 10 mg of empagliflozin were transferred into a clean, dry 50 mL volumetric flask. About 20 mL of diluent was added, and the mixture was sonicated for 10 minutes to ensure complete dissolution. The solution was then diluted to volume with the same diluent to obtain a standard stock solution containing 40 µg/mL of alogliptin and 200 µg/mL of empagliflozin.

From this stock solution, 1 mL was transferred into a 10 mL volumetric flask and diluted to volume with diluent to obtain an intermediate working solution. Further appropriate dilutions were made from this solution to prepare working standards in the concentration ranges of 1–6 µg/mL for alogliptin and 5–30 µg/mL for empagliflozin.

PREPARATION OF SAMPLE SOLUTION

Ten tablets , each containing 20 mg of alogliptin (Aloja) and 100 mg of empagliflozin(Glempa), were accurately weighed to determine the average tablet weight and then finely powdered using a mortar and pestle. A portion of the powder equivalent to 20 mg of alogliptin and 100 mg of empagliflozin was transferred into a 100 mL volumetric flask. An appropriate volume of diluent was added, and the mixture was sonicated for 20 minutes to ensure complete extraction of the analytes. The solution was filtered through a 0.22 µm nylon membrane filter, and the filtrate was collected and diluted to volume with diluent to obtain a stock sample solution containing 200 µg/mL of alogliptin and 1000 µg/mL of empagliflozin. Further dilution was performed by transferring 0.4 mL of this solution into a 10 mL volumetric flask and diluting to volume with diluent to obtain the final sample solution containing 4 µg/mL of alogliptin and 20 µg/mL of empagliflozin, suitable for analysis.

METHOD OPTIMIZATION BY EXPERIMENTAL DESIGN

The chromatographic conditions were optimized using a Box–Behnken Design (BBD) to evaluate the main, interaction, and quadratic effects of critical method variables on analytical responses. The design consisted of three independent factors studied at three levels, and the experiments were generated using Design-Expert® software (Version 11.1.0.1, Stat-Ease Inc., USA). Based on preliminary trials, a mobile phase comprising 0.1 N potassium dihydrogen phosphate buffer and acetonitrile was selected. The independent variables included flow rate (X_1), percentage of acetonitrile (X_2), and column temperature (X_3). The dependent responses were retention time of empagliflozin (Y_1) and alogliptin (Y_2), resolution (Y_3), theoretical plate count of empagliflozin (Y_4) and alogliptin (Y_5), and tailing factors of empagliflozin (Y_6) and alogliptin (Y_7). A total of 17 experimental runs were performed as per the BBD matrix. A standard mixture containing 20 µg/mL of empagliflozin and 4 µg/mL of alogliptin was used for all runs. The Method Operable Design Region (MODR) was established to define optimal conditions ensuring robust chromatographic performance.

METHOD VALIDATION

The developed RP-UPLC method based on the Quality by Design (QbD) approach was validated in accordance with ICH Q2 (R1) guidelines, evaluating system suitability, linearity, limit of detection (LOD), limit of quantification (LOQ), precision, accuracy, and robustness. System suitability was assessed by injecting six replicate injections of standard solutions containing alogliptin (4 µg/mL) and empagliflozin (20 µg/mL), and evaluating parameters such as theoretical plates, resolution, tailing factor, %RSD of retention time, and peak area.

LINEARITY

Linearity was established by constructing calibration curves of peak area versus concentration over six concentration levels. The LOD and LOQ were calculated based on the standard deviation (σ) of the response and the slope (S) of the calibration curve, using the expressions 3.3σ/S and 10σ/S, respectively.

S.NO	Method precision of Empagliflozin		Method precision of Alogliptin		Day by Day precision		SST	
1	28773	54078	27912	54395	27615	53579	27642	53953
2	28321	54570	27449	54029	27348	53797	27403	54206
3	28811	55073	27748	54321	27671	53826	27679	53778
4	28606	54480	27635	54099	27328	53568	27648	54033
5	28803	54613	27764	54738	27679	53543	27730	53572
6	27313	53990	27973	54053	27563	53630	27655	54155
Average	28437	54467	27746	54273	27534	62585	27625	53950
SD	241.1	397	190.1	273.4	160.3	131.6	113.1	233.2
%RSD	0.8	0.7	0.7	0.5	0.6	0.21	0.4	0.4

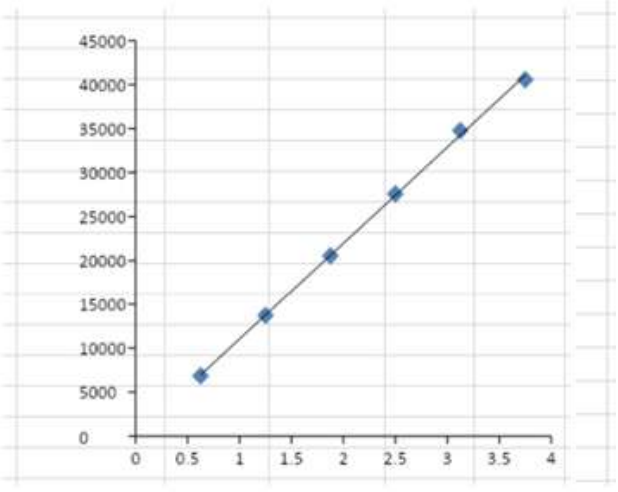


Figure:2 Calibration curve for Empagliflozin and Alogliptin

PRECISION

Method precision was evaluated by intra-day and inter-day studies. Intra-day precision (repeatability) was determined by analyzing three concentrations of alogliptin (2, 4, and 6 µg/mL) and empagliflozin (10, 20, and 30 µg/mL) in triplicate within the same day. Inter-day precision (intermediate precision) was assessed by analyzing the same concentration levels over three consecutive days.

Table:2 Precision Results for Empagliflozin and Alogliptin

LIMITS OF DETECTION AND QUANTIFICATION

The ICH standard recommendations were followed to determine the limits of detection (LOD) and quantification (LOQ). Known analytes at low concentrations were compared with the signals of blank samples, and the resulting chromatograms were analyzed. Signal-to-noise ratios of 3:1 and 10:1 were used to calculate the LOD and LOQ, respectively. As shown in Table 3, the method exhibited LOD and LOQ values of 0.04 µg/mL and 0.12 µg/mL, respectively, for Empagliflozin, and 0.07 µg/mL and 0.21 µg/mL, respectively, for Alogliptin.

Table:3Linear regression data for Empagliflozin and Alogliptin

S.NO	Statistical parameter	UPLC
1.	Correlation coefficient of Empagliflozin($R^{2)}$	0.999
2	Correlation coefficient of Alogliptin($R^{2)}$	0.999
3	Limit of Detection (LOD) µg/mL Empagliflozin	0.04µg/mL
4	LimitofQuantification(LOQ)µg/mL Empagliflozin	0.12µg/mL
5	LimitofDetection(LOD)µg/ Alogliptin	0.07µg/mL
6	LimitofQuantification(LOQ)µg/mL Alogliptin	0.21µg/mL

ACCURACY

Accuracy was determined through recovery studies performed at 50%, 100%, and 150% spiking levels of pre-analyzed samples, and the percentage recovery of empagliflozin and alogliptin was calculated. Specificity was confirmed by comparing chromatograms of blank, placebo, and standard

solutions, demonstrating no interference at the retention times of the analytes.

Table4: Accuracy Data Of Empagliflozin and Alogliptin

Spiked level ./.	Sample area of Empagliflozin	Sample area of Alogliptin	µg/ml	Percentage recovery of Empagliflozin	Percentage recovery of Alogliptin	Percentage mean recovery of Empagliflozin	Percentage mean recovery of Alogliptin
50 ./.	61373	80973	50	99.78	99.08	99.92	99.03
	61445	80949	50	100.35	98.98		
	61323	80960	50	99.63	99.05		
100./.	81396	108877	100	99.20	100.16	99.01	100.30
	81129	108349	100	98.60	100.20		
	81476	108628	100	99.23	100.61		
150./.	102487	134382	150	100.64	98.71	100.30	99.35
	102284	135055	150	100.39	99.96		
	102038	134684	150	99.89	99.39		

ROBUSTNESS

Robustness was evaluated by intentionally varying chromatographic conditions, including flow rate (±0.3 mL/min), percentage of acetonitrile in the mobile phase (±3%), and column temperature (±3 °C), to assess the reliability of the method under small deliberate changes.

TABLE5: RESULTS OF ROBUSTNESS STUDY

Parameter	Modification	Retentiontime of Empagliflozin	Retention time of Alogliptin	RS	Theoretical plates of Empagliflozin	Theoretical plates of Alogliptin	Tailingfactor of Empagliflozin	Tailing factor of Alogliptin
Flow rate	0.2700	1.214	1.599	3.1	2884	2962	1	0.89
	Optimized	1.262	1.643	3	3110	3507	1.04	0.9
	0.33 mL/min	1.234	1.580	3	2960	3596	1	0.9
% mobile phase	27.00	1.124	1.395	2.4	2651	3409	1	0.93
	Optimized	1.120	1.435	2.7	2675	3228	1.03	0.94
	33.00% acetonitrile	1.186	1.474	2.5	2867	3850	1	0.98
Column temperature	27.00°C	1.013	1.353	2.7	2456	2698	1	1
	Optimized	1.053	1.343	2.8	2558	3112	1	1.03
	33.00°C	1.022	1.319	2.6	2399	3041	1	1.04

SAMPLE SOLUTION STABILITY

The stability of Empagliflozin and Alogliptin in the prepared sample solution was assessed by storing the solution at room temperature in a volumetric flask. The retention time and peak area were recorded after 24 hours and compared with the initial values to evaluate any significant variation in analyte stability over time.

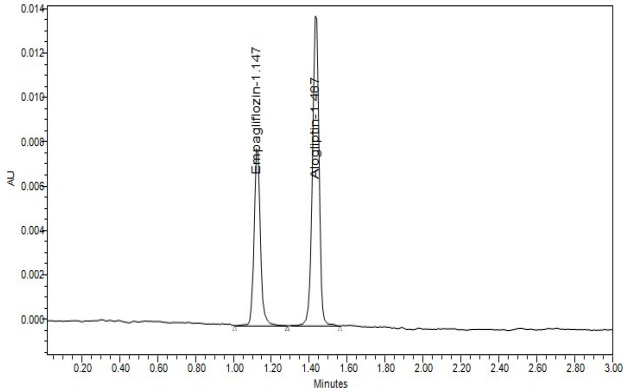


Figure:3 solution stability Chromatogram

FORCED DEGRADATION STUDIES

Forced degradation studies were conducted to evaluate the stability-indicating capability of the developed RP-UPLC method for empagliflozin and alogliptin under various stress conditions. The drug solutions were subjected to thermal degradation (105 °C for 1 hour), photolytic exposure (UV light for 24 hours), neutral hydrolysis (water at 60 °C for 1 hour), alkaline hydrolysis (2 N NaOH at 60 °C for 30 minutes), and acidic hydrolysis (2 N HCl at 60 °C for 30 minutes). Following exposure to stress conditions, the samples were appropriately diluted to obtain final concentrations of 4 µg/mL for Alogliptin and 20 µg/mL for Empagliflozin. Subsequently, 5 µL of each solution was injected into the UPLC system, and chromatograms were recorded to assess drug stability and degradation behavior.

STATISTICAL ANALYSIS

All experimental data were expressed as mean ± standard deviation (SD). Statistical parameters, including mean, SD, percentage relative standard deviation (%RSD), and regression coefficients, were calculated using Microsoft Excel. Analysis of variance (ANOVA) was applied to evaluate the significance of the model and individual terms. A p-value of less than 0.05 was considered statistically significant.

RESULTS

METHOD DEVELOPMENT STUDIES

The RP-UPLC method was developed through systematic evaluation of various chromatographic conditions, including different mobile phase compositions (acetonitrile, methanol, ethanol, water, and phosphate buffer at specific pH values), flow modes (isocratic and gradient), stationary phases (C18 and C8 columns), and column temperatures. Based on preliminary optimization studies, a mobile phase consisting of 0.1 N potassium dihydrogen phosphate buffer and acetonitrile was selected as optimal. This combination provided efficient separation of empagliflozin and alogliptin with minimal peak tailing, reduced retention time, and improved peak symmetry.

Table6: Central composite design along with the observed values for optimization of RP-UPLC method for Empagliflozin and alogliptin

Run No	X1: Flow rate (ml/min)	X2: %ORGANIC PHASE	X3: Ten (°C)	Y1: Retention time Empagliflozin	Y2: Retention time Alogliptin	Y3: Resolution factor	Y4: Theoretical plates Empagliflozin	Y5: Theoretical plates Alogliptin	Y6: Tailing factor Empagliflozin	Y7: Tailing factor Alogliptin
1	0.27	40	27	1.282	1.655	3	3111	3526.9	1.04	1
2	0.33	35	30	1.024	1.353	2.9	2466.7	2704	1	1
3	0.27	35	30	1.214	1.608	3.1	2893.8	2971.8	1	0.98
4	0.33	40	33	1.015	1.303	2.6	2381.8	3105.3	1.04	0.9
5	0.3	45	33	1.132	1.404	2.5	2636.9	3438.4	1.03	0.98
6	0.3	40	30	1.137	1.442	2.7	2696	3271	1.03	0.96
7	0.3	40	30	1.133	1.442	2.8	2706	3307	1.03	0.95

8	0.3	40	27	1.129	1.442	2.8	2684	3236	1.03	0.96
9	0.3	35	30	1.142	1.514	3.0	2819.5	2860.2	1	0.96
10	0.33	45	33	1.031	1.327	2.7	2408.9	3050.8	1	1.04
11	0.27	40	30	1.208	1.545	2.9	2928.8	3503.8	1	1.03
12	0.27	45	33	1.234	1.589	3	2958.7	3594.3	1	0.8
13	0.3	35	27	1.124	1.392	2.3	2649.5	3407.3	1	0.92
14	0.33	40	30	1.053	1.354	2.7	2558.3	3109	1	1.02
15	0.3	40	30	1.141	1.446	2.7	2716	3279	1.03	0.96
16	0.3	40	30	1.137	1.457	2.8	2707	3266	1.03	0.97
17	0.3	45	27	1.186	1.482	2.6	2867.4	3850.2	1.02	0.97

Method Optimization by Experimental Design :A risk estimation matrix was applied to identify critical method parameters, including the proportion of acetonitrile, pH of the mobile phase, and flow rate. Additionally, an Ishikawa fishbone diagram was utilized as a systematic risk assessment tool to further elucidate potential sources of variability affecting method performance. In the present investigation, a Box–Behnken Design (BBD) comprising 17 experimental runs was employed to comprehensively evaluate the influence of three independent variables on seven critical analytical responses. The independent variables investigated were flow rate (X_1), percentage of acetonitrile in the mobile phase (X_2), and column temperature (X_3). The corresponding dependent responses included retention time of empagliflozin (Y_1), retention time of alogliptin (Y_2), resolution (Y_3), theoretical plate count of empagliflozin (Y_4), theoretical plate count of alogliptin (Y_5), tailing factor of empagliflozin (Y_6), and tailing factor of alogliptin (Y_7), as presented in Table 9.The Method Operable Design Region (MODR) was established to define optimal operating conditions that ensure robust and consistent chromatographic performance by using box behnken method and Ishikawa fish bone diagrams for prior risk assessments in the method. Subsequent method validation encompassed the evaluation of key analytical parameters, including linearity and retention characteristics, thereby confirming the reliability and suitability of the optimized method for routine analysis.

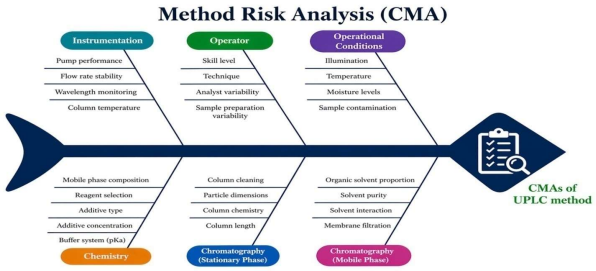


Figure:4TheIshikawa(fishbone)diagramtoidentify potentialvariablesinUPLCmethoddevelopment.

ANALYTICAL TARGET PROFILE (ATP) AND CRITICAL METHOD ATTRIBUTES (CMAS)

The Analytical Target Profile (ATP) for the RP-UPLC method developed for the simultaneous estimation of empagliflozin and alogliptin was established with appropriate scientific justification. Critical Method Attributes (CMAs) are defined as measurable characteristics of the chromatographic system that must be maintained within predefined limits to ensure the desired performance and quality of the analytical method.In this study, the selected CMAs included retention time for each analyte, tailing factor, resolution, peak height, theoretical plates, capacity factor, and peak area.

RISK ASSESSMENT AND PRELIMINARY EXPERIMENTATION

An Ishikawa (fishbone) diagram was constructed to systematically identify potential risk factors affecting the analytical method, based on prior experimental knowledge and chromatographic principles. A risk assessment matrix was then developed to evaluate the impact of these factors on CMAs. The risks were categorized as high, medium, or low depending on their severity and likelihood of affecting method performance.

ANALYTICAL TARGET PROFILE REQUIREMENTS

The developed UPLC method for the determination of empagliflozin and alogliptin must prioritize high sensitivity and selectivity to ensure precise quantification and the clear separation of analytes from potential interferences. By optimizing chromatographic conditions, the method should achieve a rapid analysis time and cost-effectiveness through reduced solvent consumption and high-throughput efficiency. Furthermore, the procedure must demonstrate significant robustness against minor operational variations, consistently delivering reliable assay results within the specified range to meet stringent pharmaceutical quality control standards.

Table:7 Critical Method Attributes (CMAs)

Method Attribute	Target	Justification
Retention Time	Optimum	Should be minimized to enable faster analysis without compromising separation
Peak Height	Optimum	Ensures proper peak symmetry and accurate quantification
Tailing Factor	NMT 2	Maintains acceptable peak symmetry for reliable quantification
Theoretical Plates	>1500	Indicates adequate column efficiency for better separation
Capacity Factor	>2	Ensures sufficient retention for accurate quantification
Resolution	>2	Provides clear separation between peaks
Peak Area	Consistent/Optimum	Should remain consistent as it directly reflects analyte concentration and method precision

RISK ASSESMENT

Risk assessment of the developed RP-UPLC method was systematically performed using an Ishikawa (fishbone) diagram to identify potential factors that could influence the Critical Method Attributes (CMAs). Based on prior knowledge of chromatographic principles and preliminary experimental trials, various sources of variability were categorized into key domains such as method parameters (e.g., mobile phase composition, flow rate, column temperature), instrumentation (e.g., detector performance, pump pressure fluctuations), materials (e.g., column characteristics, reagents), environment (e.g., temperature, humidity), and analyst-related factors. Each identified factor was further evaluated through a risk assessment matrix to determine its potential impact on CMAs, including retention time, resolution, and tailing factor. The risks were classified as high, medium, or low based on their severity and likelihood of occurrence. High-risk factors were prioritized for further optimization and control during method development to ensure robustness, reliability, and consistent analytical performance. The experimental data were subjected to mathematical modeling, and second-order polynomial equations were developed to elucidate the relationship between independent and dependent variables. The statistical significance of the model and its individual terms was assessed using analysis of variance (ANOVA). Furthermore, three-dimensional response surface plots and two-dimensional contour plots were generated to better understand the interaction effects among the variables. The optimal chromatographic conditions were established using a numerical optimization technique based on predefined acceptance criteria.

RESPONSE SURFACE MODELING ALIGNMENT

All experimental responses obtained from the 17 BBD runs were analyzed using Design-Expert® software to determine the most appropriate mathematical model for each response. The suitability of the models was evaluated using statistical parameters such as the coefficient of determination (R^2), adjusted R^2, predicted R^2, predicted residual sum of squares (PRESS), standard deviation, and coefficient of variation (CV), as

presented in Table 3.Model selection was guided by higher R^2 values, lower standard deviation, CV, and PRESS values, along with strong agreement between adjusted and predicted R^2 values. Based on these criteria, all responses (Y_1–Y_7) were best described by a quadratic model, confirming its adequacy in accurately representing the experimental data and predicting system behavior.

Table :8 Regression analysis for different responses Y1 to Y7 for fitting to different polynomial models

Response(Y_1):Retentiontimeof Empagliflozin (min)						
Linear	0.0151	0.9685	0.9613	0.9359	0.0059	
2FI	0.0139	0.9791	0.9667	0.9006	0.0091	
Quadratic	**0.0095**	**0.9935**	**0.9847**	**0.9205**	**0.0073**	**Suggested**
Cubic	0.0068	0.9982	0.9923			Aliased
Response(Y_2):Retentiontimeof Alogliptin(min)						
Linear	0.0160	0.9825	0.9782	0.9675	0.0063	
2FI	0.0153	0.9881	0.9798	0.9565	0.0080	
Quadratic	**0.0074**	**0.9992**	**0.9959**	**0.9884**	**0.0030**	**Suggested**
Cubic	0.0067	0.9989	0.9955			Aliased
Response(Y_3):Resolutionfactor						
Linear	0.1535	0.5689	0.4691	0.1377	0.6053	
2FI	0.1613	0.6296	0.4067	-0.7375	1.22	
Quadratic	**0.0538**	**0.9731**	**0.9373**	**0.8028**	**0.1398**	**Suggested**
Cubic	0.0558	0.9839	0.9324			Aliased
Response(Y_4): Theoretical Plates of Empagliflozin						
Linear	40.53	0.9680	0.9599	0.9351	4249.03	
2FI	38.99	0.9774	0.9633	0.8929	69671.42	
Quadratic	**19.65**	**0.9968**	**0.9915**	**0.9515**	**31919.40**	**Suggested**
Cubic	13.74	0.9998	0.9963			Aliased
Response(Y_5): Theoretical Plates of Alogliptin						
Linear	190.88	0.6372	0.5532	0.2416	9.877E+05	
2FI	146.42	0.8367	0.7382	0.1658	1.098+06	
Quadratic	**29.96**	**0.9953**	**0.9880**	**0.9538**	**61469.85**	**Suggested**
Cubic	26.33	0.9989	0.9921			Aliased
Response(Y_6): Tailing factor of Empagliflozin						
Linear	0.0183	0.0074	-0.2239	-0.7579	0.0079	
2FI	0.0173	0.3202	-0.0888	-1.1554	0.0088	
Quadratic	**0.0040**	**0.9741**	**0.9396**	**0.8657**	**0.0008**	**Suggested**
Cubic	0.0055	0.9799	0.9191			Aliased
Response(Y_7): Tailing factor of Alogliptin						
Linear	0.0088	0.9755	0.9696	0.9581	0.0019	Suggested
2FI	0.0090	0.9799	0.9679	0.9388	0.0029	
Quadratic	**0.0099**	**0.9817**	**0.9569**	**0.8420**	**0.0059**	
Cubic	0.0099	0.9899	0.9595			Aliased

IMPACT OF INDEPENDENT VARIABLES ON EMPAGLIFLOZIN RETENTION TIME (Y_1)

The relationship between the independent variables and the retention time of empagliflozin (Y_1) was expressed using the following quadratic regression equation:

$$Y_1 = 0.1008A + 0.0089B - 0.0341C - 0.0043AB + 0.0076AC - 0.0136BC - 0.0102A^2 - 0.0024B^2 + 0.0146C^2 \quad (1)$$

The equation indicates that the retention time of empagliflozin exhibits a negative correlation with all three independent variables, namely flow rate (X_1), column temperature (X_3), and percentage of acetonitrile in the mobile phase (X_2). This suggests that an increase in any of these variables leads to a corresponding reduction in retention time. Among the studied factors, column temperature (X_3) demonstrated the most pronounced influence on the retention time, as evidenced by its comparatively higher coefficient value.

The interaction terms (AB, AC, and BC) exhibited varying effects on the retention behavior of empagliflozin. The analysis of variance (ANOVA) results for Y_1 are presented in Table 9. The model F-value of 112.4 confirms the statistical significance of the model, with a probability of less than 0.01% that such a high F-value could occur due to random variation. This indicates that the model terms are highly significant and effectively describe the response surface within the studied design space.

The effects of the independent variables on the retention time of empagliflozin (Y_1) are further illustrated through three-dimensional response surface plots and corresponding two-dimensional contour

plots (Fig. 5A–B). These graphical representations clearly demonstrate that increasing flow rate, column temperature, and acetonitrile concentration collectively results in a reduction in retention time, confirming their inverse relationship with the The equation indicates that the retention time of empagliflozin is negatively correlated with all three independent variables, namely flow rate (X_1), column temperature (X_3), and acetonitrile concentration in the mobile phase (X_2). This implies that an increase in any of these factors leads to a corresponding decrease in retention time. Of all the variables examined, column temperature (X_3) exerted the most significant effect, as reflected by its comparatively higher coefficient.

The interaction effects (AB, AC, and BC) exhibited varying degrees of influence on the retention behavior of empagliflozin. The ANOVA results for Y_1 are presented in Table 9. The model F-value of 112.4 confirms that the regression model is statistically significant, with a probability of less than 0.01% that such a high F-value could arise due to random variation. This indicates that the model terms are highly significant and provide a robust representation of the response surface within the investigated design space.

The impact of the independent variables on the retention time of empagliflozin (Y_1) is further illustrated through three-dimensional response surface plots and corresponding two-dimensional contour plots (Fig. 5A–B). These plots demonstrate that a simultaneous increase in flow rate, column temperature, and acetonitrile percentage consistently leads to a reduction in retention time, thereby confirming their inverse relationship with the response.

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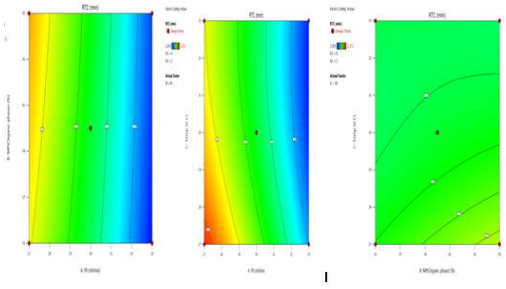


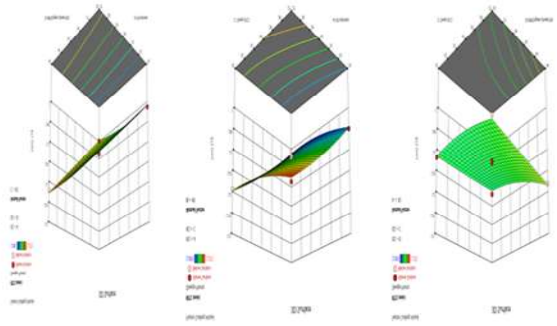


FIGURE.5:THE2DCONTOURPLOTS(A)ANDTHE3DRESPONSESURFACEPLOTS(B)ILLUSTRATINGTHEEFFECTOFINDEPENDENT VARIABLES ON THE RETENTION TIME OF EMPAGLIFLOZIN (Y1)

Table:9Analysisofvariancetestsolutionsandadequateprecisionforvariousresponses.

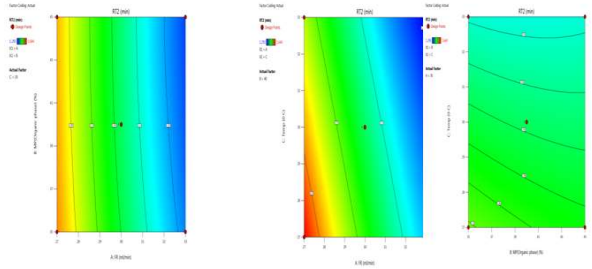
Source	Y1		Y2		Y3		Y4		Y5		Y6		Y7	
	F-value	P-value	F-value	P-value	F-value	p-value	F-value	p-value	F-value	p-value	F-value	p-value	F-value	p-value
Model	112.4	<0.0001	4440.94	<0.0001	28.14	0.0001	196.49	<0.0001	173.83	<0.001	27.15	0.0001	175.37	<0.0001
A-FR	949.48	<0.0001	3401.38	<0.0001	79.83	<0.0001	143.843	<0.0001	382.74	<0.001	0.9333	0.3817	466.25	<0.0001
B-MP(Organicphase)	9.08	0.0298	18.71	0.0049	22.99	0.0023	0.9015	0.3840	577.99	<0.001	0.0000	1.0000	28.94	0.0001
C-Temp	54.86	0.0003	396.29	<0.0001	46.87	0.0003	192.52	<0.0001	1.777	0.2247	0.8433	0.3817	10.14	0.0082
AB	0.	0.5	0.3	0.5	0.	0.3	11.	0.0	24	0.0	0.	1.0	0.	1.0

	5888	0800	0233	9866	87940	7500	54	1116	.36	018	00000	0000	00000	00000	0000
AC	2.211	0.18888	15.881	0.00073	0.00000	1.00000	0.12122	0.838382	0.00001	0.94355	82.68	<0.0001	72.73	>0.0001	
BC	9.443	0.02228	6.557	0.00369	15.466	0.00078	5.254	0.007866	273.27	<0.0001	0.000000	1.00000	0.00000	1.00000	
A²	4.999	0.00612	39.711	0.00006	71.60	<0.0001	1.25	0.31933	89.67	<0.0001	7.994	0.0282	6.74	0.00382	
B²	0.09885	0.77555	1.883	0.24044	0.47229	0.61881	0.96554	0.39333	13.97	0.0084	145.28	<0.0001	139.28	<0.0001	
C²	11.455	0.01554	0.12022	0.83922	18.47	0.0042	32.663	0.00008	146.29	<0.0001	7.84	0.0273	6.84	0.00382	
Adeq Precision	**36.7718**		**74.0918**		**17.8946**		**49.7134**		**52.5358**		**14.0352**		**39.7873**		

IMPACT OF INDEPENDENT VARIABLES ON ALOGLIPTIN RETENTION TIME (Y_2)

The relationship between the independent variables and the retention time of alogliptin (Y_2) was described using the following quadratic regression equation:

$$Y_2 = 1.45 - 0.1314A - 0.0096B - 0.0458C - 0.0018AB + 0.0123AC + 0.0083BC + 0.0198A^2 + 0.0051B^2 + 0.0021C^2 \quad (2)$$



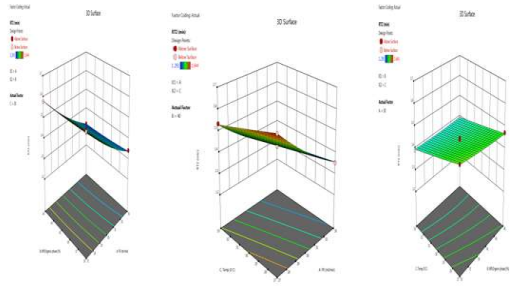


Figure.6The2Dcontourplots(A)andthe3Dresponsesurfaceplots(B)illustratingtheeffectofindependentvariables on the retention time of Alogliptin (Y2)

The model indicates that flow rate (X_1), column temperature (X_3), and acetonitrile concentration in the mobile phase (X_2) all significantly influence the retention behavior of alogliptin. An increase in any of these parameters results in a decrease in retention time, confirming an inverse relationship. Among the studied factors, flow rate (X_1) had the most dominant effect on retention time, as shown by its relatively higher coefficient.

The interaction effects (AB, AC, and BC) showed varying influences on retention behavior, with AB contributing negatively, while AC and BC exhibited minor positive effects. The ANOVA results for Y_2 are summarized in Table 9, where the model F-value of 440.94 confirms that the regression model is highly statistically significant. The terms A, B, C, AB, BC, A^2, and C^2 were identified as significant contributors to the response.

The robustness of the model is further validated by the close agreement between the adjusted R^2 (0.9837) and predicted R^2 (0.9313), indicating strong predictive capability. Moreover, the adequate precision value exceeded the recommended limit of 4, confirming a strong signal-to-noise ratio and demonstrating that the model is well-suited for navigating the experimental design space.

IMPACT OF INDEPENDENT VARIABLES ON RESOLUTION FACTOR (Y_3)

The influence of independent variables on the resolution factor (Y_3) was represented by the following quadratic equation:

$$Y_3 = -0.1725A - 0.0975B - 0.1350C - 0.0350AB + 0.0000AC + 0.1000BC + 0.3175A^2 + 0.0175B^2 - 0.1076C^2 \quad (3)$$

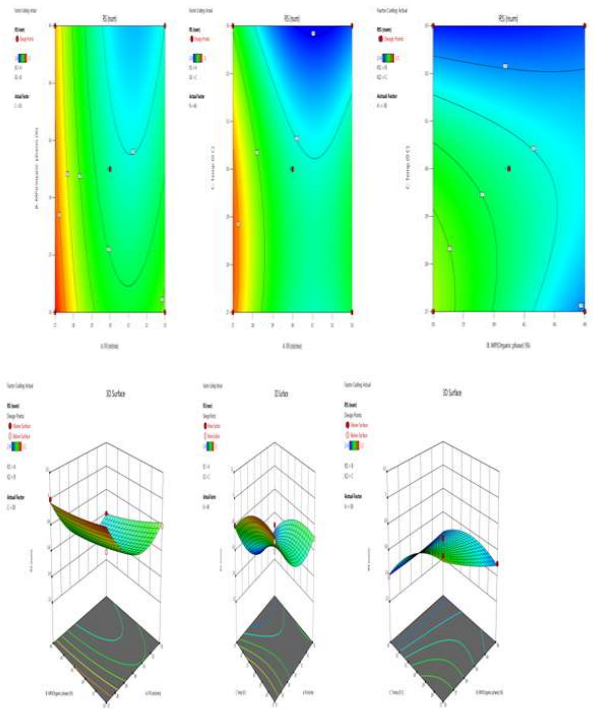


Figure.7.Theassociated2Dcontourplots(A)andtheassociated3Dresponsesurfaceplots(B)showingtheeffectof independent variables on the resolution factor (Y3)

The equation shows that the resolution between empagliflozin and alogliptin is adversely affected by flow rate (X_1), column temperature (X_3), and acetonitrile concentration in the mobile phase (X_2). An increase in any of these factors leads to a reduction in resolution, indicating an inverse relationship. Of all the variables studied, flow rate had the most pronounced effect on resolution, as reflected by its relatively higher coefficient.

The interaction terms (AB, AC, and BC) exhibited different influences on the response. The ANOVA results for the resolution factor are presented in Table 9, where the F-value of 28.14 confirms the statistical significance of the model. Model terms with p-values less than 0.0500, including A, B, C, AC, A^2, B^2, and C^2, were identified as significant contributors to the response.

The robustness of the model is supported by the strong agreement between the adjusted R^2 (0.9889) and predicted R^2 (0.9529), indicating high predictive accuracy. Furthermore, the adequate precision value of 17.8946 demonstrates a strong signal-to-noise ratio, confirming that the model is reliable and suitable for navigating the design space.

The three-dimensional response surface plots and corresponding two-dimensional contour plots (Figs. 7A–B) further illustrate the influence of the independent variables on resolution. These plots clearly indicate that simultaneous increases in flow rate, temperature, and acetonitrile percentage consistently lead to a decrease in the resolution between empagliflozin and alogliptin.

IMPACT OF INDEPENDENT VARIABLES ON THEORETICAL PLATE COUNT (Y_4) OF EMPAGLIFLOZIN

The influence of the selected independent variables on the theoretical plate count (Y_4) of Empagliflozin is described by the following quadratic polynomial equation:

$$Y_4 = -272.08A + 7.58B - 96.77C - 34.30AB + 3.41AC - 21.13BC - 11.34A^2 - 8.96B^2 + 53.69C^2$$

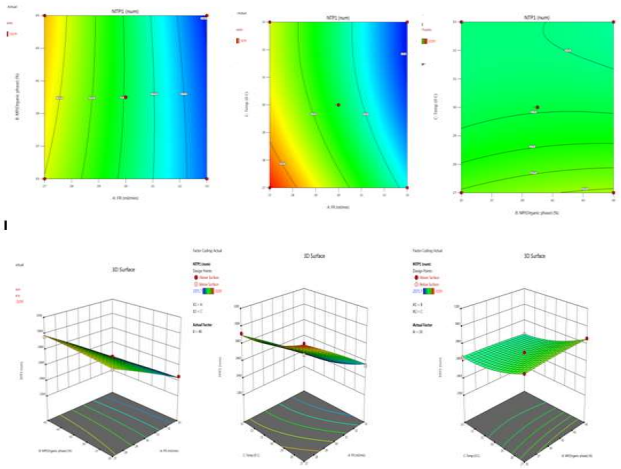


Figure:8.The2Dcontourplots3Dresponsesurfaceplots(A)andtheassociated3Dresponsesurfaceplots(B)showing the effect of independent variables on the theoretical plates of empagliflozin (Y4)

The model shows that acetonitrile proportion (X_2) positively influences the theoretical plate count, whereas flow rate (X_1) and column temperature (X_3) have negative effects. Increasing the acetonitrile content enhances chromatographic efficiency, resulting in a greater number of theoretical plates. In contrast, higher flow rates and temperatures decrease plate count, likely due to reduced interaction between the analyte and the stationary phase.

Among the investigated factors, flow rate (X_1) has the most pronounced effect on theoretical plate count, as indicated by its comparatively higher coefficient. Regarding interaction effects, X_1X_2 and X_1X_3

negatively influence the response, while X_2X_3 shows a positive contribution.

The ANOVA results Table 9 confirm that the model is statistically significant, with an F-value of 196.49. All linear, interaction, and quadratic terms (A, B, C, AB, AC, BC, A^2, B^2, and C^2) were found to be significant based on p-values below 0.05.

The model also demonstrates strong predictive ability, as evidenced by the close agreement between the predicted R^2 (0.9600) and adjusted R^2 (0.9962). Furthermore, the adequate precision value of 49.7134, which is well above the recommended threshold of 4, indicates a robust signal-to-noise ratio and confirms the model's suitability for navigating the design space.

The three-dimensional response surface plots and corresponding two-dimensional contour plots (Fig. 8A–B) further support these findings, showing that higher theoretical plate counts are achieved at increased acetonitrile levels, while elevated temperature and flow rate lead to a decline in plate count.

IMPACT OF INDEPENDENT VARIABLES ON THEORETICAL PLATE COUNT (Y_5) OF ALOGLIPTIN

The effect of the selected independent variables on the theoretical plate count (Y_5) of Alogliptin is represented by the following quadratic polynomial equation:

$$\mathbf{Y_5 = 3373.61 - 203.46A + 251.81B + 14.98C - 71.69AB + 0.1350AC - 245.70BC - 137.80A^2 - 54.83B^2 + 175.23C^2}$$

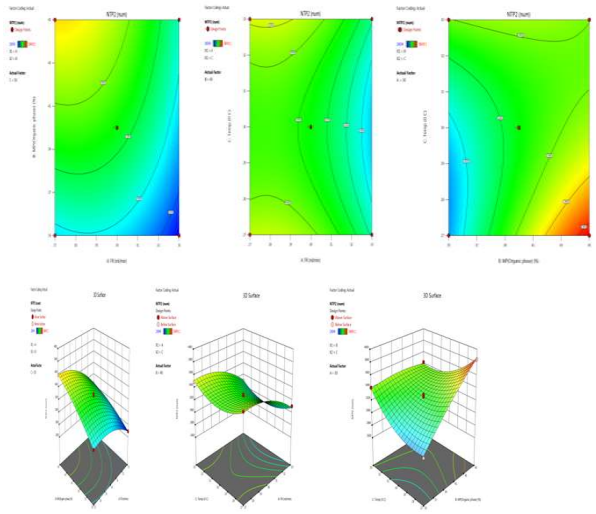


Figure:9The2Dcontourplots(A)andtheassociated3Dresponsesurfaceplots(B)showingtheeffectofindependent variables on the theoretical plates of alogliptin (Y5)

The equation demonstrates that acetonitrile percentage (X_2) positively affects the theoretical plate count of alogliptin, whereas flow rate (X_1) and column temperature (X_3) exert negative effects. Consequently, increasing acetonitrile content enhances chromatographic efficiency by increasing the number of theoretical plates, while higher flow rates and temperatures reduce performance.

Among the investigated variables, flow rate (X_1) has the strongest influence on theoretical plate count, as indicated by its relatively larger coefficient. Regarding interaction effects, AB and AC negatively impact the response, whereas BC shows a positive contribution.

The ANOVA results in Table 9 confirm that the model is statistically significant, with an F-value of 173.83. All terms with p-values below 0.05, including A, B, C, AC, BC, and the relevant linear and quadratic components (X_1, X_2, X_3, X_1X_3, X_2X_3, A^2, and B^2), were identified as significant factors.

The model also exhibits strong predictive performance, as evidenced by the close agreement between the adjusted R^2 (0.9875) and predicted R^2 (0.9259). Furthermore, the adequate precision value of 52.5358, which is substantially higher than the recommended minimum of 4, confirms a strong signal-to-noise ratio and supports the robustness of the model.

The three-dimensional response surface plots and two-dimensional contour plots (Fig. 9A–B) further substantiate these results, showing that higher acetonitrile levels increase the theoretical plate count of alogliptin, while increased flow rate and temperature lead to a decline in chromatographic efficiency.

IMPACT OF INDEPENDENT VARIABLES ON EMPAGLIFLOZIN TAILING FACTOR (Y_6)

The effect of the selected independent variables on the tailing factor (Y_6) of Empagliflozin is described by the following quadratic polynomial equation:

$$\mathbf{Y_6 = -0.0013A + 0.0000B - 0.0014C + 0.0000AB + 0.0185AC + 0.0000BC - 0.0063A^2 - 0.0328B^2 - 0.0062C^2}$$

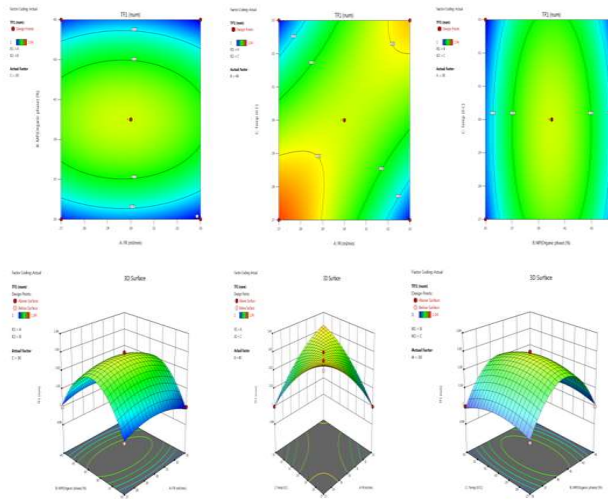


Figure:10The2Dcontourplots(A)andtheassociated3Dresponsesurfaceplots(B)showingtheeffectofindependent variables on the on the tailing factor of empagliflozin (Y6)

The model shows that acetonitrile percentage (X_2) positively affects the tailing factor of empagliflozin, whereas flow rate (X_1) and column temperature (X_3) have negative effects. Accordingly, increasing acetonitrile content leads to higher tailing factor values, while increases in flow rate and temperature reduce peak tailing.

Among the studied variables, flow rate (X_1) has the most significant influence on the tailing factor, as indicated by its relatively higher coefficient. The interaction terms AB, AC, and BC all show negative effects on the response.

The ANOVA results presented in Table 9 confirm that the model is statistically significant, with an F-value of 27.15. All terms with p-values below 0.05, including A, B, C, AB, AC, BC, A^2, B^2, and C^2, were identified as significant contributors.

The model also demonstrates good predictive performance, supported by the close agreement between the predicted R^2 (0.9374) and adjusted R^2 (0.9843). In addition, the adequate precision value of 14.0352, which is well above the minimum acceptable threshold of 4, indicates an adequate signal-to-noise ratio and confirms the reliability of the model.

The effects of the independent variables on the tailing factor are further visualized using 2D contour and 3D response surface plots (Fig. 10A–B), which show that the tailing factor of empagliflozin increases with higher acetonitrile concentration, while it decreases as flow rate and temperature increase.

IMPACT OF INDEPENDENT VARIABLES ON ALOGLIPTIN TAILING FACTOR (Y_7)

The relationship between the independent variables and the tailing factor (Y_7) of Alogliptin is described by the following quadratic polynomial equation:

$$\mathbf{Y_7 = 0.0687A + 0.0250B - 0.0097C + 0.0000AB + 0.0175AC + 0.0000BC - 0.0053A^2 - 0.0239B^2 - 0.0072C^2}$$

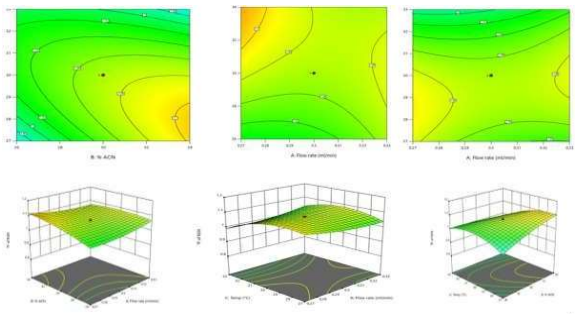


Figure:11The2Dcontourplots(A)andtheassociated3Dresponsesurfaceplots(B)showingtheeffectofindependent variables on the on the tailing factor of Alogliptin (Y7).

The model indicates that flow rate (X_1) and column temperature (X_3) positively influence the tailing factor of alogliptin, while acetonitrile percentage (X_2) has a negative effect. Thus, an increase in temperature and flow rate leads to higher tailing values, whereas increasing the proportion of acetonitrile reduces peak tailing.

Among the investigated factors, acetonitrile concentration (X_2) shows the most pronounced effect on the tailing factor, as suggested by its comparatively larger coefficient. The interaction terms (AB, AC, and BC) all contribute positively to the response.

The ANOVA results presented in Table 9 confirm that the model is statistically significant, with an F-value of 175.37. All terms with p-values below 0.05, including A, B, C, AB, AC, BC, A^2, B^2, and C^2, were found to be significant contributors to the model.

The model demonstrates strong predictive ability, supported by the close agreement between the predicted R^2 (0.9768) and adjusted R^2 (0.9763). In addition, the adequate precision value of 39.7873, which is well above the recommended minimum of 4, indicates an excellent signal-to-noise ratio and confirms the robustness of the model.

The influence of the independent variables on the tailing factor is further illustrated using 2D contour plots and 3D response surface plots (Fig. 11A–B). These plots show that the tailing factor of alogliptin decreases with increasing acetonitrile concentration, while it increases with higher temperature and flow rate.

Selection of Optimized Chromatographic Conditions

The optimal chromatographic conditions were determined using the numerical optimization function of Design Expert® software. The software predicted the best conditions for the simultaneous separation of empagliflozin and alogliptin. The optimized parameters included a flow rate of 0.3 mL/min, a column temperature of 30 °C, a detection wavelength of 252 nm, and a mobile phase consisting of 63:37 v/v (0.1 N potassium dihydrogen phosphate buffer: acetonitrile).

Standard solutions of empagliflozin (20 µg/mL) and alogliptin (4 µg/mL) were injected into the UPLC system under these optimized conditions, and the observed responses were recorded. Table 10 presents the predicted, observed, and percentage residual values for each response. The low residual values confirmed good agreement between predicted and experimental results, indicating that the optimized conditions effectively achieved satisfactory separation of empagliflozin and alogliptin. This demonstrates the suitability and reliability of the QbD-based design approach. The method was subsequently validated under these optimized conditions, and the low percentage error further confirmed the validity of the central composite design.

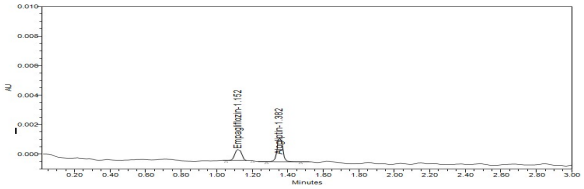


FIGURE:12. OPTIMIZED CHROMATOGRAM

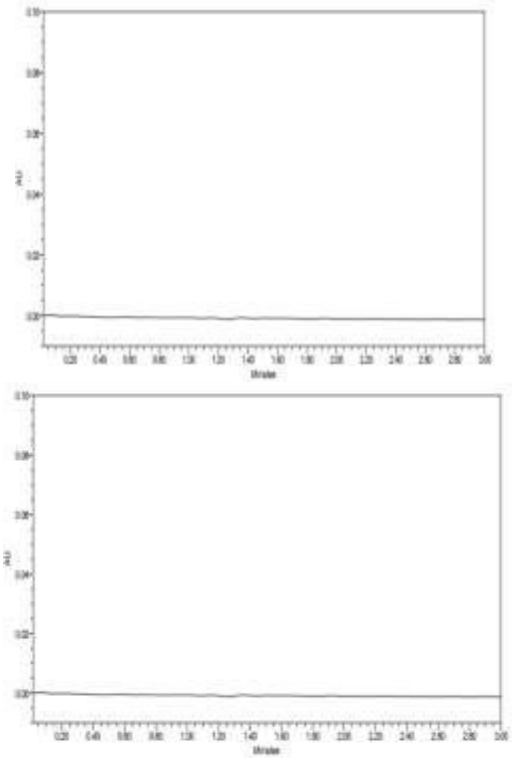
Table10:Thepredictedandobservedvaluesoftheresponsesobtainedfromtheoptimizedchromatographic conditions

	Predicted	Observed	Residual values
Retention time of Empagliflozin	1.087	1.099	0.012
Retention time of Alogliptin	1.357	1.350	0.007
Resolution Factor	2.433	2.512	0.079
Theoretical plates of Empagliflozin	2556	2594	38
Theoretical plates of Alogliptin	3288	3378	90
Tailing factor of Empagliflozin	1.06	1.08	0.02
Tailing factor of Alogliptin	0.97	0.99	0.02

METHOD VALIDATION

The developed RP-UPLC method was validated in accordance with ICH guidelines, demonstrating excellent analytical performance across all evaluated parameters. Linearity was established over the concentration range of 5–30 µg/mL for EGN and 1–6 µg/mL for AGN, with strong correlation coefficients indicating a direct proportional relationship between analyte concentration and detector response. The sensitivity of the method was confirmed by low limits of detection (LOD) and quantification (LOQ), determined to be 0.04 µg/mL and 0.12 µg/mL for EGN, and 0.07 µg/mL and 0.21 µg/mL for AGN, respectively. These values reflect the method's capability for trace-level quantification.

Precision studies revealed excellent repeatability and intermediate precision, with %RSD values below 1% for both intra- and inter-day analyses (0.8% and 0.7% for EGN; 0.7% and 0.5% for AGN). Accuracy was confirmed through recovery studies, yielding mean recoveries of 99.9% for EGN and 100.3% for AGN, indicating minimal systematic error and high reliability of the method. System suitability parameters, including retention time, theoretical plates, and tailing factors, were within acceptable limits, confirming the robustness and efficiency of the chromatographic system. Overall, the validation results demonstrate that the method is precise, accurate, sensitive, and suitable for routine quantitative analysis.



Blank(A) placebo(B)

Figure:13 ChromatogramsofBlank(A),placebo(B)andstandard empagliflozin and alogliptin showingthe specificity of the established UPLC method

FORCED DEGRADATION STUDIES

Forced degradation studies were conducted under a range of stress conditions, including acidic, alkaline, oxidative, neutral, photolytic, and thermal environments, to evaluate the stability-indicating capability of the method. Both analytes, EGN and AGN, exhibited measurable degradation under all tested conditions, confirming their susceptibility to stress-induced breakdown.

The degradation behavior showed consistent patterns, with EGN and AGN exhibiting degradation values of 1.114 and 1.446 under acidic conditions, 1.116 and 1.438 under alkaline conditions, and 1.104 and 1.437 under oxidative conditions. Under neutral, photolytic, and thermal stress, similar degradation trends were observed, indicating that both compounds are moderately sensitive to environmental factors.

Importantly, the developed method effectively resolved degradation products from the parent compounds, with no significant interference observed at the retention times of EGN and AGN. This confirms the specificity and stability-indicating nature of the method, making it suitable for stability testing and degradation profiling.

Table:11.Stabilitystudiesof Empagliflozin and Alogliptin

	Stress Conditions	Empagliflozin	Alogliptin
Acidhydrolysis	2NHCl,60°C for30min	1.114	1.446
Alkalinehydrolysis	2NNaOH,60 °Cfor30min	1.116	1.438
peroxidedegradation	20%H_2O_2 60°Cfor30min	1.104	1.437
Thermaldegradation	105°Cfor1 hour	1.136	1.436
Photolyticdegradation	UVlightfor1 hour	1.111	1.441
NeutralHydrolysis	Waterat60°C for1h	1.117	1.449

RESULTS AND DISCUSSION

The present study demonstrates the successful application of an Analytical Quality by Design (AQbD) framework for the development and optimization of a robust RP-UPLC method. The integration of the Ishikawa fishbone diagram enabled systematic identification of critical method parameters, while the use of Design-Expert® software facilitated multivariate optimization through statistical modeling. The influence of key chromatographic variables flow rate, organic phase composition, and column temperature on critical quality attributes such as retention time, resolution, efficiency, and peak symmetry was effectively modeled. The optimized conditions provided rapid separation with short retention times (<1.5 min), improved resolution, and high column efficiency, highlighting the method's suitability for high-throughput analysis. Validation results confirmed that the method meets stringent analytical requirements, with excellent linearity, precision, accuracy, and sensitivity. Compared to conventional chromatographic methods, the proposed RP-UPLC approach offers reduced analysis time, lower solvent consumption, and enhanced resolution. Furthermore, the forced degradation studies demonstrated the method's capability to distinguish analytes from their degradation products, reinforcing its application as a stability-indicating method. The consistent degradation patterns observed across stress conditions also provide valuable insights into the intrinsic stability characteristics of the analytes.

CONCLUSION

A robust, rapid, and stability-indicating RP-UPLC method was successfully developed and validated for

the simultaneous estimation of EGN and AGN in pharmaceutical dosage forms using an AQbD-driven framework. The method development strategy was systematically guided by risk assessment tools, including the Ishikawa fishbone diagram, to identify and evaluate critical method parameters and their potential impact on analytical performance. Subsequent multivariate optimization using statistical design of experiments enabled precise control and fine-tuning of critical method variables, ensuring method robustness within the established design space. The optimized method was comprehensively validated in accordance with regulatory guidelines, demonstrating excellent performance characteristics in terms of accuracy, precision, linearity, sensitivity, and specificity. Furthermore, the method exhibited strong stability-indicating capability, effectively resolving analytes from degradation products under various stress conditions. The short run time, reduced solvent consumption, and high separation efficiency highlight its suitability for high-throughput environments. Owing to its robustness and reliability, the proposed method is well-suited for routine quality control analysis, stability studies, and pharmaceutical formulation assessment. Overall, this study underscores the significance of AQbD principles in the development of scientifically sound, efficient, and regulatory-compliant analytical methods, thereby providing a comprehensive framework for future advancements in pharmaceutical analytical research.

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