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Article

Chromatographic study of sitagliptin and ertugliflozin under quality-by-design paradigm

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The present study entails the systematic development and validation of a stability-indicating RP-HPLC method for the analysis of sitagliptin and ertugliflozin in a fixed-dose combination. Analytical quality by design (AQbD) concepts were used to define critical method variables, employing Pareto risk assessment and a Placket-Burman screening design, preceded by a Box-Behnken design with response surface analysis to optimise critical method parameters such as % acetonitrile (X1), buffer pH (X2) and column oven temperature (X3). Multiple response optimisation (Derringer's desirability) of variables was accomplished by studying critical analytical attributes, such as resolution, retention time and theoretical plates. The title analytes were separated effectively on a PRONTOSIL C18 column at 37 °C using a mobile phase of acetonitrile:acetate buffer, pH 4.4 (36:64 percent v/v), pumped at a flow rate of 1 mL/min, and UV detection at 225 nm. Linearity was observed over a concentration range of 25-150 µg/mL and 3.75- 22.5 µg/mL at retention times of 2.82 and 3.92 min for sitagliptin and ertugliflozin, respectively. The method obeyed all validation parameters of the ICH Q2(R1) guidelines. The proposed robust method allows the study of the selected drugs in pharmaceutical dosage forms as well as in drug stability studies under various stress conditions.

Key words: AQbD. HPLC. Sitagliptin. Ertugliflozin. Stability-indicating.

INTRODUCTION

Sitagliptin (SIT) is a hypoglycaemic agent belonging to the family of dipeptidyl peptidase-4 inhibitors that decrease glucagon-like peptide insulinotropic hormone-1 breakdown for improved glycaemic regulation. It is a white to off-white, crystalline, non-hygroscopic powder with a pKa of 8.78 (log P = 1.5). SIT is prescribed alone (monotherapy) or in combination with either ertugliflozin (ERT) or metformin (Barnard, Cox, Green, 2010). ERT is a potent and selective inhibitor of sodium-dependent glucose cotransporter-2, which is responsible for glucose reabsorption from the glomerular filtrate in the kidney (Markham, 2018). It is a white to off-white powder with a pKa of 11.98 (log P = 2.21). The FDA have approved a fixed-dose combination of SIT and ERT (Steglujan®, 2017), which is indicated as an adjunct to diet and exercise to improve glycaemic control in adults with type 2 diabetes mellitus.

The development of a rapid, sensitive and highly robust analytical method is desirable for the simultaneous estimation and routine chromatographic analysis of SIT and ERT in bulk, pharmaceutical formulations and stability evaluation samples. A few literature reports have documented the simultaneous quantification of SIT and ERT using UV (Anjali *et al*., 2019), HPLC (Suneetha, Mounika, Shaik, 2020; Anjaneyulu, 2019; China, 2018; Harshalatha, 2018; Amtul, Yunoos, 2018) and LC-MS/MS (Venkateswara, Lakshmana, Prasad, 2021) techniques in active pharmaceutical ingredients and pharmaceutical dosage forms. Few methods report the analysis of SIT and ERT separately (Jiu *et al*., 2011;

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Reddy *et al.,* 2013) and in combination with other drugs (Karimulla *et al*., 2013; Kumari, Bandhakavi, 2020). The literature methods utilise a conventional experimentation strategy, *i.e.*, varying one factor at a time (OFAT), which may deliver ambiguous and inept optimisation in analytical method development and must therefore be avoided (Ramalingam, Bhadraya, Reddy, 2015; Sangshetti *et al*., 2017). This necessitates the use of a systematic and statistical approach for the optimisation of method variables to attain consistent results (Vogt, Kord, 2011; Hemant *et al*., 2014), which can be achieved through an analytical quality by design (AQbD) strategy. In method development, AQbD facilitates the simultaneous assessment of significant variables through the design of experiments (DoE) and response surface analysis (Sahu *et al*., 2018; Deidda *et al.,* 2018; Giordani *et al.,* 2018) to accomplish enhanced method performance.

The contemplated research aimed to exploit the AQbD approach in the development of an HPLC method for the simultaneous estimation of SIT and ERT, using a multivariate approach for HPLC parameter optimisation.

MATERIAL AND METHODS

Chemicals and reagents

The reference standards of SIT and ERT were obtained from Hetero Laboratories, Hyderabad, India. The marketed dosage form (Steglujan®), consisting of SIT (100 mg) and ERT (15 mg), was procured from the local pharmacy. All solvents used for the mobile phase were of HPLC grade and were obtained from Merck, Mumbai, India.

Instrumentation

A Waters alliance 2695 HPLC system (Waters Corporation, UK) was connected to Empower 2 software, consisting of a quaternary pump, autosampler, column heater and photodiode array (PDA-2996) detector. An ultra-sonicator (RK 106, Spincotech), millipore (0.45 µm) filters and digital pH meter (LI-120, Elico) were also used in this work.

Software

Design-Expert version 11.0.5.0 software (Stat-Ease Inc. Minneapolis) was employed for the design of experiments and response-modelling to generate the design space for an optimised robust analytical method.

Chromatographic conditions

Chromatographic separation of the title analytes was achieved using a PRONTOSIL column C18 (150 X 4.6 mm, 5µ) at 37 °C with isocratic elution. The mobile phase consisted of acetonitrile:acetate buffer pH 4.4 (36:64 % v/v), pumped at a flow rate of 1 mL/min. A volume of 10 µL was withdrawn from the sample vials by autosampler for all injections. Analytes were detected with a PDA detector at a UV wavelength of 225 nm.

Preparation of standard solutions

A stock solution of standard drug mixture was prepared by transferring accurately weighed quantities of 100 mg SIT and 15 mg ERT into a 100 mL volumetric flask and dissolving in methanol by sonication. The volume was made up to the mark with diluent and the flask was shaken well to obtain a concentration of 1000 µg/mL SIT and 150 µg/mL ERT.

A working standard solution (100% test concentration of each drug) was prepared by diluting 1 mL of the stock solution to 10 mL with mobile phase. This solution (100 µg/mL SIT, 15 µg/mL ERT) was used for the chromatographic method development through experimental design approach.

Sample solution preparation

Twenty tablets, each containing 100 mg of SIT and 15 mg of ERT, were taken and weighed accurately. The average weight of the tablets was determined, and the tablets were then crushed into fine powder. An accurately weighed quantity of powder equivalent to 100 mg SIT/15 mg ERT was transferred to a 100 mL volumetric flask containing 50 mL methanol and sonicated for 15 min. The flask was jiggled, and the volume was made up to the

mark with methanol. The above solution was filtered, and 1 mL of the filtrate was transferred to a 10 mL volumetric flask. The volume was made up to the mark with mobile phase (acetonitrile:buffer pH 4.4, 36:64 %v/v) to give a solution containing 100 µg/mL SIT and15 µg/mL ERT. This solution was analysed for the targeted drugs using the planned HPLC method.

Method development by analytical quality by design (AQbD) approach

AQbD follows a series of steps for systematic method development (Hubert *et al.,* 2015; Gurrala *et al.,* 2019), which includes defining the analytical target profile (ATP), identifying critical analytical attributes (CAAs) and critical method variables (CMVs) with risk assessment, and generating an analytical method working space known as a design space (DS).

The *ATP* of the proposed method is liquid chromatographic separation of SIT and ERT from each other as well as from their degradant peaks/formulation excipients. The aim is to obtain the best possible separation between the analytes in the shortest possible run time.

The *CAAs* are measurable attributes of the chromatogram that should be within specified limits to ensure the desired quality of the method (Musters, Bos, Kellenbach, 2013). CAAs such as resolution (a measure of the separation between eluted peaks), retention time (an indicator of analyte interaction with the column), selectivity factor (an indicator of analyte separation) and theoretical plate number (which embodies the suitability of mobile phase and column) were chosen from various chromatographic system responses (embodying the suitability of the mobile phase and column).

The *CMVs* were selected from preliminary risk assessment and factor screening studies. Three factors, namely, % acetonitrile in the mobile organic phase (X1), aqueous phase buffer pH (X2) and column temperature (X3), were identified as CMVs, as shown in Table I. Factor screening and optimisation studies were premeditated using the DoE tool Design-Expert software.

TABLE I - Factors and responses of optimisation studies

Variable	Unit	Code	Level		
			Low (-1)	Mid-Point (0)	High (+1)
Acetonitrile	%	X_1	30	35	40
pH of buffer	Number	X_2	3	4	5
Column temperature	ºC	X_3	30	35	40
Responses					
Resolution of SIT/ ERT (Rs)		Y1		-	
Retention time of SIT (Rt1), min		Y2		-	
Retention time of ERT (Rt2), min		Y3		-	
Theoretical plates of SIT (Tp1)		Y4		-	
Theoretical plates of ERT (Tp2)		Y5		-	

A Placket-Burman experimental design (Politis *et al.,* 2017; Ferey *et al.,* 2018) was employed in the factor screening studies. Seven variables, column oven temperature (A), % organic component of mobile phase (B), type of organic modifier (C), stationary phase with diverse chemistry (D), pH of aqueous phase (E), flow rate (F) and injection volume (G), were screened using the Placket-Burman experimental design under twelve experimental runs. A Box-Behnken design (BBD) with response surface methodology was exploited in the factor optimisation studies. BBD, a multivariate optimisation technique (Candioti *et al.,* 2014; Lin *et al.,* 2018), was used to evaluate the effect of the CMVs on the CAAs. Systematic chromatographic experimentation was considered using 18 experimental runs under a BBD, including 6 centre points and CMVs at 3 levels (low, midpoint and high). A randomised experimentation order was followed to abate the bias effects of uncontrolled variables. The statistical confirmation of the experimental findings was carried out using ANOVA and multiple regression analysis.

The *DS* was generated by numerical optimisation, where Derringer's desirability function was used to attain high method performance criteria. During the numerical optimisation, the targets of individual variables and responses were fixed, and the DS was produced as a multi-dimensional combination between CMVs and CAAs for the maximum desirability function (Aruna, Prasad, Bharathi, 2019). Graphical optimisation was also performed to obtain the method operable design region (MODR) from the model for the selected responses.

Method validation

The validation parameters, such as system suitability test, specificity, selectivity, linearity, precision, accuracy, sensitivity and robustness, were premeditated in accordance with ICH Q2(R1) guidelines (1994).

System suitability test

The system suitability test was monitored for the optimised method. It was carried out via the injection of freshly prepared working standard solution (100% test concentration) containing 100 µg/mL SIT and 15 µg/mL ERT, which was performed six times under the optimised HPLC conditions. The chromatographic responses (analytical attributes) were retention time, resolution, capacity factor, theoretical plate number, peak area, selectivity factor and tailing factor of the analytes.

Linearity and range

Linearity was studied using 0.25, 0.5, 0.75, 1.0, 1.25 and 1.5 mL aliquots of the standard stock solution, which were pipetted into six different 10 mL volumetric flasks and the volume made up to the mark with diluent prior to injection. These solutions were in the concentration range of 25-150 µg/mL SIT and 3.75- 22.5 µg/mL ERT. Three replicate injections were analysed at each concentration level. Concentration of drug *vs.* peak area plots were drawn, and correlation coefficients were obtained for the calibration curves by linear regression analysis.

Precision

The precision of the optimised method was evidenced by repeatability and intermediate precision studies. The repeatability of the method was verified by analysing six replicate injections of freshly prepared working standard solution (100% test concentration) containing 100 µg/mL SIT and 15 µg/mL ERT in mobile phase on the same day (intra-day precision). Intermediate (inter-day) precision was performed by analysing replicates of the same concentration solution prepared on three consecutive days. The peak area of the analytes was determined and the % RSD was calculated.

Accuracy

The accuracy of the method was determined based on the recovery of the standard spiked to a target concentration at three levels (50%, 100% and 150%). Pre-analysed tablet samples were spiked with the standard solution (50 µg/mL SIT and 7.5 µg/mL ERT). These samples were prepared in triplicate and analysed using the proposed method, resulting in nine determinations. The % recovery of the standard drug was calculated by measuring the peak area of the chromatogram.

Specificity

The specificity of the method was established by comparing the chromatogram of blank (mobile phase) and placebo solutions with test solutions (analytes with mobile phase). The placebo solution comprised all the excipients commonly used in the manufacture of tablet dosage forms. The selectivity study was performed to demonstrate the effective separation of the title analyte peaks from the matrix (degradants) and was validated by measuring the (% RSD) retention times of the analytes. Drugs were exposed to diverse stress conditions in order to generate their degradants.

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

The detection limit of the method was investigated by injecting diluted standard solution into the HPLC system. The peak-to-peak noise around the analyte retention times were measured using the signal-to-noise (S/N) method. A S/N of 3 was generally accepted for estimating the limit of detection (LOD) and a S/N ratio of 10 was used to estimate the limit of quantification (LOQ). Two types of solution were analysed, a blank solution and a test solution with progressively decreasing concentrations of the drugs.

Robustness

Experimental conditions were deliberately altered within the DS. The variation in the parameters included organic phase (± 2%), pH (± 0.2) and flow rate (± 0.2 mL/min) of the mobile phase. A working standard solution at 100% test concentration of the analytes (100 µg/mL SIT and 15 µg/mL ERT) was used during the experiment. The % RSD of the retention time and peak area of the chromatogram was calculated for every variation.

Forced degradation studies

The stability testing of drugs was performed by forced degradation studies, as per ICH Q1A (R2) guideline (2003). The degradation behaviour of the drugs was studied under various stress conditions. Sample solution (5 mL) and hydrochloric acid solution (1 N, 5 mL) were mixed and heated at 60 °C for 30 min. The solution was cooled and neutralised with sodium hydroxide solution (1.0 N). Similarly, alkali (1 N sodium hydroxide solution), neutral (water) and peroxide (6% H_2O_2) degradation studies were carried out. Photolytic (UV-light, 200 Watt/m^2, 18 h) and thermal degradation (80 °C, 24 h) studies were also carried out. All stressed samples were diluted with mobile phase and analysed using the optimised HPLC method (Zhang, Hu, 2017; Lange *et al.,* 2012a; Lange *et al.,* 2012b). Controlled samples and stressed samples were concurrently analysed.

Assay of pharmaceutical dosage form

The proposed method was used for the simultaneous analysis of labelled analytes in the tablet dosage form (Steglujan®) containing 100 mg of SIT and 15 mg of ERT. The drugs were initially extracted into methanol using an ultra-sonicator, and the solution was prepared as previously mentioned (sample preparation step), with subsequent dilution with mobile phase to attain a suitable concentration within the linearity range. Solutions, prepared in triplicate, were analysed by the optimised method and the percentage drug content of each analyte was determined.

RESULTS AND DISCUSSION

Selection of UV detection wavelength

The standard solutions of SIT and ERT showed an iso-absorptive point at 225 nm in the mobile phase [S1]. Hence, chromatographic peak integration was performed at 225 nm using a PDA detector.

AQbD-assisted method development

Initial risk identification was performed using Ishikawa analysis for the identification of factors. Based on the literature reports (Jain *et al.,* 2019; Reid *et al.,* 2013) and past experience in the development of HPLC methods for the estimation of selected drugs, an Ishikawa fish-bone

diagram was constructed [S2]. The risk assessment was performed by Pareto charts. In the initial stage of the risk assessment, various mobile phase combinations were attempted with buffers employing ammonium formate, acetate and potassium (or sodium) phosphate (each at 10 mM and 20 mM strength) at varying pH (between 3 and 6), and acetonitrile and methanol as organic modifiers. Various flow rates (0.8-1.2 mL/min), diverse column chemistry (C_8, C_{18}), elution modes (isocratic, gradient) and temperature settings were studied. The preliminary studies recommended the selection of a 150 x 4.6 mm column over a 250 x 4.6 mm column, owing to faster elution (*i.e.*, low R_t values) and adequate peak symmetry. An acetate buffer was found to be appropriate owing to low peak tailing and high peak symmetry, although phosphate and acetate are the most commonly used buffers for HPLC with UV detection. Isocratic elution was selected over gradient elution due to its low cost and simple instrumentation, and to avoid re-equilibration of the column between successive injections.

Screening of variables

Seven factors were screened using a Placket-Burman experimental design under twelve experimental runs (Patel, Kothari, 2020). The chromatographic method responses were recorded, and the design [S3] was analysed statistically to understand the influence (main effects) of the studied factors on the analytical attributes. Experimental data fitted a first-order polynomial model, maintained a hierarchical model at each step of analysis and generated the following linear equation, where β_0 is the intercept and β_1 to β_7 are the coefficients of model terms.

$$Y=\beta_0+\beta_1A+\beta_2B+\beta_3C+\beta_4D+\beta_5E+\beta_6F+\beta_7G$$

Pareto risk ranking analysis of the variables was performed. The Pareto charts, Figure 1, show the risk ranking matrix, where variables were ranked 1 to 7 based on their prioritised risk towards CAAs. The variable that crosses the line (t-value limit 2.44) was considered to be significant (Bonferroni limit set to 4.40); it was also found that few variables were affirmative on the outcome of the method attributes, while others were antagonistic. The length of each bar in the Pareto chart is proportional to the magnitude of the regression coefficient of that factor in the standard Pareto charts (Suryawanshi *et al.,* 2019), which are derivatives of multivariate regression analysis.

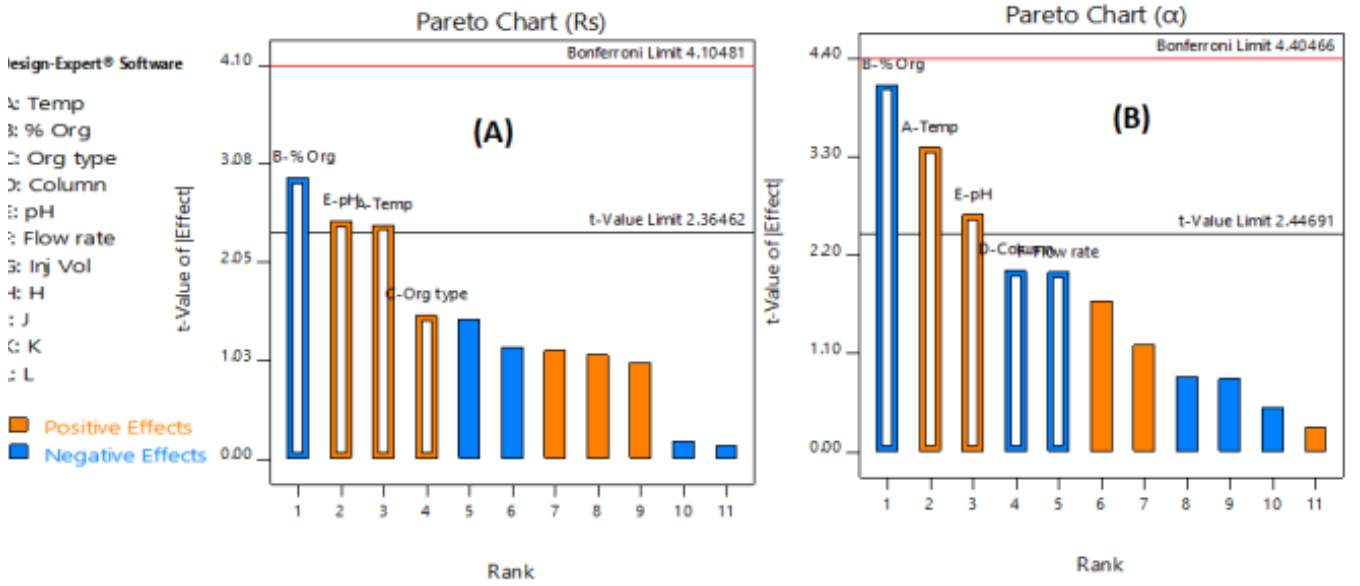

FIGURE 1 - Pareto charts - risk ranking analysis of responses: Resolution (A) and selectivity factor (B).

Figure 1 indicates that variables A, B and E were found to have the most influential effect on the method attributes, followed by D and F, while C and G were found to be insignificant. The temperature of the column oven will influence the viscosity of the mobile phase, which affects the analyte separation efficiency. In the mobile phase, the % organic factor caused a major change in resolution and retention times. The organic modifier acetonitrile showed selectivity and good separation of target analytes within a short run time compared to methanol. Hence, acetonitrile was selected as the organic modifier for economical method development. Resolution was moderately influenced by the column chemistry. An ODS Column (C18) was preferred and fixed, providing superior analyte selectivity with good peak shape compared to the C8 column. Selectivity and capacity factor values were most readily modified by the pH of the aqueous phase and different pKa values of (three) drugs recommended the optimisation of desirable pH of mobile phase. Peak symmetry and stationery-mobile phase interactions were altered with the mobile phase flow rate. Hence, the flow rate was maintained at an optimum level (*i.e.*, 1.0 mL/min). The injection volume was also fixed at a specified level (10 µL).

The risk assessment via Pareto analysis designated that % organic component, pH and column temperature were the most significant factors ($p<0.05$). Hence, the % acetonitrile, bufrfer pH and column oven temperature were identified as typical CMVs and chosen for subsequent chromatographic optimisation studies.

Optimisation of variables

A BBD was employed to study 3 CMVs (X1, X2, X3 - independent variables) and 5 CAAs (Y1, Y2, Y3, Y4, Y5 - dependent variables); the results for all 18 designed experimental runs are abridged in Table II. The chromatograms were recorded. Experimental observations were subjected to ANOVA (analysis of variance) and regression analysis to assess the significant factors ($p<0.05$) and are summarised in Table III. The highest r^2 (>0.991) of the responses was obtained for the selected model (Pradipbhai *et al*., 2017). The coefficient of variation (CV) was found to be less than 5%, indicating the reproducibility of the model. The high adjusted R^2 value (~1) indicated a good relationship between the experimental data in the fitted (quadratic) model. The aptness of the model was endorsed by the lowest predicted residual sum of squares value, the non-significant lack of fit ($p>0.05$) and the agreement of the predicted R^2 with the adjusted R^2 (difference<0.2). The signal (response) to noise ratio is measured by adequate precision; the ratios were higher than desirable for all responses (should be more than 4), indicating an adequate signal. Hence, the model can be chosen for the DS. Models for each critical analytical attribute were used to produce the second-order polynomial equations mentioned below. The coefficient values in the polynomial equations and the positive/negative sign of the coefficient indicate a synergistic/antagonistic influence.

Y1 (Rs) = $5.93 - 1.65X_1 - 0.24X_2 - 0.56X_3 + 0.25X_1X_2 + 0.001X_1X_3 - 0.07X_2X_3 + 0.12X_1^2 - 0.004X_2^2 + 0.19X_3^2$

Y2 (Rt_1) $= 2.94 - 0.21X_1 - 0.02X_2 - 0.64X_3 + 0.04X_1X_2 + 0.05X_1X_3 - 0.01X_2X_3 + 0.05X_1^2 - 0.18X_2^2 + 0.13X_3^2$

Y3 (Rt_2) $= 4.33 - 0.86X_1 - 0.19X_2 - 1.0X_3 + 0.18X_1X_2 + 0.21X_1X_3 - 0.01X_2X_3 + 0.21X_1^2 - 0.14X_2^2 + 0.25X_3^2$

Y4 (Tp_1) $= 3669 - 31X_1 + 292X_2 - 653X_3 + 231X_1X_2 + 193X_1X_3 - 442X_2X_3 - 230X_1^2 - 499X_2^2 + 244X_3^2$

Y5 (Tp_2) $= 4172 - 297X_1 + 326X_2 - 752X_3 + 256X_1X_2 - 272X_1X_3 - 347X_2X_3 - 124X_1^2 - 374X_2^2 + 152\ X_3^2$

where X_1, X_2, X_3 represent the main effects from the % organic component in the mobile phase, aqueous mobile phase pH and column temperature, respectively; X_1X_2, X_1X_3, X_2X_3 represent the effects from two-factor interactions; and X_1^2, X_2^2, X_3^2represent quadratic effects.

TABLE II - Box- Behnken design and experimental results

Run	Variables			Responses				
	X_1	X_2	X_3	Y_1	Y_2	Y_3	Y_4	Y_5
	Acetonitrile (%)	pH	Column temperature (□C)	Rs	Rt1	Rt2	Tp1	Tp2
1	30	3	35	8.2	3.073	5.613	2883	3930
2	40	3	35	4.4	2.574	3.593	2348	2686
3	30	5	35	7.2	2.94	4.846	3070	4148
4	40	5	35	4.4	2.64	3.542	3459	3929
5	30	4	30	8.4	4.078	6.953	4496	4859
6	40	4	30	5.1	3.504	4.756	4059	4946
7	30	4	40	7.4	2.625	4.411	2923	3997
8	40	4	40	4.1	2.275	3.04	3257	2995
9	35	3	30	6.9	3.518	5.536	3426	4117
10	35	5	30	6.6	3.503	5.206	4830	5386
11	35	3	40	5.8	2.313	3.677	2883	3208
12	35	5	40	5.2	2.244	3.323	2519	3088
13	35	4	35	6	2.959	4.372	3788	4361
14	35	4	35	5.7	2.891	4.222	3675	4126
15	35	4	35	5.8	2.939	4.299	3563	4118
16	35	4	35	6	2.943	4.338	3687	4094
17	35	4	35	6	2.952	4.361	3611	4217
18	35	4	35	6.1	2.968	4.401	3693	4117

Rs- resolution of SIT/ERT peaks; Rt1- Retention time of SIT; Rt2- Retention time of ERT; Tp1- Theoretical plates of SIT; Tp2- Theoretical plates of ERT

TABLE III - ANOVA and regression analysis of selected models

Response	Rs	Rt1	Rt2	Tp1	Tp2
ANOVA					
SS	25.29	3.92	15.07	6.805E+06	7.898E+06
Df	9	9	9	9	9
Mean square	2.81	0.43	1.67	7.561E+05	8.775E+05
F-value	154	300	211	90	58
*p-value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

TABLE III - ANOVA and regression analysis of selected models

Response	Rs	Rt1	Rt2	Tp1	Tp2
Regression analysis					
SD	0.14	0.04	0.09	91.5	122
Mean	6.07	2.94	4.47	3453	4017
% CV	2.22	1.30	1.99	2.65	3.06
r^2	0.9943	0.9970	0.9958	0.9903	0.9849
Adjusted R^2	0.9878	0.9937	0.9911	0.9793	0.9679
Predicted R^2	0.9731	0.9664	0.9526	0.9071	0.8528
Adequate precision	43.9	63.9	56.1	35.4	28.1703
PRESS	0.68	0.13	0.72	6.385E+05	1.180E+06

*p< 0.05 (significant); SS- Sum of squares; Df- Degrees of freedom; SD- Standard deviation; CV- Coefficient of variation; Rs- resolution of SIT/ERT peaks; Rt1- Retention time of SIT; Rt2- Retention time of ERT; Tp1- Theoretical plates of SIT; Tp2- Theoretical plates of ERT

Response surface analysis

The factor–response relationship and probable interaction effects were studied by response surface analysis. Contour (2D) plots [S5] and response surface (3D) plots were generated as a function of significant variables, while the third variable was held constant at a specified level (Awotwe-Otoo *et al.,* 2012). The occurrence of a high degree of interaction among the studied CMVs on the method CAAs is evidenced by the large curvatures formed by most of the response surfaces. These plots are used to find out the response for a given set of input variables. Significant interaction between the factors was observed for the studied responses; their response surface plots are shown in Figure 2(A), which reveals that X1X2 interaction is highly significant, with a linear descending trend on resolution (Rs). Factor X1 (% acetonitrile) has a significant effect on resolution, and factor X3 (column temperature) is moderately affected. The maximum resolution is observed at low extremes of X1, and low levels of X2 and X3. Figure 2 (B1, B2) signifies that the retention times of SIT (Rt1) and ERT (Rt2) are markedly affected by X1X3 interaction and are curvilinear. A non-linear interaction of the X1X2 variable is observed which affected responses moderately. X1 and X3 have a significant effect on Rt1 and Rt2. Low retention time values were observed with high values of X1, X3 and intermediate levels of X2. Theoretical plates of both drugs (Tp1 and Tp2) are highly influenced by X2X3 interaction (non-linear), as shown in Figure 2 (C1, C2). A significant effect of X3 is observed on Tp1 and Tp2. A high theoretical plate number is observed for both drugs at high levels of X2, low levels of X3 and intermediate levels of X1.

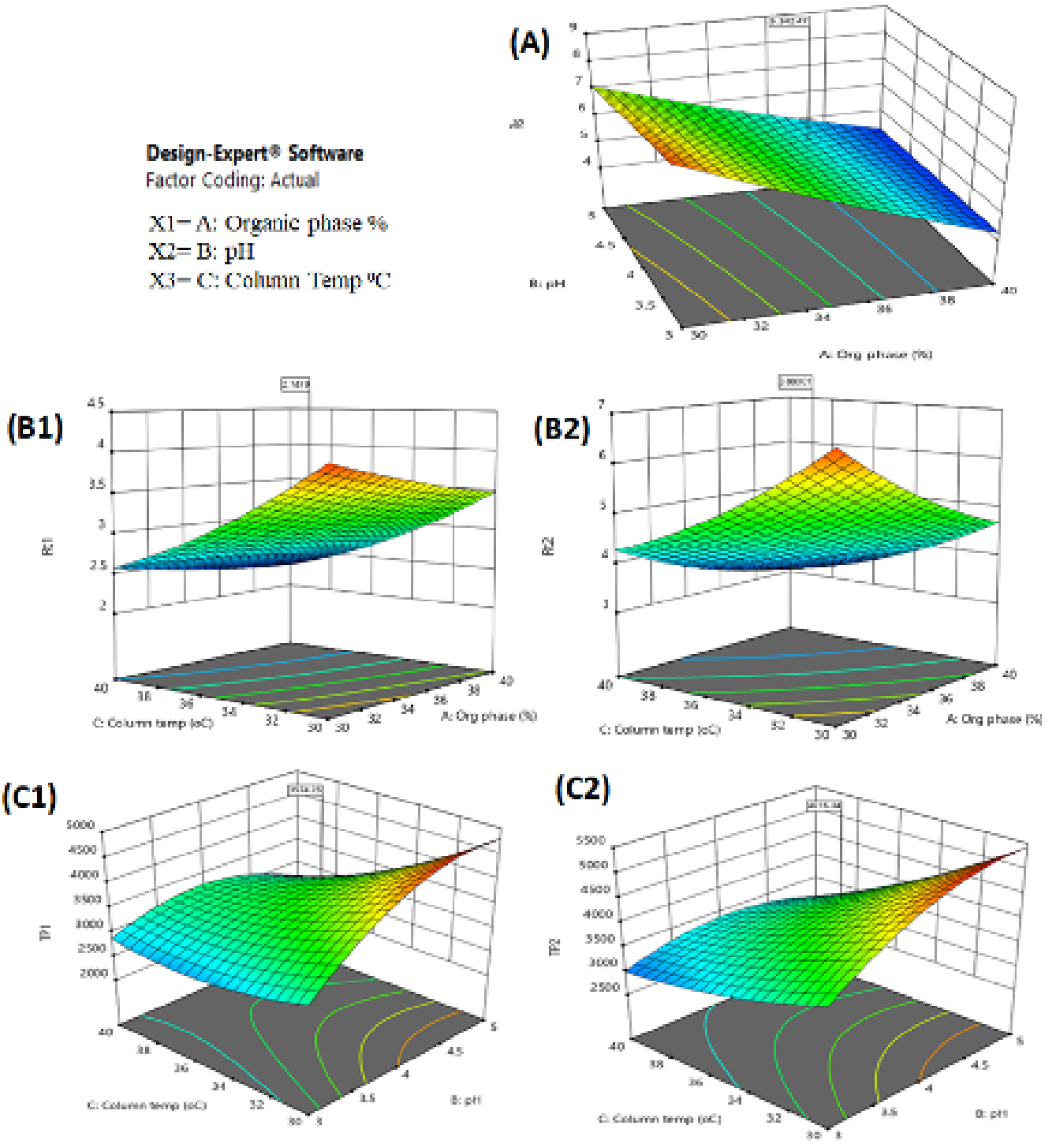

FIGURE 2 - Response surface plots: Resolution (A); retention time of SIT (B1); retention time of ERT (B2); theoretical plates of SIT (C1); theoretical plates of ERT (C2).

Design space (DS) identification

Multiple response optimisation of the variables for chromatographic separation of the target analytes was carried out using numerical optimisation by setting up CAAs at the desired goals, *i.e.*, the maximisation of resolution (range: 2-8), minimisation of retention time (range: 1-5) and maximisation of theoretical

plates (range: 2000-5000). The DS generated through Derringer's desirability function is portrayed in Figure 3(A) and indicates the high method performance (Costa, Lourenço, Pereira, 2011) owing to the maximum desirability value (equal to 1). The overlay plot obtained from graphical optimisation is illustrated in Figure 3(B), exhibiting the MODR and location of the optimised solution for the studied design. The MODR for the proposed method can be defined as organic phase (34-40%), pH (3.4-4.9) and column temperature (34-39 ºC).

The accuracy of predictions within the DS is verified by experimentation (Ganorkar, Dhumal, Shirkhedkar, 2017) [S10]. Results obtained from the robustness verification are compared between the predicted space and the observed; the % prediction error was calculated and was less than ±5%. This assures the consistency of the method performance, as per the intended use.

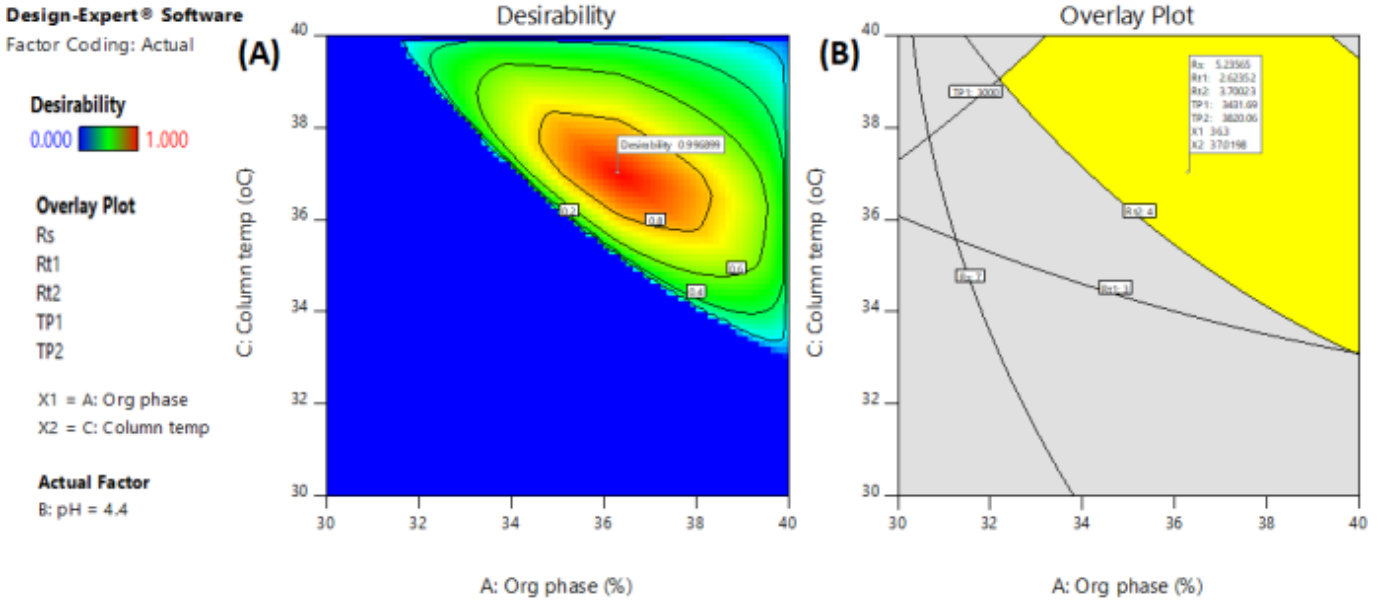

FIGURE 3 - Design space and location of optimised solution: Desirability plot (A) and overlay plot (B).

Optimised chromatographic conditions

The CMVs (within the DS) were optimised as acetonitrile (36%), acetate buffer, pH 4.4 and column temperature 36 ºC, where all CAAs were attained in the desired range. The chromatographic conditions were optimised, and the chromatogram is given in Figure 4. The retention times of SIT and ERT were found at 2.829 and 3.927 minutes, respectively.

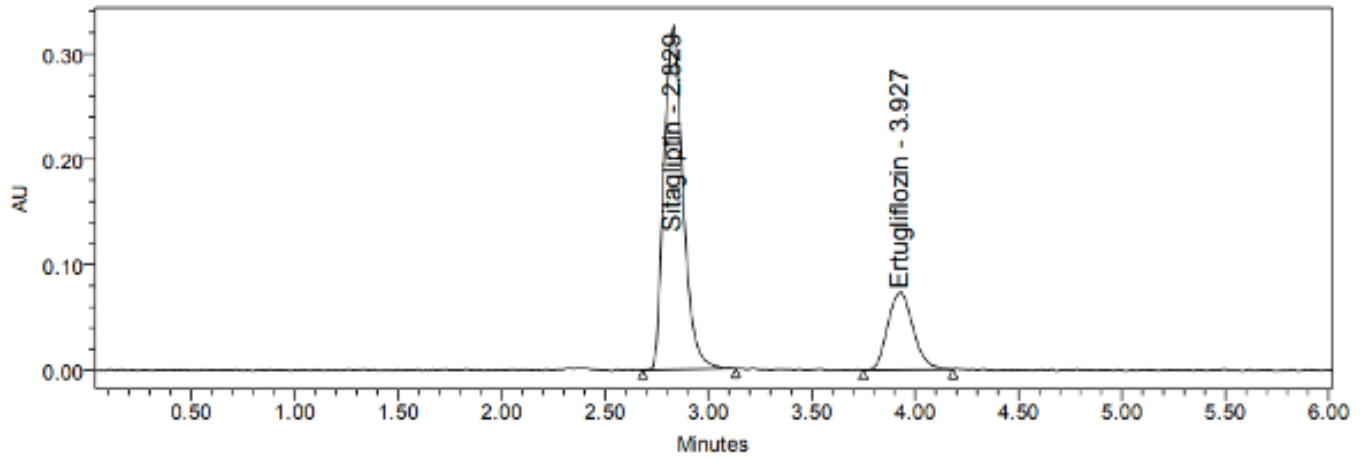

FIGURE 4 - HPLC chromatogram under optimised conditions: mobile phase acetonitrile:acetate buffer, pH 4.4 (36:64 % v/v) at flow rate 1 mL/min, column temperature 37 ^{0}C.

Method Validation

System suitability test

The system suitability test results are shown in Table IV, indicating that the chromatographic responses obtained under the optimised conditions were analogous with specifications. The % RSD was found to be less than 2 for all chromatographic attributes, indicating that the optimised chromatographic conditions allow simultaneous determination of the target analytes.

TABLE IV - System suitability parameters

Parameter	Sitagliptin AM ± SD; % RSD (n=6)	Ertugliflozin AM ± SD; % RSD (n=6)	Specification
Retention time (min)	2.82 ± 0.01; 0.34	3.91 ± 0.02; 0.49	% RSD ≤ 2
Peak area	2312158 ± 23116; 0.99	645571 ± 5417; 0.83	% RSD ≤ 2
Tailing factor	1.26 ± 0.008; 0.633	1.16 ±0.011; 0.95	<1.5
Theoretical plates	4336± 58; 1.33	4861± 63; 1.29	>2000
Capacity factor	1.82±0.01; 0.53	2.91±0.02; 0.67	>1.0
Selectivity factor	1.6± 0.002; 0.17		>1.0
Resolution	5.53 ± 0.08; 1.45		>2.0

AM- Arithmetic mean; SD- standard deviation; RSD- Relative standard deviation

Linearity

The linearity was verified quantitatively using optimised conditions at six concentrations. The linearity was assessed by the regression equation of the calibration data. Linearity occurred over the concentration range of 25-150 µg/mL for SIT and 3.75-22.5 µg/mL for ERT [S6, S7]. Higher values of the regression coefficient (r^2=0.999) indicated a strong correlation between the concentration of the analytes and their peak area. The linearity of the method was represented by the following linear regression equations:

Y_{SIT} = 23775X + 17128 (r^2=0.9991)

Y_{ERT} = 42715X + 3918 (r^2=0.9999)

Precision

The precision of the method was established through repeatability and intermediate precision studies (Table V). No significant difference between intra- and inter-day precision values was observed, as the acceptance criteria (RSD) was less than 2%. The precision study results corroborated the reproducibility of the proposed method.

TABLE V - Method validation results

S. No.	Parameter			Sitagliptin	Ertugliflozin
1	**Precision** (Peak area) Mean ± SD; % RSD (n=6)	Repeatability		2303923 ± 21095; 0.91	643837 ± 3025; 0.46
		Intermediate precision		2159206 ± 24148; 1.11	637472 ± 2736; 0.42
2	**Accuracy** (%Recovery) Mean± SD; % RSD (n=3)	50 % level		101.79 ± 0.29; 0.28	101.93 ± 0.75; 0.73
		100% level		99.04 ± 0.46; 0.46	98.69 ± 0.93; 0.94
		150% level		102.15 ± 0.72; 0.70	98.97 ± 0.89; 0.89
3	**Robustness** Mean± SD; % RSD (n=3)	pH (± 0.2)	Rt	2.84 ± 0.015; 0.54	3.93 ± 0.010; 0.25
			PA	2298683 ± 21975; 0.96	640947 ± 3054; 0.48
		Organic phase (± 2%)	Rt	2.86 ± 0.031; 1.07	3.92 ± 0.015; 0.39
			PA	2314823 ± 26267; 1.13	642281 ± 9843; 1.53
		Flow rate (± 0.2 mL/min)	Rt	2.87 ± 0.047; 1.65	3.92 ± 0.045; 1.15
			PA	2342747 ± 35846; 1.53	647902 ± 8912; 1.38

RSD- Relative standard deviation; Rt- Retention time; PA- Peak area

Accuracy

When used for extraction and subsequent estimation of target drugs from pharmaceutical formulation, the proposed method afforded recoveries of 99.04-102.15% and 98.69-101.93%, respectively, for SIT and ERT, after spiking with additional standard drug at three different levels. The results presented in Table V indicate good recovery of both drugs, with % RSD less than 2 at all levels studied, designating the fair accuracy of the proposed method.

Specificity

Specificity is the extent to which the procedure applies to the analyte of interest and is checked by examining the formulation sample for any interfering

peaks from the excipients. The chromatograms of the blank and placebo sample indicated the absence of peaks representing the targeted analytes at the identified retention times [S8]. The excipients used in the formulation did not interfere with the drug peaks; however, the formulation chromatogram gave characteristic peaks for metformin, empagliflozin and linagliptin. Thus, the specificity of the proposed analytical method was confirmed.

LOD and LOQ

The sensitivity of the proposed method was determined by the LOD and LOQ values, which were found to be 0.80 and 2.41 µg/mL, respectively, for SIT, and 0.13 and 0.39 µg/mL, respectively, for ERT.

Robustness

The robustness of the method was determined as per the guidelines, under different conditions including changes in buffer pH, organic mobile phase composition and different column temperatures. The data are presented in Table V. The % RSD values for peak area and retention time obtained for both drugs under the varied experimental conditions were consistent with the acceptance criteria (% RSD<2), signifying the robustness of the proposed method to deliberate variations.

Forced degradation studies

The stability-indicating property of the method was determined by forced degradation studies. The developed RP-HPLC approach was used to investigate drug behaviour under a variety of stress conditions, including acidic, alkaline, oxidative, photolytic and thermal. A summary of the degradation studies is reported in Table VI. Extensive degradation of both drugs was observed in basic conditions, and significant degradation was observed in acidic and oxidative hydrolysis conditions, whereas degradation was insignificant in UV light, neutral hydrolysis and thermal conditions. The analytes and degradation products had well-separated peaks in the chromatograms of the stressed samples, with no modification in the analyte peak retention times. Chromatograms in certain stressed environments did not indicate different peaks relating to degradation products, but rather a reduction in the height and area of the analyte peak. Peak purity testing was performed on stressed samples (acid, base and oxidative) using a photodiode array detector and UV scans of SIT and ERT, represented in Figure 5. The purity angle was found to be less than the purity threshold value, and no purity flag was observed for any of the stressed samples, indicating that the target analytes had attained their peak homogeneity. As a result, the proposed method was comprehensive for determining SIT and ERT without degradant intervention.

TABLE VI - Data of forced degradation studies

Stress Condition	Sitagliptin			Ertugliflozin		
	% degraded	Purity angle	Purity threshold	% degraded	Purity angle	Purity threshold
Acidic (1N HCl, 60 ˚C)	11.04	1.029	1.365	6.12	1.085	1.415
Alkali (1N NaOH, 60 ˚C)	14.19	0.914	1.230	7.55	1.104	1.361
Oxidative (6 % v/v H_2O_2)	10.82	1.003	1.326	7.21	1.163	1.426
UV light	5.03	0.983	1.270	2.27	1.105	1.411
Neutral (H_2O, 60 ˚C)	3.14	1.095	1.388	1.14	1.195	1.483
Thermal (80 °C)	4.17	1.072	1.418	2.33	1.095	1.393

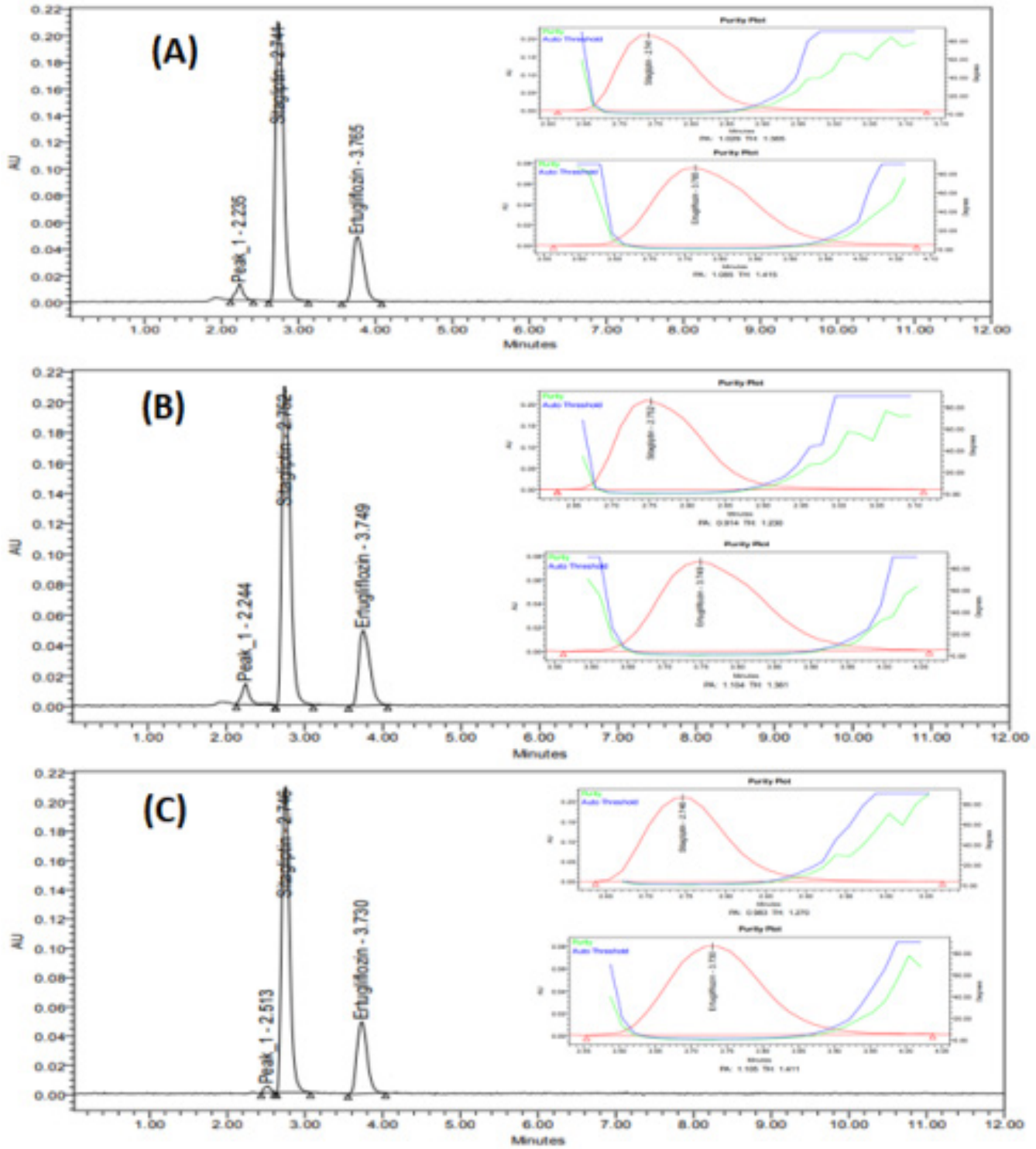

FIGURE 5 - HPLC chromatograms of drugs under stress conditions: acid (A), base (B), oxidative (C) and peak purity plots.

Assay of pharmaceutical dosage form

The proposed RP-HPLC method was applied to assay a tablet formulation (Steglujan®) containing two drugs. The HPLC chromatograms of the sample (tablet solution) are given in Figure 6 and show identical retention times for the analytes, indicating that the selected drugs were clearly separated and showed no interfering peaks

due to excipients within the retention time ranges. Hence the proposed method is selective. The assay was found to be 99.47% for SIT and 99.73% for ERT, suggesting that the proposed method is suitable for the analysis of drugs in tablet dosage form and may be equally applicable to other dosage forms

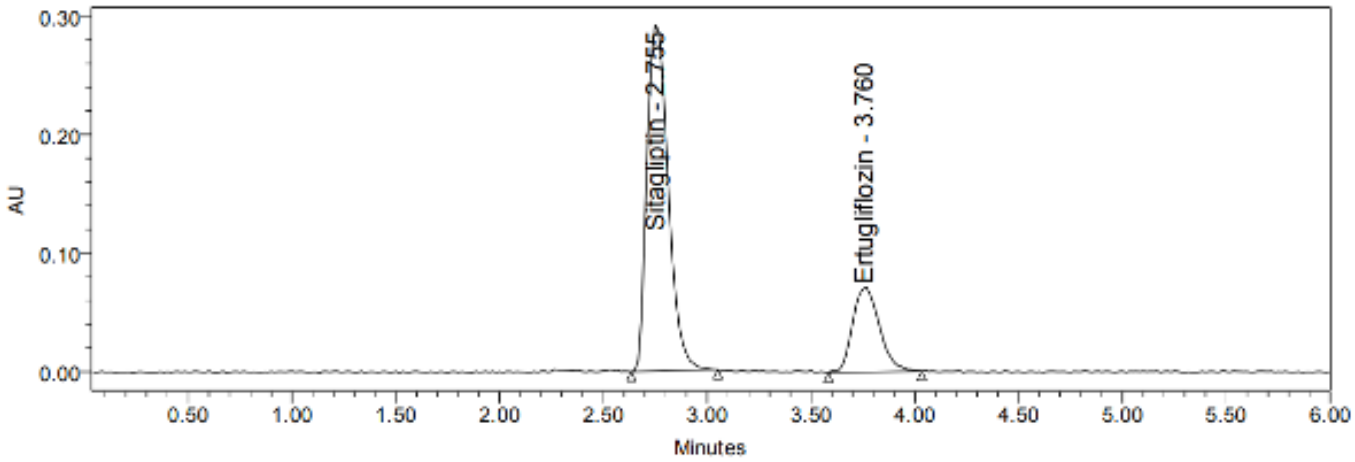

FIGURE 6 - HPLC chromatogram of formulation (Steglujan® tablets) solution.

CONCLUSION

The quality by design approach was utilised for the simultaneous analysis of SIT and ERT, helping to develop a robust and cost-effective method that is applicable throughout the product lifecycle and facilitates regulatory flexibility. Screening and multifactorial optimisation of the chromatographic method parameters was carried out using experimental designs with statistical analysis. The response surface plots facilitated an improved understanding of the factor–response relationship and the associated interactions. Diminutive changes in organic (%) mobile phase, buffer pH and column temperature resulted in a distinct change in method attributes, and these factors needed to be strictly controlled. The method conformed with all validation parameters, as per ICH guidelines, satisfying the precision, accuracy, LOD, LOQ and robustness requirements. The specificity of the method was confirmed. The proposed RP-HPLC method can be applied for the analysis of API, assays in dosage forms, and drug analysis in stability samples without matrix interference. It has a short analysis time (run time 5.0 min) and can be employed in the quality control testing of drugs. An aqueous mobile phase, acetate buffer, pH 4.4, is highlighted in this work. The use of acetate buffer has the added benefit of extending the present method for LC-MS applications. The proposed method is cost effective due to the low % organic mobile phase (36%) and is sensitive compared to previous reports (Suneetha, Mounika, Shaik, 2020; Anjaneyulu, 2019; China, 2018). Moreover, it has reduced analysis time, resources and solvent consumption, whilst generating the maximum information with a small number of experiments.

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