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Advances in Bioresearch

ORIGINAL ARTICLE

Development and Validation of a Chemometric Assisted Analytical Method for the Simultaneous Estimation of Ertugliflozin and Sitagliptin in Pharmaceutical Dosage Forms by RP-HPLC

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ABSTRACT
Using a quality by design and multi-criteria decision-making approach, this work aims to describe a recently created, optimized, and validated isocratic RP-HPLC technique for the separation of two anti-diabetic medicines (Ertugliflozin and Sitagliptin) in bulk and pharmaceutical formulations. The effective chromatographic separation was accomplished by utilizing the Monolithic C_{18} segment (100×4.6 mm id, 5µm molecule size) and PDA-UV- detection at 210nm.Methanol was the range of independent variables used for the streamlining. 30- 45% v/v, pH: 3 to 5 and buffer strength: 0.01 to 0.5. Methanol, Acetonitrile, a pH balance of 5 to 0.5, a flow rate of 1 ml/min, and buffer strength of 0.499 were chosen as the ideal test conditions. The peak area ratio of the analyte was used to evaluate pharmaceutical formulation tests. The total chromatographic analysis time per sample was approximately 2 minutes, with Sitagliptin and Ertugliflozin eluting with retention times of 2.6 and 3.1 minutes, respectively. For the quantitative analysis of commercially available tablets containing Ertugliflozin and Sitagliptin, the optimized assay setup was validated in accordance with ICH guidelines.The validation study supported the determination of the assay conditions by confirming that the assay was precise, accurate, linear, specific, and robust. As a result, this RP-HPLC method can be used as a routine quality control analysis of sodium-glucose co-transporter 2 (SGLT2) inhibitors like Ertugliflozin in combination with Sitagliptin.
Key words: Multi-Criteria Decision-Making Approach, SGLT2, RP- HPLC, Sitagliptin, Ertugliflozin.

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INTRODUCTION

According to the International Diabetes Federation's global cartographic picture of diabetes, type 2 diabetes mellitus (T2DM) is a global pandemic. Diabetes mellitus is a chronic, progressive metabolic condition characterized primarily by hyperglycemia [1]. Impaired insulin secretion, tissue resistance to insulin effect, or a combination of both, is believed to be the most common causes contributing to the pathophysiology of T2DM. Secretion is a prominent activity of pancreatic beta cells [2-3]. T2DM is a progressive disease, for the treatment of many patients they require combination therapy to maintain over time glycemic levels [4-5].Including Ertugliflozin to people with type 2 diabetes who were not adequately overseen with Metformin and Sitagliptin is compelling and secure [6–8]. A verbal sodium glucose transporter 2 inhibitor is Ertugliflozin. The study compared the safety and efficacy of starting Ertugliflozin and Sitagliptin together with placebo in patients with T2DM who were well-controlled with exercise and diet [9-10].Ertugliflozin (ERTU) Figure 1, is chemically known as Ertugliflozin L-pyroglutamic acid is (1S,2S,3S,4R,5S)-5-(4-chloro-3-(4ethoxybenzyl)phenyl)-1-(hydroxymethyl)-6,8 di-oxabicyclo [3.2.1] octane-2,3,4-triol, compound with (2S)-5oxopyrrolidine-2-carboxylicacid. A white to off-white powder, ERTU is. The molecular weight is 566.00 and the chemical formula is $C_{22}H_{25}ClO_7$. The drug ingredient is soluble in organic solvents like DMSO, ethanol, and dimethyl formamide; it is just

marginally soluble in methanol; and it is somewhat soluble in acetone, ethanol, and acetonitrile. ERTU is a selective and potent inhibitor of sodium-dependent glucose co-transporters (SGLT), specifically type 2, which is responsible for approximately 90% of glomerular glucose reabsorption [11]. Ertugliflozin dosing increases urine glucose excretion, leading to negative balance and osmotic diuresis. As a result, this diabetes medication has been demonstrated to drastically lower weight and blood pressure in diabetic individuals [12].

Sitagliptin (SITG) Figure 2, chemically (S)-3-amino-1-(3- (trifluoromethyl)-5,6-dihydro-[1,2,3] triazolo[4,3-a]pyrazin-7(8H)- yl)-4-(2,4,5-trifluorophenyl)butane-1-one. SITG is a white to off-white powder. It has a molecular formula of $C_{16}H_{15}F_6N_5O•H_3PO_4•H_2O$ with molecular weight 407.320 g/mol. The solubility of drug substance is soluble in water and N, N-diethyl formamide, marginally soluble in methanol, somewhat soluble in ethanol, acetone and acetonitrile. SITG is the first of a new class of medications for the treatment of type II diabetes, and it is a well-known hypoglycemic agent in current therapy. It lowers blood glucose levels by improving the effect of incretins, resulting in a significant increase in insulin secretion [13]. Recently reverse phase high performance chromatography (RP-HPLC). The methods for simultaneous determination of ERTU and SITG in pharmaceutical dosage forms and biological fluids have been published, but they are either long delayed or expensive. To the best of our knowledge, as of now, there is no high performance liquid chromatography (HPLC) system using advancement strategies utilizing numerous criteria decision-making approach have been represented for the concurrent estimation of ERTU and SITG. As a result, synchronous backup of these specimens is empowering and essential. The creation and upgrading of isocratic HPLC techniques is an interesting system that requires simultaneous estimation of several components: natural stage type and synthesis, flow rate, pH, fixed stage type, section temperature, etc. For a long time, HPLC separations have been based on experimental philosophies, but the application of long-term experimental approaches has only led to a clear ideal, data on elemental susceptibility to analyte distribution and inter factor communication are not readily available. All chemometric techniques including multi-criteria decision making (MCDM), factorial outlines, and reaction surface systems can be combined to achieve this goal. The best test configuration approach to model and advancement are the reaction surface design.

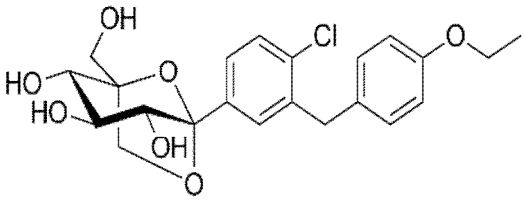

Figure: 1 Structure of ERTU

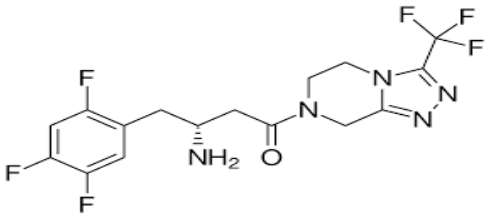

Figure: 2 Structure of SITG

MATERIAL AND METHODS

Apparatus

A Shimadzu RP-HPLC model (Tokyo, Japan) with an LC-20AD solvent supply module, a Rheodyne injector (model 7125, USA) valve with a 20-loop, and an SPD-M20A prominence diode array detector was used for chromatographic measurements. Shimadzu chromatography software (LC Solution, Release 1-11SP1) and a personal computer running the SCL-10A system controller were used to operate the system. To degas the mobile phase, the Branson sonicator (Branson Ultrasonic Corporation, USA) was used. The absorbance spectra were recorded using a UV-double beam spectrophotometer. (Japan, Systronices 2202 Model UV-1601PC) having a quartz cell with a route length of 1 cm. Design-Expert® Software 2017 trial version 11 was used for the experimental design, data analysis, and desirability function calculations. The analysis was carried out using Microsoft Excel 2007 programme (Microsoft, USA).

Chemicals and reagents

Chemicals and reagents for working standards of ERTU and SITG were procured from Lara drugs private limited, Dharmajigudem Telangana. Acetonitrile (ACN), Methanol (MeOH) of HPLC grade and Potassium dihydrogen orthophosphate (KH_2 PO_4) and Orthophosphoric acid was of analytical- reagent grade provided by M/S SD Fine Chemicals, Mumbai, India. Using Milli-Q Academic, Millipore HPLC quality water was created. The pharmaceutical drugs were acquired from Whitehouse Station, USA-based Merck and Co. Inc.

Standard solutions

Utilizing the mobile phase, stock standard solutions of ERTU and SITG (1 mg/mL) were created. The prepared stock arrangements were then covered from light and stored at 4°C + 0.05. During analysis day, working standard solutions were freshly arranged by dilution stock solutions with mobile phase. ERTU and SITG peak area ratio calibration curves were constructed in the 01-06 g/ml and 20-120 g/ml ranges, respectively. A standard solution prepared for the optimization procedure constituted ERTU and SITG at 10.0µg/mL and 10.0µg/mL respectively.

Sample preparation

20 tablets of (ERTU-5mg and SITG-100mg) should be weighed and ground up. The crushed tablet powder, which is equal to 1mg of ERTU and 20mg of SITG, should then be added to a 10mL volumetric flask along with 8mL of mobile phase and sonicated for at least 30 minutes while shaking the flask occasionally. Mobile phase (MeOH, ACN, 0.01 mM KH_2 PO4 at pH 5 0.5; 45 mL: 10: 45% v/v) should be added to the volume to make it up to 10 mL. Use a 0.2-m membrane filter (Gelman-Science, India) to filter the solution. 1mL of the above solution is transferred to a 10mL volumetric flask, diluted to volume with mobile phase, and mixed.

Chromatographic procedure

Chromatographic separations were carried out on a C_{18} Monolithic column (100mm× 4.5mm i.d., 5µm) connected with a C_{18} guard cartridge (4mm×3mm i.d., 5µm). The mobile phase consisted of MeOH, ACN, 0.01M KH_2 PO_4 (pH 5 ±0.5), adjusted with freshly prepared 10% orthophosphoric acid. A wavelength of 210 nm was selected for detection. The injection volume of the sample was 20µl. The HPLC system was employed in an air-conditioned laboratory atmosphere (20±2°C).

Validation

Validation studies were conducted utilizing the optimized assay conditions in light of the standards of approval portrayed in the ICH guidelines "Text on validation of Analytical Procedures" **[14]**. And "Q2B, Validation of Analytical Procedure: Methodology" **[15]**. Key analytical parameters, including linearity, precision, accuracy, detection limit, quantization limit were evaluated. The calibration curves were tested utilizing one-way analysis of variance (ANOVA) at a 5% significance level. Calibration curves were inherent in a low region of 10-50% of the target analytes concentration for the limit of detection and quantification. Additionally, the suggested method's robustness was assessed for slight changes in the pH, buffer concentration, and MeOH concentration.

RESULTS

Analysis and design of optimization during the process of streamlining technique, it is necessary to research the shape term in the centre points utilizing Factorial design. ANOVA for a 2k Factorial outline reveals that arch is significant for all responses (K_1, R_2, and S (1, 2), tR_2), with a p-value of less than 0.05. This result in a quadratic model, and cubic models should also be taken into account to illustrate the separation technique. For Capacity factor, resolution and separation models we picked quadratic and for retention time we picked linear models. Remembering the ultimate objective to get a second request prescient model, a central composite design (CCD) is used, which is an outline composed under response surface methodology (RSM). CCD is picked because of its adaptability and can be connected to upgrade a RP-HPLC separation gaining better comprehension of variables fundamental and communication impacts. The choice of key elements analyzed for improvement relied upon preparatory trials and prior data from the literature. The variables decided for enhancement process were MeOH concentration (A), buffer strength (B) and pH (C). As reactions, the limit factor for the first eluted peak (K_1), the resolution and separation of the second peak (R_2, $S_{(1, 2)}$), and the retention time of the last peak (tR_2) were chosen. During the preliminary investigation, the resolution between two peaks R_2 was found to be near 0 and merging; as a result, these two peaks were identified as critical peaks and included as one of the responses for the global optimization.All experiments were conducted in a randomized order to limit the effects of uncontrolled variables that may have an influence on the estimates. Replicates (n=6) of the central points were performed to estimate the experimental error shown in **Table 1**, summarizes the

conducted experiments and responses. The quadratic and cubic mathematical model for the independent factors is specified in Eq. (1) and (2)

$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_2 X_1 X_2 + \beta_3 X_1 X_3 + \beta_3 X_2 X_3 + \beta_1 X_1^2 + \beta_2 X_2^2 + \beta_3 X_3^2$ (1)

$y = \beta_0 x^3 + \beta_1 x^2 + \beta_2 x + \beta_3$ (2)

Where Y is the response to the model, ß is the regression coefficient and X_1, X_2 and X_3 represents factors A, B and C, individually. **Table 2** shows the statistical ANOVA parameters of the compact model. In order to create a simple and realistic model, a backward elimination approach was used to remove insignificant terms ($P > 0.05$) from the model. In the current study, the adjusted R2 was well within the acceptable limits of R2 = 0.6752 which uncovered that the experimental data demonstrate a good fit with the second-order polynomial equations. In this study, the ratio was found to be in the range of 5.4799 - 10.0733, indicating that the signal is adequate and that the model is significant for the separation procedure. The coefficient of variation (C.V.) is a measure of model reproducibility, and a model is considered reasonably reproducible if it is less than 10%. The C. V for all the models was found to be less than 10% except for K1 (16.23), R2 (21.08), tR2 (27.74) Hence, the diagnostic plots, (a) normal probability plots [16] of residuals and (b) plot of residuals versus predicted values**[17]** were analyzed for response K1, R2 and tR2. Since the assumptions of normality and constant variance of residuals were observed to be satisfied, the fitted model for the K1, R2, and tR2 was accepted **[18]**.

As can be found in **Table 2**, the interaction term with the largest absolute coefficient among the fitted models is C (0.3812) of tR2 model. The positive result shown for C is statistically momentous for tR_2. The study reveals that changing the fraction of pH results in a rapid decline in the retention time of ERTU and SITG. At a low level of factor C, results in a decrease in the retention time. Therefore, when the pH has to be at its lowest level to shorten the runtime. **Figure 3** displays perturbation plots for projected models to show the impact of a single independent component on a single response while keeping all other factors constant relative to a reference point. A steepest slope or curvature shows affectability of the response to a particular factor **(Figure 3d)** shows that pH (factor C) had the most important effect on a retention time tR_2 followed by factor A and B. In (Figure 3c and 3b) the factors B (Buffer strength) had significant effect on $S_{(1, 2)}$ and R_2 followed by factor C and A. In (Figure 3a) shows that MeOH concentration (factor A) had the most important effect on a capacity factor K_1 followed by factor B and C.

Response surfaces plots for K_1, R_2 and $S_{(1, 2)}$ and tR_2 are illustrated in **Figure 4** (% Methanol concentration is plotted against the Buffer concentration and pH held at constant at the center value). Analysis of perturbation plots and response plots of optimization models uncovered that factor A and B had the huge impact on a separation of the analytes, whereas the factor C i.e. the Flow rate, is of little noteworthiness.

When the goal of experiment is optimization, FDS graph gives interpretation of sizing and precision of design. We obtained FDS value 0.99 or 99 % as shown in **Figure 5** for central composite design indicating good size and precise design to see effect of formulation parameter on the selected CQAs **[19]**.

Global optimization in the present study, the distinguished criteria for the optimization were: resolution between two critical peaks, capacity factor, separation and retention time of the last peak. Three responses with different aims were optimized using Derringer's desirability function **[20]**. The geometric mean, weighted average, or another term may be used to describe the Derringers desirability function, or D. The expression that characterizes the Derringers desirability function is:

$$D = [d_1^{p1} \, X \, d_2^{p2} \, X \, d_3^{p3} \, X \, \ldots\ldots\ldots X \, d_n^{p}]^{\frac{1}{n}}$$

Where pi is the weight of the response, n the number of responses and di is the individual desirability function of every response. Desirability task (D) can take values from 0 to 1. Weights can extend from 0.1 to 10. Weights lower than1 gives less significance to the objective, whereas a weight more than 1 gives more significance to the objective. In the present study, pi values were set 1 for all the responses. A value of D close up to 1 indicates that the amalgamation of the different criteria is matched in a global optimum shown in (**Table 3**). Criteria I have been wished-for selecting an optimum experimental circumstance for analyzing schedule quality control samples. As can be seen under criteria I, the responses tR_2, R_2, K_1 ware targeted in order to keep the value in acceptable limit. On the other hand, $S_{1, 2}$ kept unchanged to allow baseline separation ERTU and SITG. In order to separate the first eluting peak (ERTU) from the solvent front, K_1 was in range. Importance can range from 1 to 5, which gives emphasis to a target value. The significance for retention time is 3 to trim down the time of analysis. Following the conditions and restrictions above, the optimization procedure was carried out. The Graphical representation of the overall desirability function D (D=0.865)where MeOH Conc. (a) of 45, of Buffer Strength (b) 0.499 and pH

(c) 5 and individual desirability of the four responses and three factors (**Figure 6**). The predicted response values corresponding to the latter value of D were: K_1 =2.056, R_2 =2, $S_{(1, 2)}$ = 5.717 and tR_2 = 3.1 min. Figures 7 and 8 illustrate the corresponding chromatogram in comparison to the chromatogram obtained before the experiment was optimized , which proved the prediction accuracy of the model.
The agreement between experimental and anticipated responses for the projected optimums is shown in **Table 4** to evaluate the predictability of the projected model. Eq. (3) was used to get the accuracy percentage of predictions. The average error for K_1 = 2.14, R_2 = 2, $S_{(1, 2)}$ = 2.431 and tR_2 = 1.58 were respectively, indicating good correlation between the experimental and predicted responses.

Predicted Error= Experimental- Predicted/ Predicted *100 (4)

$$D = [d_1^{p1} \text{ X } d_2^{p2} \text{ X } d_3^{p3} \text{ X } \ldots\ldots\ldots \text{X } d_n^{p}]^{\frac{1}{n}}$$

Assay method validation the last step of the study was to check method validation for specificity, linearity, intra/between day precision and robustness. In comparison to the placebo used in the experiment, the optimized HPLC method was unique. Each and every placebo chromatogram showed no interference peaks. An amazing linearity was set up at six levels in the range of 1-6 µg/ml and 20-120 µg/ml for ERTU and SITG respectively with R^2 of more than 0.999 for all the analytes. The slope and intercept of the calibration curve were 98747x + 2666.4 and 53129x + 26666 for ERTU and SITG respectively. Since the correlation coefficients are not good indicators of linearity performance of an analytical procedure a one-way ANOVA was performed. For all the analytes, the calculated F-Value (F calculated) was found to be less than the theoretical F-value (F critical) at 5% significance level, indicating that there was no significant difference between replicate determinations for each concentration level. The limit of detection (LODs) and limit of quantification (LOQs) for ERTU and SITG are 6.175, 18.712 µg/ml and 123.499, 374.241 µg/ml respectively. Accuracy (n=9), assessed by spike recovery, were found to be 99.63, 100.3, 100.93 for ERTU and 99.27, 100.59, 100.83 for SITG, with were within acceptable ranges of 100 ± 2%. The intra and inter-assay precision (n=6) was established since, the %CV were well within the target criterion of ≤ 2 and ≤3 respectively. Robustness revision reveals that small changes did not alter the retention times, retention factor and resolution and wherefore it would be concluded that the method conditions are robust. Application of the method as a final step, commercial tablet product containing 5mg of ERTU and 100mg of SITG were assayed by the proposed RP-HPLC method. Representative chromatograms are presented in **Figure 7, 8 & 9.** The results achieved when analyzing marketed pharmaceutical tablets was 98.9% of ERTU and 99.25% of SITG. Good conformity was found between the assay results and the label claim of the product. The %C.V. for the tablet is < 2, indicating the precision of the analytical methodology.

DISCUSSION

The preliminary chromatographic conditions (stationary phase, pH-range, choice of buffer and wavelength) were chosen based on experience and prior knowledge from literature. The resolution was to be improved, and the analysis time was to be shortened. For the optimization, central composite design (CCD) was preferred as it is ideal for chromatographic trailing and allows relatively controlled range of experiments to outline the factors that have an effect on the chromatographic behavior of investigated substances. The method was optimized by developing the experimental methodology, which also provided a detailed understanding of the relation between factor and response and the underlining interaction between them. Numerical optimization by "trading" different variables to achieve the desired objectives, i.e. optimizing the top area and theoretical plate and reducing retention times and the height to obtain a target feature near to 1 min, has been carried out in the search for optimum condition. The graphical optimization also yielded the optimum **[21]**.In this study, Analytical quality by design (AQbD) concept was used in the development of RP-HPLC method for the simultaneous estimation of ERTU and SITG. Therefore, three parameters, pH, Buffer strength and % MeOH in the mobile phase were selected as Critical material attribute (CMA)Central Composite experimental design with three independent variables at four levels was implemented to optimize critical method parameters **[22]**. The design space presents the operable method region where the changes will not affect the quality of analysis. Specificity was assessed by percent recovery of both the drugs when analyzed in combination. The percentages of recovery for both drugs were within statistical confines. The peaks of each drug were observed to be well separated and not interfering. As a result, the method can be said to be unique to the two drugs in combination. The estimated limit of detection (LOD) and limit of quantification (LOQ) values confirmed that the methods were sensitive enough. Furthermore, the drug recovery rates were found to be

acceptable. As a result, the developed method is suitable for concurrent, quantitative analysis of ERTU and SITG. The method was validated for linearity, precision, accuracy, sensitivity, system suitability, as well as robustness. The developed method is convenient and effective for quality control as well as simultaneous routine analysis of ERTU and SITG in pharmaceutical dosage forms. Low Rt values of the DPs show that the method was extremely beneficial in terms of time economy for determining ERTU and SITG formed under stress conditions. The developed method was found to be sensitive which was evaluated in terms of LOD and LOQ. Further, the Rt of ERTU and SITG in all the dosage forms were similar with respect to the standard ERTU and SITG without any significant difference in the standard solution. Other parameters, like peak tailing and theoretical plates were found to be within the acceptable limits. This is a corroborated high degree of utility of developed method for routine estimation of ERTU and SITG in pharmaceutical formulations. The method was optimized by design of expert (DOE) technique using different variables and the method shown to be precise, accurate and linear over the concentration range. The less solvent usage and quick analytical run time result in a cost-effective procedure.

Table1: Experimental design and results of a rotatable central composition design

	Factor 1	Factor 2	Factor 3	Response 1	Response 2	Response 3	Response 4
Run	A:Methanol Con	B:Buffer Strength	C:pH	K1	R2	S2	tR2
1	45	0.5	3	2.453	1.621	5.601	1.44
2	30	0.01	3	2.114	1.225	4.459	1.298
3	30	0.01	5	2.231	1.212	4.113	1.321
4	30	0.5	5	2.345	1.321	4.231	1.45
5	37.5	0.255	4	1.482	2.724	5.014	1.32
6	45	0.01	5	1.564	1.97	4.97	3.78
7	37.5	0.255	4	1.482	2.724	5.014	1.32
8	37.5	0.255	2.31821	1.421	2.132	4.997	1.112
9	37.5	0.255	5.68179	1.524	2.156	5.002	3.789
10	24.8866	0.255	4	1.678	3.342	5.21	2.08
11	37.5	0.255	4	1.482	2.724	5.014	1.32
12	37.5	0.255	4	1.482	2.724	5.014	1.32
13	50.1134	0.255	4	2.876	3.455	5.35	1.423
14	37.5	0.255	4	1.482	2.724	5.014	1.32
15	37.5	0.667039	4	1.786	1.001	5.034	1.45
16	45	0.5	5	1.962	1.813	5.601	3.132
17	30	0.5	3	2.013	1.321	4.231	1.21
18	45	0.01	3	2.321	1.321	4.231	1.35
19	37.5	-0.157039	4	1.567	1.321	3.45	1.25
20	37.5	0.255	4	1.562	2.724	5.014	1.32

Table 2:Models and statistical parameters obtained from ANOVA for CCD

RESPONSES	**REGRESSION MODELS**	**ADJUSTED R_2**	**MODEL P VALUE**	**%C.V**	**ADEQUATE PRECISION**
K_1	+1.49+0.1180 * A+0.0667*B-0.0458* C+0.0646*AB-0.2121* AC+0.0601*BC+0.3392* A^2+0.1269* B^2+0.0547* C^2	0.4983	< 0.05	16.23	5.4799
R_2	+2.75+0.1344 *A -0.0139*B+0.0636*C-0.0077*AB+0.1068*AC-0.0555* BC+0.0846* A^2-0.7065 *B^2-0.3590* C^2	0.6752	< 0.05	21.08	8.0289
$S_{1,2}$	+5.02+0.2639*A+0.3335*B+ 0.0294*C+0.2639*AB+ 0.1356*AC-0.0491*BC+0.0623* A^2-0.3047* B^2-0.0369* C^2	0.6970	< 0.05	6.17	10.0733
TR_2	+1.32+0.0965*A-0.0132*B+0.3812*C-0.0749*AB+0.2324*AC-0.0651* BC+0.1349* A^2-0.0070*B^2+0.2053* C^2	0.6808	< 0.05	27.74	7.7240

Model P values are statistically significant (P < 0.05)

Table 3: Criteria for the optimization of the individual responses for the analysis of quality control samples (Criteria I)

RESPONSES	LOWE LIMIT	UPPER LIMIT	CRITERIA I		
			GOAL	IMPORTANCE	WEIGHTS
K_1	1.421	2.876	TARGET=2.1485	3	1
R_2	1.001	3.455	TARGET= 2	3	1
$S_{1,2}$	3.45	5.601	NONE	3	1
tR_2	1.112	3.789	TARGET= 1.5	3	1

Table 4: Comparison of observed and predictive values of different objective functions under optimal conditions

OPTIMUM CONDITIONS	MEOH (%)	BUFFER STRENGTH	PH	K_1	R_2	$S_{1,2}$	tR_2
FOR FORMULATION	DESIRABILITY VALUE (D) =0.866						
	45	0.5	5				
	EXPERIMENTAL VALUE			2.1	2.03	5.856	3.149
	PREDICTED VALUE			2.056	2.000	5.717	3.1
	AVERAGE % ERROR			2.14	2	2.431	1.58

FIGURE: 3(A)

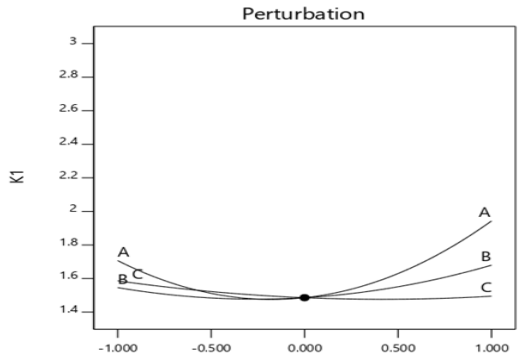

FIGURE:3 (B)

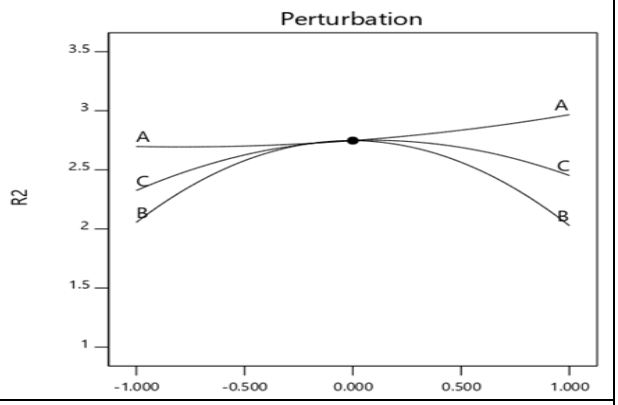

FIGURE:3 (C)

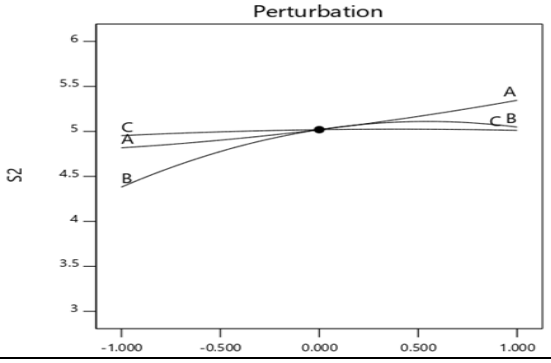

FIGURE:3 (D)

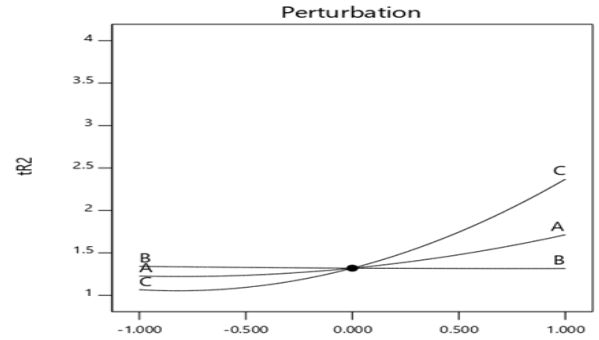

Figure:3 Perturbation plots showing the effect of the each independent variables on (a) K_1, (b) R_2, (c) $S_{1,2}$, (d) tR_2 Where A is the MeOH concentration, B the Buffer Strength, C the pH.

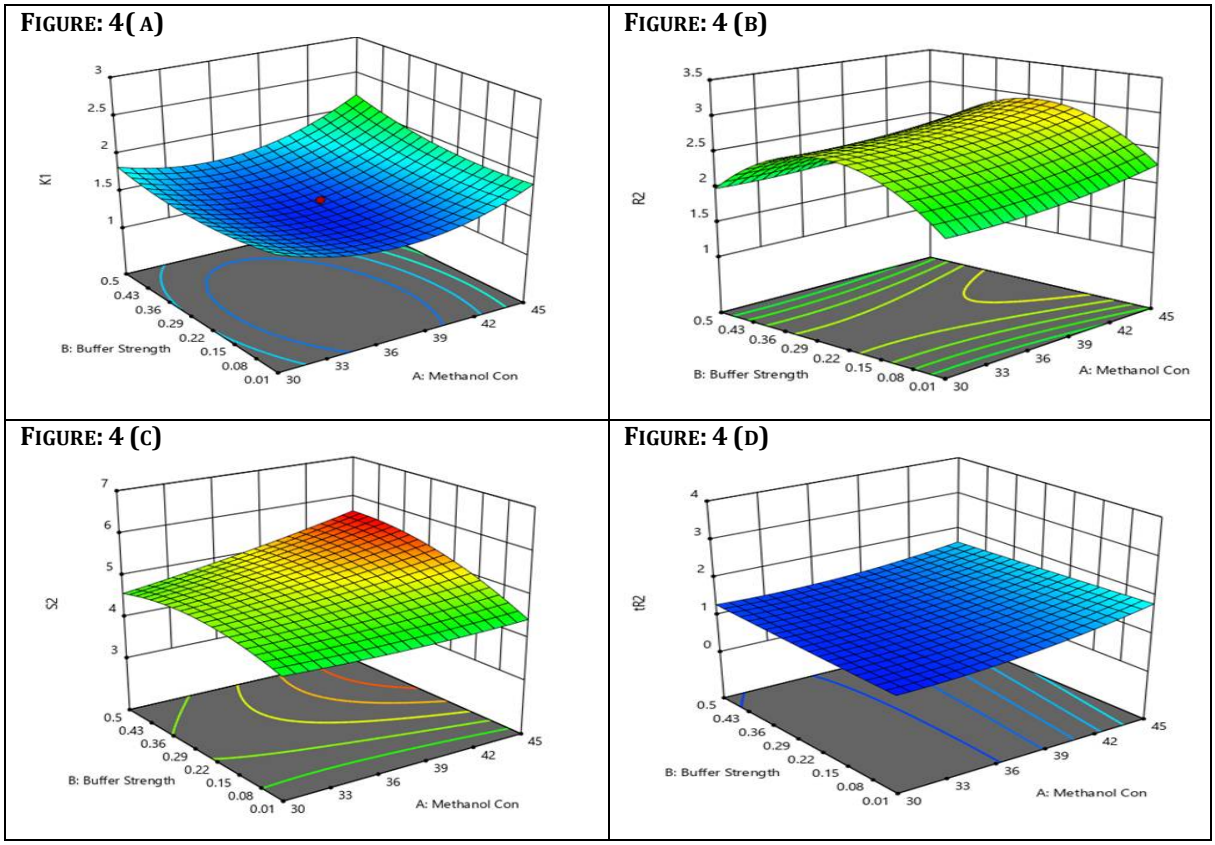

Figure 4: Response surfaces related to MeOH (A), Buffer Strength (B), Ph (C) :(a) capacity factor first peak (K_1), (b) Resolution of the critical pair (R_2), (c) Separation of two peak ($S_{1,2}$), (d) Retention time of the last peak (tR_2).

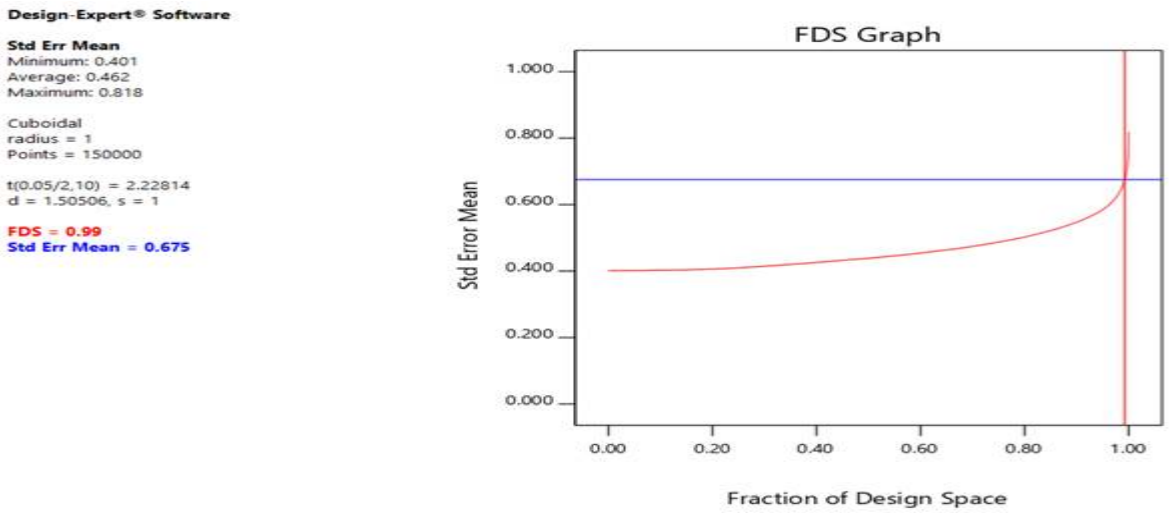

Figure 5: FDS graph for Central Composite design for ERTU and SITG in Bulk and Pharmaceutical Dosage Forms

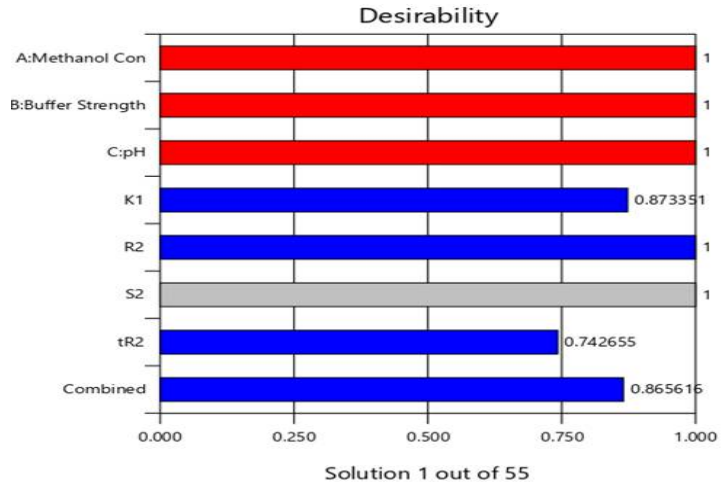

Figure 6: Graphical representation of the overall desirability function D (0.865) where MeOH Conc. (a) of 45 , Buffer Strength (b) 0.499 and pH (c)5 ± 0.5 and individual desirability of the four responses and three factors.

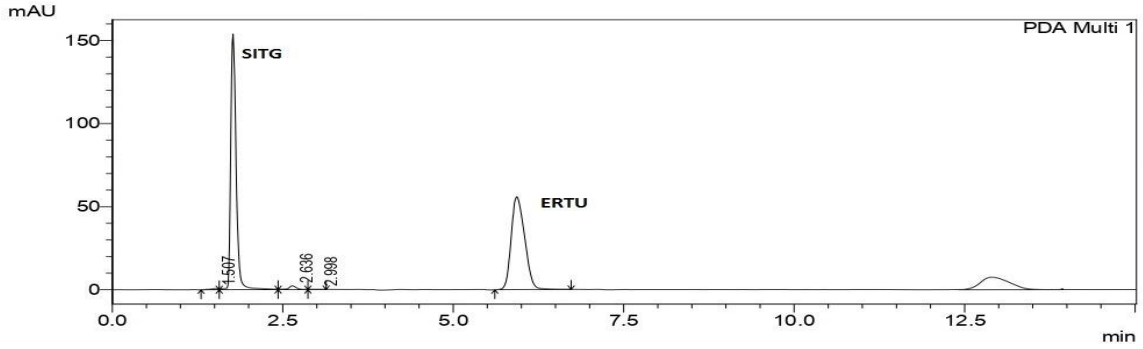

Figure 7: Chromatogram for SITG & ERTU in bulk drug solution before optimization

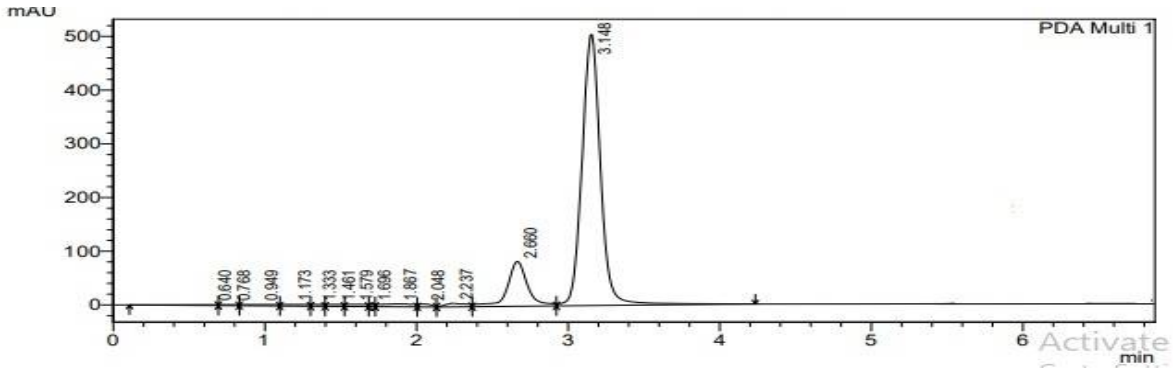

Figure 8: Chromatogram for SITG & ERTU in bulk drug solution after optimization

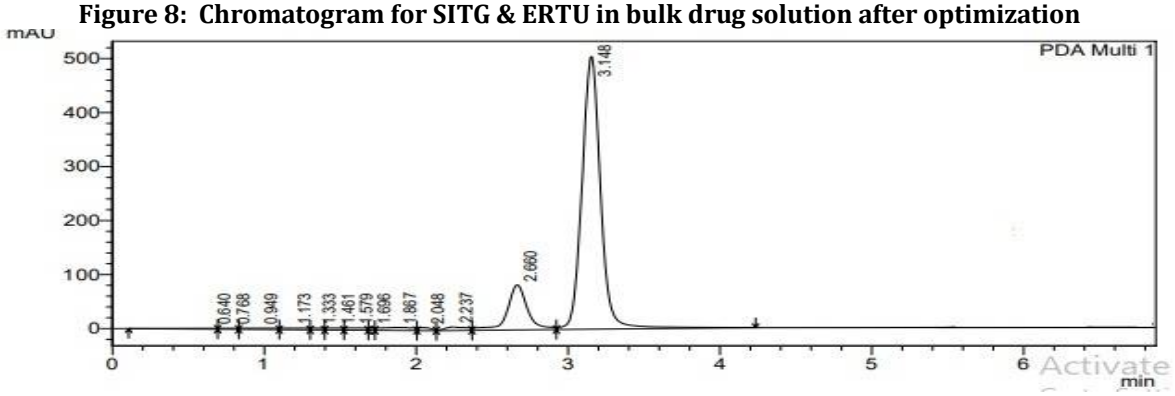

Figure 9: Chromatogram for the assay ofSITG&ERTU in Tablet formulation

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