IJPSR (2023), Volume 14, Issue 8 **(Research Article)**

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 10 December 2022; received in revised form, 16 February 2023; accepted 28 May 2023; published 01 August 2023

DEVELOPMENT AND VALIDATION OF STABILITY INDICATING RP-HPLC METHOD FOR ERTUGLIFLOZIN AND SITAGLIPTIN BY QBD APPROACH

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Keywords:

Ertugliflozin, Sitagliptin, Quality by design approach, RP-HPLC, Validation, Forced degradation studies

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ABSTRACT: The concept of quality by design (QbD), which entails understanding the essential components and their interaction effects by a desired set of tests, has lately gained relevance in developing analytical. To validate the simultaneous quantification of Ertugliflozin and Sitagliptin in bulk drugs and its pharmaceutical formulation, the present study discusses the development of the Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) method by the QbD methodology employing the Design of Experiments. The three essential elements of the RP-HPLC method-Flow rate (ml/min), Mobile phase (%), and temperature (^{o}C) are systematically explored in an effective experimental design that is provided. Statistical analysis tools were used to assess the significant impact of these parameters and their interactions with the response variables (Retention time and tailing factor). The 260nm optimized wavelength was chosen. Ertugliflozin and Sitagliptin were shown to have retention times of 3.897 and 2.527 minutes, respectively. Ertugliflozin and Sitagliptin can be estimated simultaneously using a linear method over the ranges of (1.875-11.25 g/ml) and (12.5-75 g/ml), respectively. The correlation coefficient R2 for Ertugliflozin and Sitagliptin are 0.9993 and 0.9995. The method's %RSD for precision and accuracy was discovered to be under 2%. Studies on forced degradation concluded that the strategy indicated stability. Ertugliflozin and Sitagliptin both had %Recovery values of 99.81% and 99.84%, respectively. Retention time and run time decreased, so the method developed was simple and economical and can be adopted in regular Quality control test in Industries.

INTRODUCTION: A Quality by Design approach is outlined as a system for planning, analyzing, and dominant manufacturing through timely measurements of essential quality and performance attributes of recent and in-process materials and processes, aiming to ensure the ultimate product safety.

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DOI: 10.13040/IJPSR.0975-8232.14(8).3852-67

This article can be accessed online on www.ijpsr.com

DOI link: http://doi.org/10.13040/IJPSR.0975-8232.14(8).3852-67

Quality by design (QbD) has become a crucial paradigm within the pharmaceutical industry since its introduction by the US Food and Drug Administration (USFDA) [1-2]. The idea of quality by design (QbD) has recently acquired importance in analytical methodology development by application of design of experiments approach [3].

Quality by design involves understanding the critical factors and their interaction effects by a desired set of experiments. This article describes how, statistically, QbD principles are often placed into developing optimized RP-HPLC method conditions.

The experimental runs were conducted as per the Box-Behnken statistical screening design method. Under this design, factors such as [Flow rate (ml/min), Mobile phase (%), and temperature (^{o}C)] were screened and optimized [4]. Ertugliflozin **Fig. 1** belongs to potent and selective inhibitors of the sodium-dependent glucose cotransporters (SGLT), specifically type 2, responsible for about 90% of the glucose reabsorption from the glomerulus. The mechanism of action of Ertugliflozin is a part of a normal process; the glucose from the blood is filtered for excretion and reabsorbed in the glomerulus, so less than one percent of glucose is excreted in the urine. The reabsorption is mediated by the sodium-dependent glucose cotransporter (SGLT), mainly type 2, responsible for 90% of the reabsorbed glucose. Ertugliflozin is a small inhibitor of SGLT2 and its activity will increase glucose excretion, reducing hyperglycemia while not requiring excessive insulin secretion [5-8].

Sitagliptin **Fig. 2** is a new oral hypoglycemic (anti-diabetic drug) of the new dipeptidyl peptidase-4 (DPP-4) inhibitor category of drugs. This enzyme-inhibiting drug is to be used alone or in combination with metformin or thiazolidinedione to control type 2 diabetes mellitus. Sitagliptin is an extremely selective DPP-4 inhibitor believed to exert its actions in patients with type 2 diabetes by slowing the inactivation of in cretin hormones, thereby increasing the concentration and prolonging the action of those hormones. Incretin hormones, including glucagon-like peptide-1 (GLP-1) as well as the intestine, release glucose-dependent insulin tropic polypeptide (GIP) throughout the day and increase levels in response to a meal. The enzyme DPP-4, speedily inactivates these hormones.

The incretins are an element of an endogenous system that controls glucose homeostasis physiologically. GLP-1 and GIP stimulate insulin production and release from pancreatic beta cells by intracellular signalling pathways, including cyclic AMP when blood glucose levels are normal or increased. GLP-1 also lowers glucagon secretion from pancreatic alpha cells, reducing hepatic glucose production. By increasing and prolonging active incretin levels, sitagliptin will increase insulin release and decreases glucagon levels in the circulation in a glucose-dependent manner. These changes lead to a decrease in hemoglobin A1c (HbA1c) levels and a lower fasting and postprandial glucose concentration. Sitagliptin demonstrates selectivity for DPP-4 and doesn't inhibit DPP-8 or DPP-9 activity *in-vitro* at concentrations approximating those from therapeutic doses [9, 10]. The literature survey discovered that few analytical methods, such as Ultra-performance Liquid Chromatography (UPLC), RP-HPLC (CAD) and RP-HPLC methods, were reported for the simultaneous estimation of Ertugliflozin and Sitagliptin. But until work, no Quality by Design (QbD) is applied for combination drugs that give robust, economic, and quick results. The objective of the present work was to develop easy, rapid, accurate, specific, and economic stability indicating RP-HPLC method using QbD approach for the Ertugliflozin and Sitagliptin in bulk and tablet form [11-17].

The chromatographic conditions for the proposed method were optimized with the help of design expert 11 software. Furthermore, the stability indicating RP-HPLC method was developed for stability [18, 19] studies of Ertugliflozin and sitagliptin in different stress conditions to establish inherent stability of the drugs. The method was more validated, and the analysis results were validated statistically and by recovery studies. The developed method was simple, precise, accurate, economical, and quick.

MATERIAL AND METHOD: All chromatographic measurements were made on Waters HPLC alliance 2695 model, 2996 PDA detector, PG Instruments T60 with a 2mm and 10mm special bandwidth. QbD software Design Expert 11 is used. Spectrum Laboratory, Hyderabad, supplied Ertugliflozin and standard sitagliptin drugs.

Drugs: Ertugliflozin, Sitagliptin (Procured from Rankem)

Instrumentation: The drugs were analyzed on a WATERS HPLC 2695 SYSTEM equipped with quaternary pumps, 2996 Photo Diode Array (PDA) detector, and Autosampler integrated with Empower 2 Software using RP-HPLC column. The output of signals was monitored and integrated using ChromNAV Chromatogram Software.

Electronics Balance of Denver, Ultrasonicator of BVK enterprises, p^H meter of BVK enterprises, India. UV-VIS spectrophotometer PG Instruments T60 with special bandwidth of 2mm and 10mm and matched quartz cells integrated with UV win 6 Software was used for measuring absorbances of Ertugliflozin and Sitagliptin solutions.

Chemicals and Reagents: The working standard of Ertugliflozin and Sitagliptin was provided as a gift sample from Rankem. The marketed formulation *i.e*., Steglujan 5/100 tablets containing 5mg Ertugliflozin and 100 mg Sitagliptin, Acetonitrile (HPLC grade), Methanol (HPLC grade), water (HPLC grade), Phosphate buffer, Potassium dehydrogenate ortho phosphate buffer, Ortho-phosphoric acid.

All the above chemicals and solvents are procured from Rankem. HPLC-grade water was obtained by double distillation and purification through milli-Q water purification.

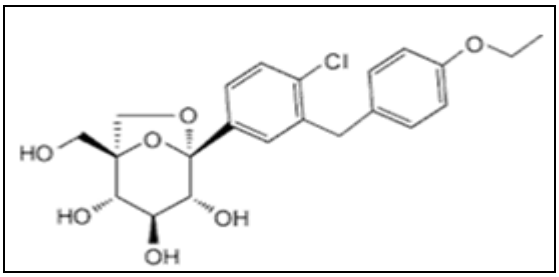


FIG. 1: STRUCTURE OF ERTUGLIFLOZIN

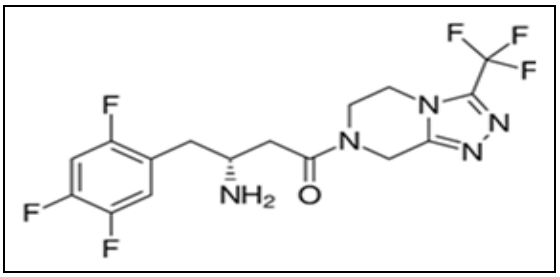


FIG. 2: STRUCTURE OF SITAGLIPTIN

Preparation of Standard Solution:

Diluent: Based upon the solubility of the drugs, diluents were selected, Acetonitrile and Water taken in the ratio of 50:50.

Preparation of Standard Stock Solutions: Take accurately weighed 3.75mg of Ertugliflozin and 25mg of Sitagliptin. Transferred it to individual 50ml volumetric flasks separately. 3/4 th of diluents were added to these flasks and sonicated for 10 minutes.

Flasks were made up with diluents and labeled as Standard stock solution 1 and Standard stock solution 2. (75µg/ml of Ertugliflozin and 500µg/ml of Sitagliptin).

Preparation of Standard Working Solutions (100% Solution): 1ml from each stock solution was pipetted out, taken into a 10ml volumetric flask, and made up with diluent. (7.5µg/ml Ertugliflozin of and 50µg/ml of Sitagliptin).

Preparation of Sample Stock Solutions: Took 5 tablets weighed accurately and the average weight of each tablet was calculated. One tablet's worth of weight was put into a 10 ml volumetric flask, 5 ml of diluents were added, and the mixture was then sonicated for 25 minutes before being made up with diluent and filtered through HPLC filters (containing 50 g/ml of ertugliflozin and 1000 g/ml of sitagliptin).

Preparation of Sample Working Solutions (100% Solution): 0.5ml of filtered sample stock solution was transferred to 10ml volumetric flask and made up with diluents (7.5µg/ml of Ertugliflozin and 50µg/ml of Sitagliptin).

Preparation of Buffer: 0.1% OPA Buffer: 1ml of Conc Ortho Phosphoric acid was diluted to 1000 ml with water.

Buffer: 0.01N Sodium dihydrogen phosphate: Take accurately weighed 1.42gm of Sodium dihydrogen phosphate in a 1000ml of Volumetric flask add about 900ml of milli-Q water added and degas to sonicate and finally make up the volume with water.

Chromatographic Conditions: The isocratic flow rate of the mobile phase was maintained at 1.0 mL/min and the analysis was carried out at an ambient column temperature at 30°C. The injection volume was 10µl. The eluted sample was monitored at 260 nm, and the run time was 6 min.

Initial Method Development:

Choice of Column: To choose the appropriate column, initial experimental trials were carried out. **Table 1** The C18 column was selected for additional trials based on the findings of the mentioned initial trials and their chromatograms **Table 2.**

TABLE 1: EXPERIMENTAL TRIALS FOR CHOICE OF COLUMN

Column	Observation	Interference
C_8	Poor retention of analyte	Broad and poor peak shape obtained.
C_{18}	Improved retention of analyte	Better peak shape obtained.

TABLE 2: CHROMATOGRAPHIC TRIALS FOR OPTIMIZED METHOD

Sr. no.	Mobile Phase	Retention time (min.)		Column	Observation	Remark
		Ertugliflozin	Sitagliptin			
1	Methanol: 0.1% OPA(50:50 v/v)	0	2.068	BDSC18 (4.6 x 150mm, 5µm)	Only Sitagliptin peak is eluted, Ertugliflozin peak was not eluted	Not satisfied
2	Acetonitrile: 0.1%OPA (50:50 v/v)	8.671	2.423	ZorbaxC18 (4.6 x 150mm, 5µm)	Both peaks were eluted but the retention of ertugliflozin is too long and less USP plate count were observed.	
3.	Acetonitriile: 0.1% OPA (50:50 v/v)	2.710	1.476	BDSC18 (4.6 x 150mm, 5µm)	Both peaks were eluted but the retention time of sitagliptin was within the voided range (<2min).	
4.	Acetonitrile : 0.1% OPA (60:40)	2.581	1.947	Kromasil C18 (4.6 x 150mm, 5µm)	Both peaks were eluted but sitagliptin peak retention time was in void range(<2mins)	

Software-Aided Method Development: A new Reverse Phase-HPLC method was developed and validated for Ertugliflozin and Sitagliptin by using QbD approach. A Quality by Design with Design of Experiments (DoE) approach to the development of an analytical method mainly involves two phases as follows:

a) Screening Phase

b) Statistical Analysis and Final Optimization

Screening Phase: A new Reverse Phase - HPLC method was developed for Simultaneous estimation of Ertugliflozin and Sitagliptin using Design Expert 11 software. In the software, Box-Behnken statistical screening design was used to optimize the Critical Process Parameters (CPP) or Critical Method Parameters (CMPs) and to evaluate the interaction effects of these parameters on the Critical Quality Attributes (CQAs).

This Box-Behnken statistical screening design is a 3 factorial level design that was specifically selected since it requires fewer experimental runs than other screening designs.

This Screening Phase includes the following steps:

Selection of Critical Method Parameters: The analytical technique that is being developed has a number of parameters that have been deliberately chosen to affect it. So, the Critical Method Parameters selected for the study are Flow rate, Mobile phase and temperature.

Selection of Critical Quality Attributes (CQAs): Critical Quality Attributes are the responses regulated to judge the quality of the developed analytical methods.

So, the Critical Quality Attributes selected for the study are Retention time and Tailing Factor. These responses were judged during the experimental trials.

Experimental Trials: In the Box-Behnken statistical screening design, low, medium and high critical method parameters were selected based on the preliminary experimentation. So, the Design summary for the Box-Behnken screening design is given in **Table 3.**

TABLE 3: DESIGN SUMMARY FOR SCREENING STUDIES

Factor	Name	Units	Type	Minimum	Maximum	Mean	Std. Dev.
A	FR	ml/min	Numeric	0.8318	1.17	1.0000	0.0848
B	MP	%	Numeric	33.18	66.82	50.00	8.48
C	T	0 c	Numeric	24.95	35.05	30.00	2.54

Evaluation of the above critical method parameters with a Box-Behnken design led to 20 experimental trials due to permutation and combination of the three parameters. These 20 experimental trials were conducted using the previously mentioned chromatographic conditions using the previously selected Phenomenex C18 (4.6 x 150mm, 5µm).

Statistical Analysis and Final Optimization: The responses obtained after carrying out the above trial runs were fed back to Design Expert software, and 3D-response surface plots and Graph plots were plotted. These plots revealed the influence of critical method parameters on the selected quality attributes *i.e.,* the effects of the factors (Flow rate, Mobile phase, temperature) on responses (Retention time, Resolution, Theoretical plate). Those plots were analyzed to estimate which method parameter gave the most acceptable responses. Thus, based on those observations, the final critical method parameters of the method were determined, and therefore, the optimized chromatographic conditions were finalized in **Table 4.**

TABLE 4: OPTIMIZED METHOD

Sr. no.	Mobile Phase	Retention time (min.)		Column	Observation	Remark
		Ertugliflozin	Sitagliptin			
5.	53.5% 0.1% OPA buffer: 46.5% Acetonitrile	3.897	2.527	Phenomenex C18 (4.6 x 150mm, 5µm)	Both peaks have good resolution, tailing factor, theoretical plate count	Satisfied

Furthermore, the evaluation of statistical analysis tools like ANOVA for each response was used to determine the significance of each method parameter selected for the study using the p-value (probability).

Validation of the Optimized Method: Analytical procedures were validated for Ertugliflozin and Sitagliptin using the following parameters.

System Suitability: System suitability testing is a core part of any analytical procedure. System suitability testing was performed by injecting 6 replicates of 10µg/ml standard Ertugliflozin and Sitagliptin solution. This evaluated system suitability parameters like retention time, number of theoretical plates and tailing factor. According to ICH guidelines, all system suitability variables were acceptable and within the acceptable range.

Robustness: Robustness conditions like flow minus (0.8ml/min), flow plus (1.0ml/min), mobile phase minus (58B:42A), mobile phase plus (42B:58A), temperature minus (25°C) and temperature plus (31°C) was maintained, and samples were injected in a duplicate manner. % RSD was within the limit.

Precision and Accuracy: The Precision is noted in terms of Relative Standard deviation (RSD) over the range of quantitation for a single experiment in which standards are assayed in replicate (Intraday) and for a series of experiments in which standards are assayed in several experiments (Interday). Precision of the developed analytical method was tested by injecting six replicate injections. Intraday and the interday precision study was carried out by estimating the corresponding responses for the solutions of the above six concentration levels on the same day and six different days, respectively.

Analysis of Marketed Formulation: 5 tablets were weighed, and the average weight of each tablet was calculated, The weight equivalent to 1 tablet was transferred into a 10ml volumetric flask, 5ml of diluents was added and sonicated for 25 min; further the volume was made up with diluent and filtered by HPLC filters (50µg/ml of Ertugliflozin and 1000µg/ml of Sitagliptin).

Then 0.5ml of filtered sample stock solution was transferred to 10ml volumetric flask and made up with diluent. (7.5µg/ml of Ertugliflozin and 50µg/ml of Sitagliptin).

Stability Indicating Assay of Ertugliflozin and Sitagliptin: To demonstrate the stability indicating nature of the method, the stock solutions of the drugs Ertugliflozin and Sitagliptin were stressed under different conditions as follows to promote degradation.

Degradation Study: Forced degradation is also known as stress testing. A substance is forcibly degraded through artificial means. It is a useful tool to predict the stability of any active pharmaceutical ingredient (API) and formulation product.

RESULT:

Software-Aided Method Optimization: DoE may be a tool for optimizing composition parameters. It is used to assess both the principle effects and their interactions. CCD may be a part of RSM, which shows quadratic response surfaces without a three-level factorial design.

The critical factors alongside the experimental levels under investigation for the optimization are on the univariate preliminary studies of the chromatographic method development.

Twenty experiments and 5 center points were studied with three factors for Ertugliflozin and Sitagliptin. **Table 5.**

A. Flow rate

B. Mobile phase

C. Temperature.

TABLE 5: MODEL OF CENTRAL COMPOSITE DESIGN (CCD)

		Factor 1	Factor 2	Factor 3	Response 1	Response 2	Response 3	Response 4	Response 5
Std	Run	A:FR	B:MP	C:T	RT1	RT2	RS	TP RT1	TP RT2
		ml/min	%	0 c	Min	Min	Num	num	Num
14	1	1	50	35.0454	2.345	3.203	5	3159.6	5751.5
4	2	1.1	60	27	2.173	4.179	9.8	3445.1	4331
5	3	0.9	40	33	2.57	3.221	4.1	5416.2	5940.4
10	4	1.16818	50	30	2.004	2.826	5.5	2826.7	6156.9
8	5	1.1	60	33	2.168	3.883	9.7	2946.6	7550.7
12	6	1	66.8179	30	2.367	6.517	17.3	3065.9	7295.2
1	7	0.9	40	27	2.541	3.234	4.3	4719.9	5833.7
2	8	1.1	40	27	2.174	2.762	4.1	4564.9	5865.6
18	9	1	50	30	2.314	3.208	4.8	3044.9	4471.8
16	10	1	50	30	2.318	3.227	4.8	3018.3	4381.4
19	11	1	50	30	2.32	3.226	4.9	2931.7	4351.1
11	12	1	33.1821	30	2.333	2.828	3.7	7740.6	5406.8
7	13	0.9	60	33	2.57	3.221	4.1	5416.2	5940.4
20	14	1	50	30	2.315	3.238	5	3110.4	4625.7
17	15	1	50	30	2.316	3.236	5.1	3181.1	4594
6	16	1.1	40	33	2.167	2.74	4.1	4883.7	5334.4
13	17	1	50	24.9546	2.346	3.425	6.1	3014.5	5886.4
9	18	0.83182	50	30	2.806	3.986	5.8	3060.9	6931.4
3	19	0.9	60	27	2.574	5.129	11.8	3011.3	7501
15	20	1	50	30	2.318	3.234	5	3035.8	4626.3

Optimization of Chromatographic Conditions using CCD: CCD has flexibility and can be applied for the optimization of HPLC separation for the view of factors main effects as well as it's interactions. A three-factorial, CCD was taken with 20 experimental runs and 5 centre points. The independent variables, such as flow rate (A), mobile phase (B) and temperature (C) and the responses for 20 experimental runs. The responses were analyzed, and a backward elimination process eliminates the insignificant terms from the model to make the model simpler and application-oriented. ANOVA and other descriptive statistics of responses. The P value < 0.05 shows the statistical significance of model terms. **Table 6** The polynomial terms showed a P value less than 0.5 indicating their significant influence on the responses.

TABLE 6:

Response	Type of Model	R- Square	Model P- Value	% CV	Adequate precision
RT1	Quadratic	0.9905	< 0.0001	1.05	41.1186
RT2	Quadratic	0.8812	< 0.0001	12.12	11.7164
RS	Quadratic	0.9031	0.0005	23.31	12.4322
TP-RT1	Quadratic	0.8677	< 0.0001	17.02	9.8353
TP-RT2	Quadratic	0.7971	0.0154	11.78	5.5435

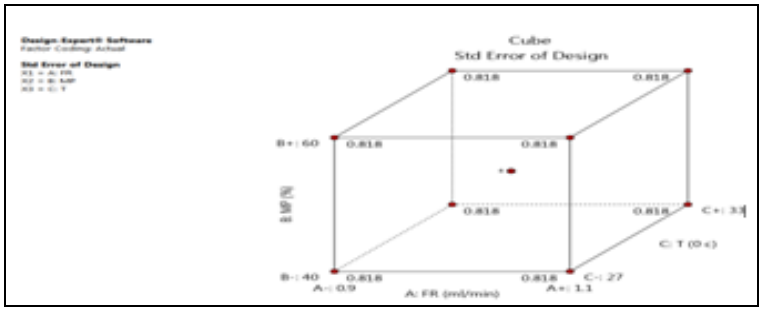


FIG. 3: DESIGN SUMMARY FOR BOX-BEHNKEN SCREENING DESIGN

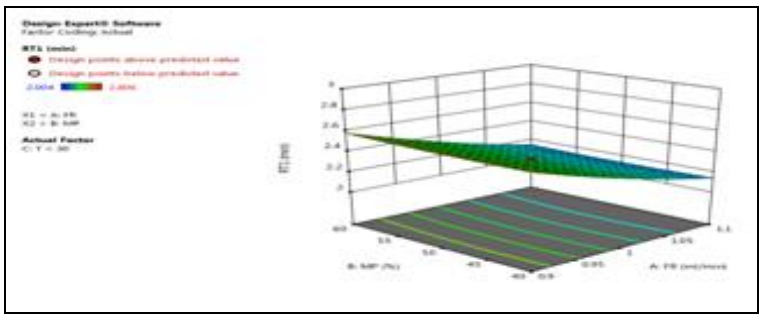


FIG. 4: 3D RESPONSE-SURFACE GRAPHS FOR RETENTION TIME

Three responses had R2 values that were more than 0.8. These substantial R2 values indicate that the chosen quadratic model fits the data and may be used to interpolate with reliability. The key to moving the model closer to optimum is adequate precision. The model must have a value greater than 4 to generate an optimization that can be replicated. All the responses exhibited and adequate precision of more than 4. Percentage CV determines the reproducibility of the model after optimization. Low percentage CV is always an added advantage for producing reproducible results with minimum variations. The ANOVA calculation helps build a polynomial equation with the model terms to make predictions about the response at a given factor level. The parturbation graphs of response allows the simultaneous comparison all the factors with respective response. **Fig. 5, 6, 7, 8.**

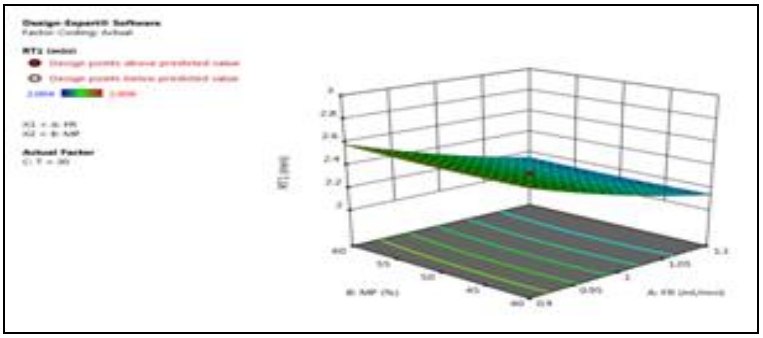


FIG. 5: 3D SURFACE PLOT FOR THE EFFECT OF A COMBINATION OF FACTORS ON RT1 OF ERTUGLIFLOZIN AND SITAGLIPTIN BY USING A CENTRAL COMPOSITE DESIGN

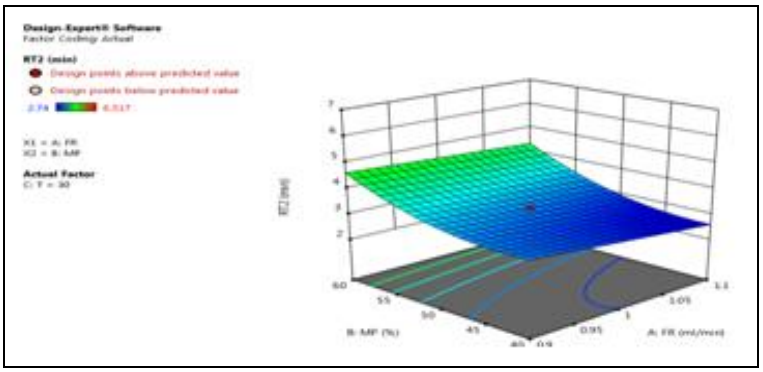


FIG. 6: 3D SURFACE PLOT FOR THE EFFECT OF A COMBINATION OF FACTORS ON RT2 OF ERTUGLIFLOZIN AND SITAGLIPTIN BY USING CENTRAL COMPOSITE DESIGN

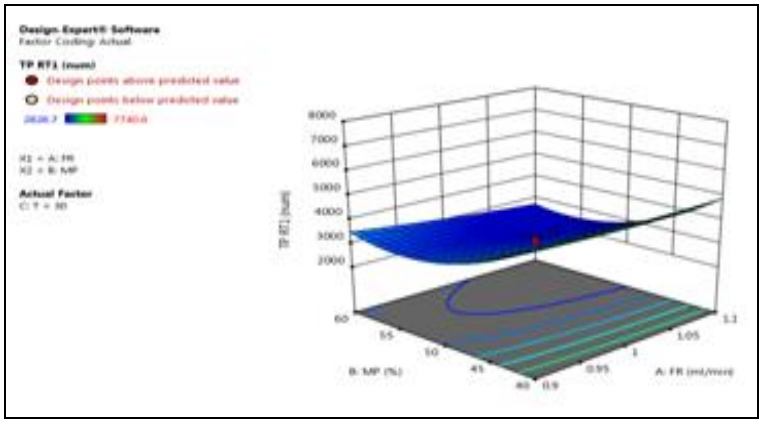


FIG. 7: 3D SURFACE PLOT FOR THE EFFECT OF A COMBINATION OF FACTORS ON TP-RT1 OF ERTUGLIFLOZIN AND SITAGLIPTIN BY USING A CENTRAL COMPOSITE DESIGN

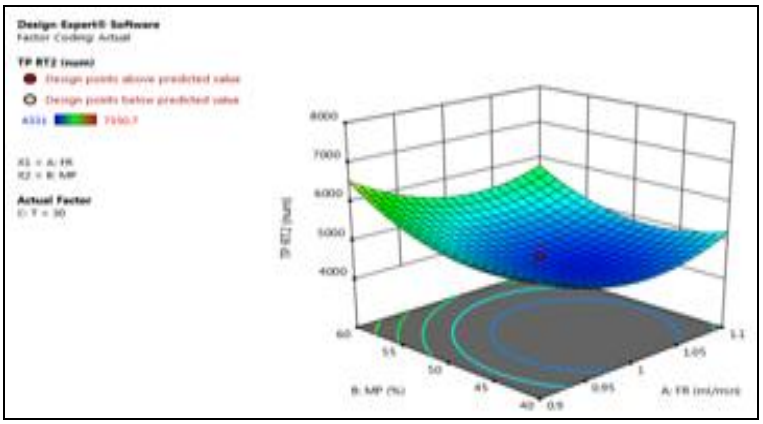


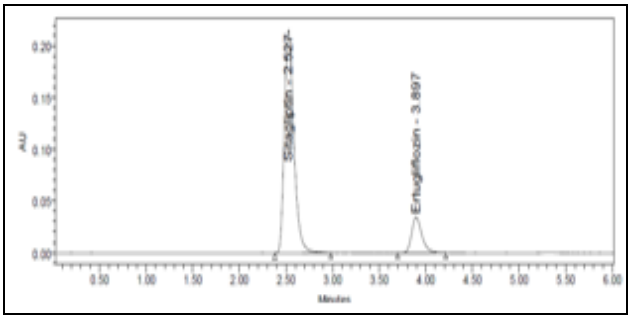
FIG. 8: 3D SURFACE PLOT FOR THE EFFECT OF A COMBINATION OF FACTORS ON TP-RT2 OF ERTUGLIFLOZIN AND SITAGLIPTIN BY USING A CENTRAL COMPOSITE DESIGN

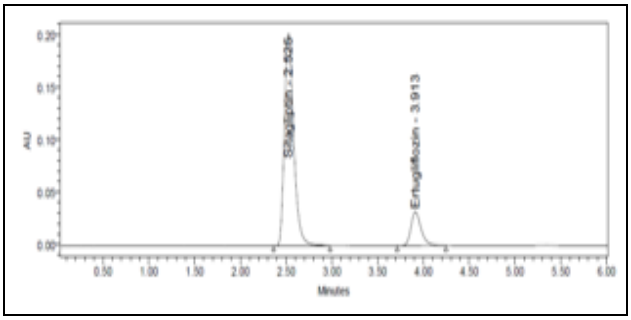
DISCUSSION:

System Suitability Chromatogram: As per the ICH guidelines, plate count should be more than 2000; tailing factor should be less than 2 and the resolution must be more than 2. All the system-suitable parameters were passed and within the limits **Table 7 Fig. 9.**

TABLE 7: SYSTEM SUITABILITY CHROMATOGRAM

Sitagliptin				Ertugliflozin			
Injection	**RT (min)**	**USP Plate Count**	**Tailing**	**RT (min)**	**USP Plate Count**	**Tailing**	**Resolution**
1	2.522	3024	1.36	3.894	6374	1.29	7.1
2	2.523	3024	1.36	3.897	6270	1.30	7.0
3	2.524	3033	1.36	3.902	6192	1.29	7.1
4	2.525	3033	1.34	3.905	6328	1.30	7.2
5	2.525	3077	1.34	3.907	6279	1.29	7.1
6	2.527	3037	1.33	3.913	6185	1.28	7.1





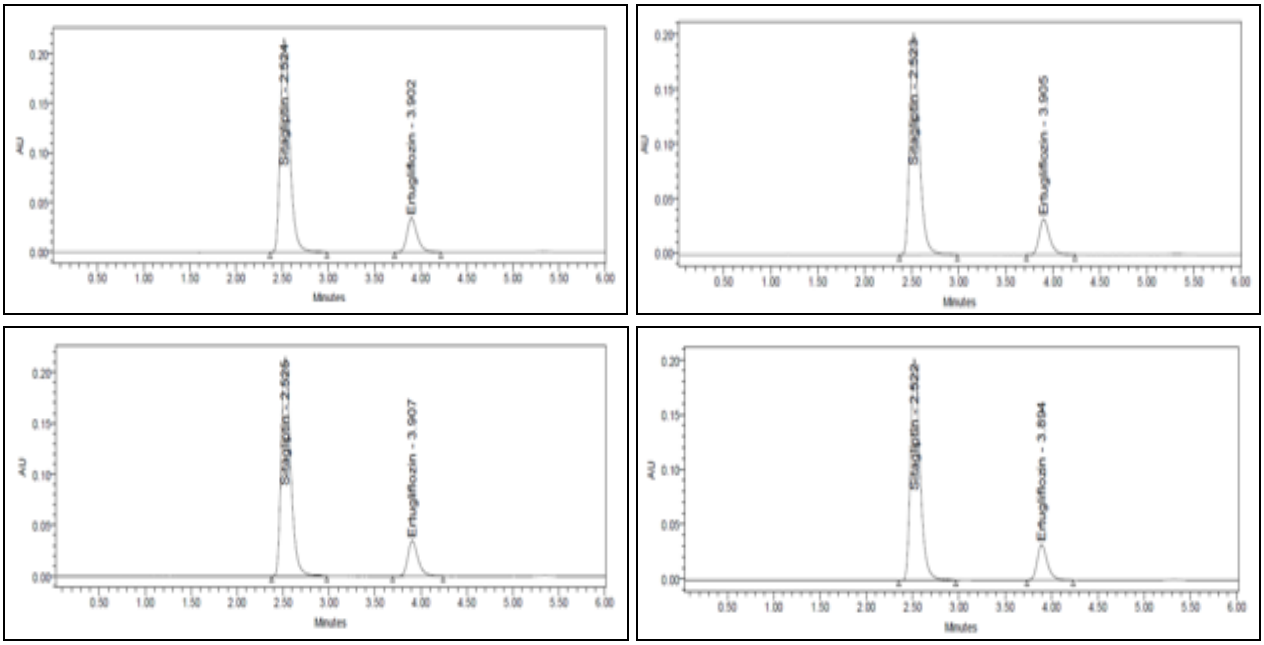


FIG. 9: SYSTEM SUITABILITY CHROMATOGRAM

Limit of Detection (LOD) and Limit of Quantification (LOQ): The LOD and LOQ of the developed method were determined by injecting progressively low concentrations of the standard solution of Ertugliflozin and Sitagliptin using the developed HPLC method **Table 8 Fig. 10** & **11.**

TABLE 8: LOD & LOQ

Name of the drug	LOD	LOQ
Sitagliptin	0.62	1.88
Ertugliflozin	0.06	0.18

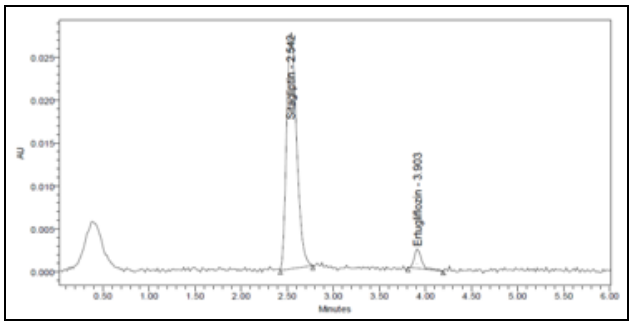


FIG. 10: LOD CHROMATOGRAM OF STANDARD

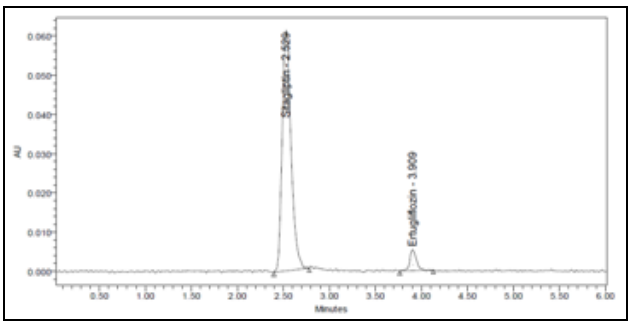


FIG. 11: LOQ CHROMATOGRAM OF STANDARD

Linearity and Range: Discussion: Six linear concentrations of Sitagliptin (12.5-75µg/ml) and Ertugliflozin (1.875-11.25µg/ml) were injected in duplicate manner. The Average areas mentioned above and linearity equations obtained for Sitagliptin was y = 29364x + 6443.7 and Ertugliflozin was y = 32315x + 3240.5. For two drugs Correlation coefficient obtained was 0.999 **Table 9, Fig. 12** & **13.**

TABLE 9: LINEARITY AND RANGE

Sitagliptin		Ertugliflozin	
Conc (µg/mL)	Peak area	Conc (µg/mL)	Peak area
0	0	0	0
12.5	366585	1.875	62482
25	727663	3.75	129956
37.5	1142555	5.625	184458
50	1484254	7.5	247295
62.5	1842376	9.375	308537
75	2189758	11.25	362365

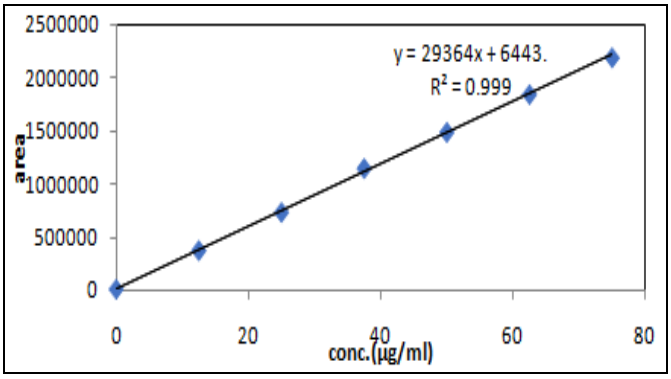


FIG. 12: CALIBRATION CURVE OF SITAGLIPTIN

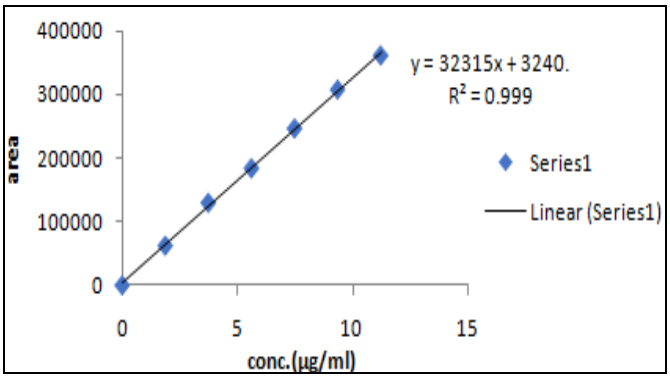
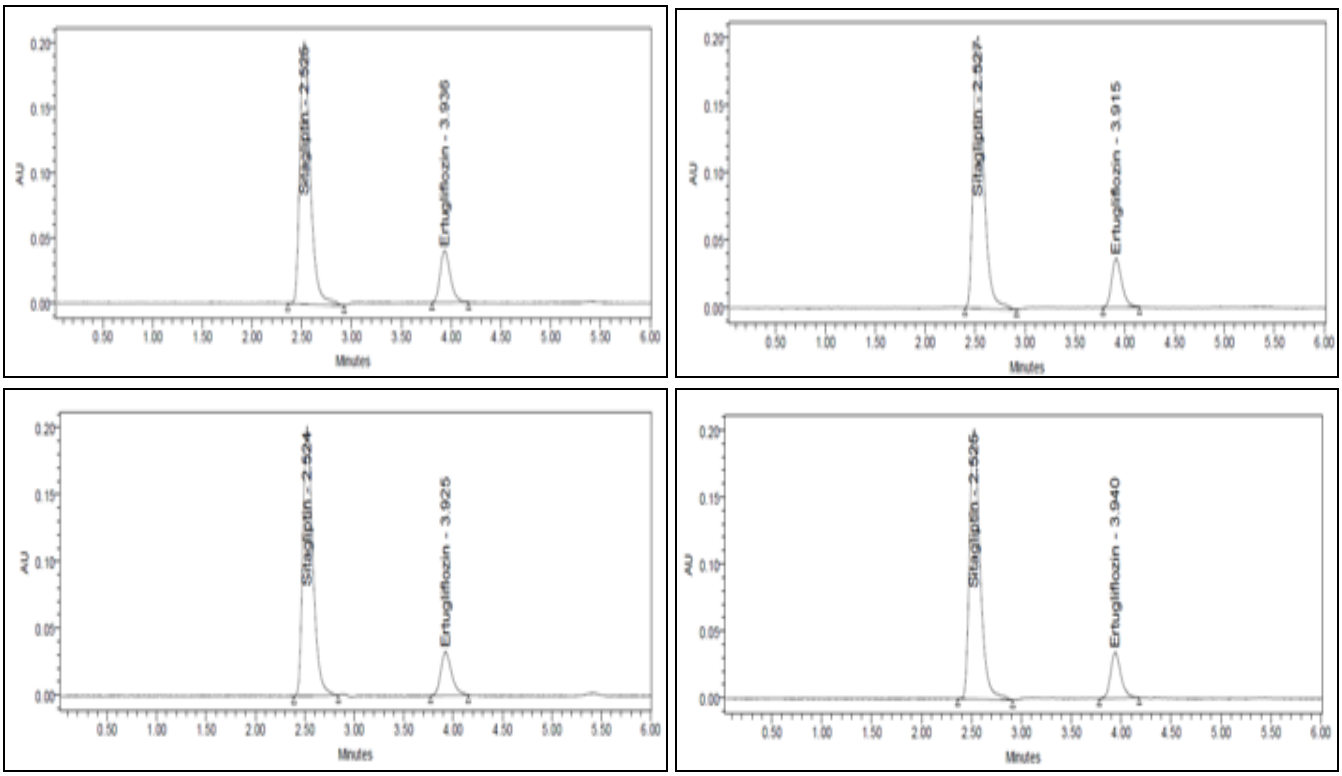


FIG. 13: CALIBRATION CURVE OF ERTUGLIFLOZIN

Repeatability: Discussion: Multiple samples were taken from a sample stock solution, six working sample solutions with identical concentrations were made, an injection was administered from each working sample solution, and obtained areas were mentioned in the table. **Table 10** Average area, standard deviation, and % RSD were calculated for two drugs and obtained as 0.9% and 1.2%, respectively for Sitagliptin and Ertugliflozin. The system precision was achieved using this method even if the precision limit was less than "2". **Fig. 14.**

TABLE 10: REPEATABILITY TABLE OF SITAGLIPTIN AND ERTUGLIFLOZIN

Sr. no.	Area of Sitagliptin	Area of Ertugliflozin
1.	1425322	242808
2.	1433417	249911
3.	1444705	242607
4.	1412580	246401
5.	1427427	248192
6.	1449523	248543
Mean	1432162	246410
S.D	13512.7	3079.6
%RSD	0.9	1.2



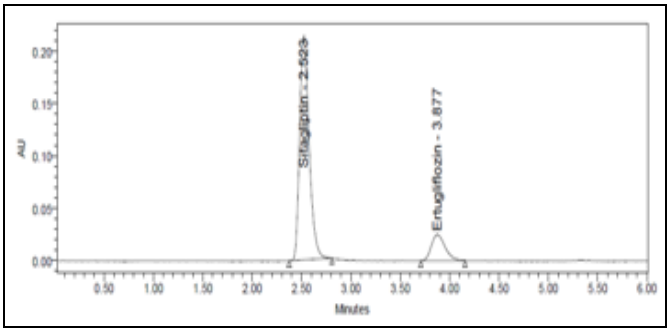


FIG. 14: REPEATABILITY CHROMATOGRAM

Intermediate Precision (Day_Day Precision): Multiple sampling from a sample stock solution was done and six working sample solutions of same concentrations were prepared, each injection from each working sample solution was given on the next day of the sample preparation, and obtained areas were mentioned in table. **Table 11** Average area, standard deviation and % RSD were calculated for two drugs and obtained as 1.3% and 1.8%, respectively for Sitagliptin and Ertugliflozin **Fig. 15.** The limit of precision was less than "2" system precision was passed in this method.

TABLE 11: INTERMEDIATE PRECISION TABLE OF SITAGLIPTIN AND ERTUGLIFLOZIN

Sr. no.	Area of Sitagliptin	Area of Ertugliflozin
1.	1384716	241155
2.	1424665	238642
3.	1418961	239262
4.	1383982	235823
5.	1418167	241235
6.	1419459	239610
Mean	1408325	239288
S.D	18713.2	1990.2
%RSD	1.3	1.8

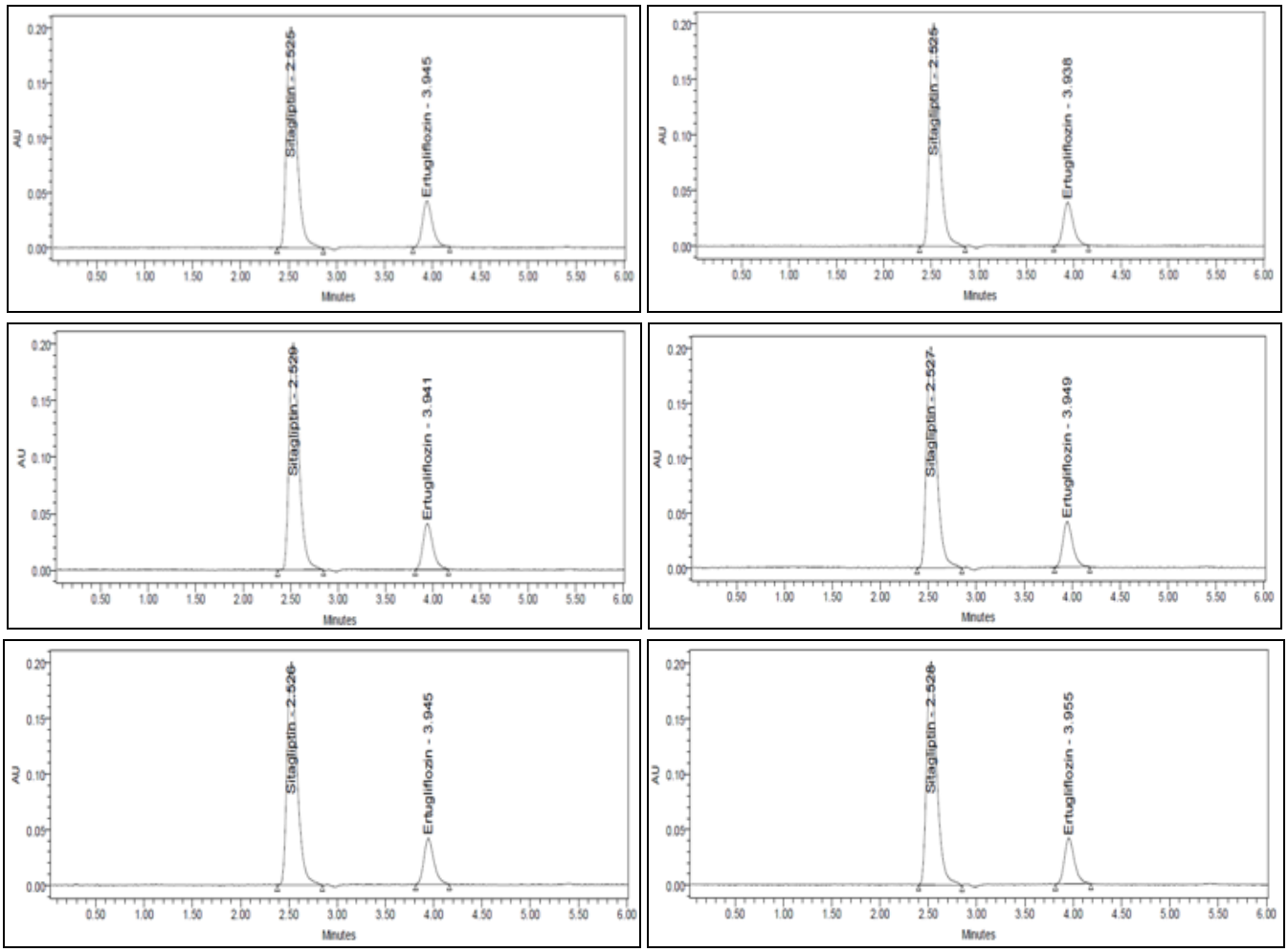


FIG. 15: INTER DAY PRECISION CHROMATOGRAM

Accuracy: Three sets of accuracy samples were prepared using the conventional addition approach. Triplicate injections were given for each level of accuracy, and mean %Recovery was obtained as 99.81% and 99.84% for Ertugliflozin and Sitagliptin, respectively **Tables 12** & **13.**

TABLE 12: ACCURACY TABLE OF ERTUGLIFLOZIN

% Level	Amount Spiked (µg/mL)	Amount recovered (µg/mL)	% Recovery	% RSD
50%	3.75	3.7358	99.62	0.4329 %
	3.75	3.7557	100.15	
	3.75	3.7680	100.48	
100%	7.5	7.4412	99.22	0.958 %
	7.5	7.4991	99.99	
	7.5	7.5842	101.12	
150%	11.25	11.1794	99.37	0.8649 %
	11.25	11.2548	100.04	
	11.25	11.0631	98.34	

TABLE 13: ACCURACY TABLE OF SITAGLIPTIN

% Level	Amount Spiked (µg/mL)	Amount recovered (µg/mL)	% Recovery	Mean % Recovery
50%	25	24.997	99.99	0.7082 %
	25	24.805	99.22	
	25	24.646	98.58	
100%	50	50.013	100.03	0.4212 %
	50	50.434	100.87	
	50	50.188	100.38	
150%	75	74.621	99.49	1.0584 %
	75	75.752	101.00	
	75	74.225	98.97	

Robustness: Discussion: Samples were injected under robustness parameters, including flow minus (0.8 ml/min), flow plus (1.0 ml/min), mobile phase minus (58B:42A), mobile phase plus (42B:58A), and temperature minus (25°C) and temperature plus (31°C) was maintained and samples were injected in a duplicate manner. The parameters for system suitability were not significantly impacted, and all of the parameters were met in **Table 14.**

TABLE 14: ROBUSTNESS DATA FOR SITAGLIPTIN AND ERTUGLIFLOZIN

Sr. no.	Condition	%RSD of Sitagliptin	%RSD of Ertugliflozin
1	Flow rate (-) 0.9ml/min	1.3	0.3
2	Flow rate (+) 1.1ml/min	0.4	0.8
3	Mobile phase (-) 60B:40A	0.5	1.1
4	Mobile phase (+) 50B:50A	0.7	1.5
5	Temperature (-) 25°C	1.7	1.5
6	Temperature (+) 35°C	1.0	1.2

Assay: Discussion: Sitagliptin 15mg 100mg, Ertugliflozin 15mg. Assay was performed with the formulation. Average % Assay for Sitagliptin and Ertugliflozin obtained was 99.91% and 99.96%respectively. **Fig. 16** & **17**, **Table 15** & **16.**

TABLE 15: ASSAY DATA OF SITAGLIPTIN

Sr. no.	Standard Area	Sample area	% Assay
1	1473164	1425322	99.43
2	1443797	1433417	100.00
3	1407004	1444705	100.79
4	1410495	1412580	98.55
5	1426751	1427427	99.58
6	1422131	1449523	101.12
Avg	1430557	1432162	99.91
STDE	24630.7	13512.7	0.9
%RSD	1.7	0.9	0.9

TABLE 16: ASSAY DATA OF ERTUGLIFLOZIN

Sr. no.	Standard Area	Sample area	% Assay
1	243841	242808	98.50
2	245808	249911	101.38
3	248705	242607	98.41
4	247136	246401	99.95
5	242010	248192	100.68
6	245686	248543	100.82
Avg	245531	246410	99.96
STDE	2366.6	3079.6	1.25
%RSD	1.0	1.2	1.2

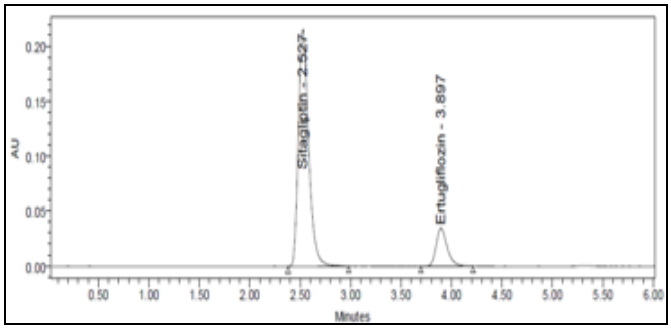


FIG. 16: CHROMATOGRAM OF WORKING STANDARD SOLUTION

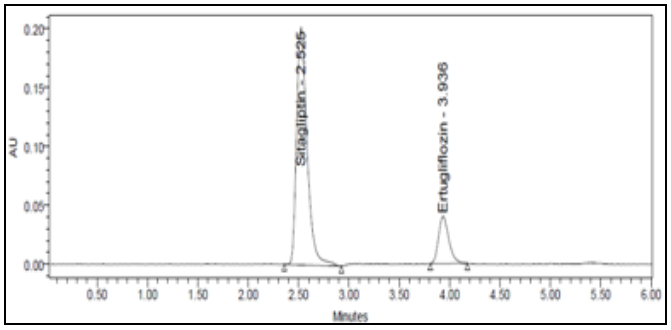


FIG. 17: CHROMATOGRAM OF WORKING SAMPLE SOLUTION

Degradation Study: Degradation study was performed with the above formulation **Table 17.**

TABLE 17: DEGRADATION STUDY

Type of degradation	Sitagliptin			Ertugliflozin		
Condition	Area	% Recovered	% Degraded	Area	% Recovered	% Degraded
Acid	1364933	95.22	4.78	234278	95.04	4.96
Base	1366474	95.33	4.67	235677	95.60	4.40
Peroxide	1347942	94.04	5.96	232374	94.26	5.74
Thermal	1397346	97.48	2.52	240693	97.64	2.36
Uv	1408384	98.25	1.75	241713	98.05	1.95
Hydrolytic	1419065	99.00	1.00	244451	99.16	0.84

Degradation Chromatograms:

Peroxide Degradation: To 1 ml of stock solution of Ertugliflozin and Sitagliptin, 1 ml of 20% hydrogen peroxide (H_2O_2) was added separately. At 60°C, the solution was maintained for 30 minutes. For HPLC study, the resultant solution was diluted to obtain 7.5µg/ml & 50µg/ml solution and 10µl were injected into the system and the chromatograms were recorded to assess the stability of sample **Fig. 18.**

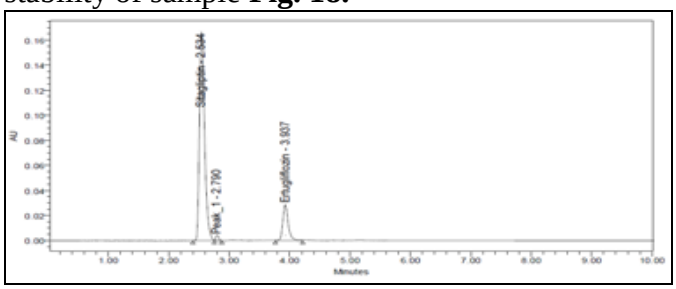


FIG. 18: PEROXIDE DEGRADATION CHROMATOGRAM

Acid Degradation Studies: To 1 ml of stock solution Ertugliflozin and Sitagliptin, 1ml of 2N Hydrochloric acid was added and refluxed for 30min at 60°C.

The resultant solution was diluted to obtain 7.5µg/ml & 50µg/ml solution and 10µl solutions were injected into the system and the chromatograms were recorded to assess the stability of sample **Fig. 19.**

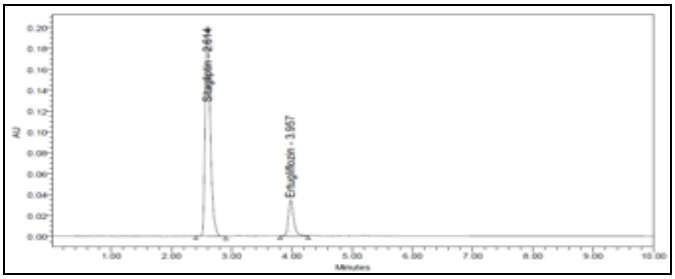


FIG. 19: ACID DEGRADATION CHROMATOGRAM

Base Degradation Studies: To 1 ml of stock solution Ertugliflozin and Sitagliptin, 1 ml of 2N sodium hydroxide was added and refluxed for 30 min at 60°C.

The resultant solution was diluted to obtain 7.5µg/ml & 50µg/ml solution and 10µl were injected into the system and the chromatograms were recorded to assess the stability of sample **Fig. 20.**

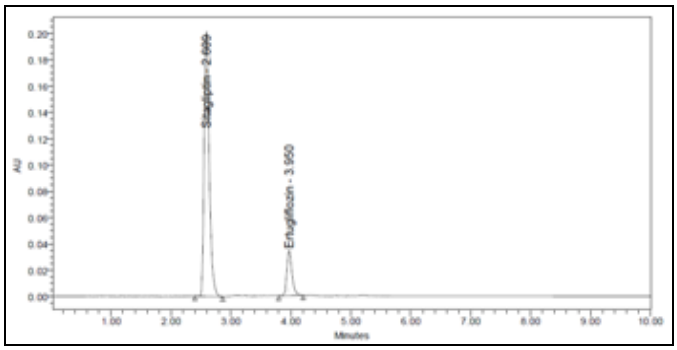


FIG. 20: BASE DEGRADATION CHROMATOGRAM

Thermal Degradation Studies: The standard drug solution placed in oven at 105°C for 6h to study dry heat degradation.

The final solution was diluted to 7.5 g/ml and 50 g/ml for HPLC research and 10 l were injected into the system. Chromatograms were recorded to determine the sample's stability **Fig. 21.**

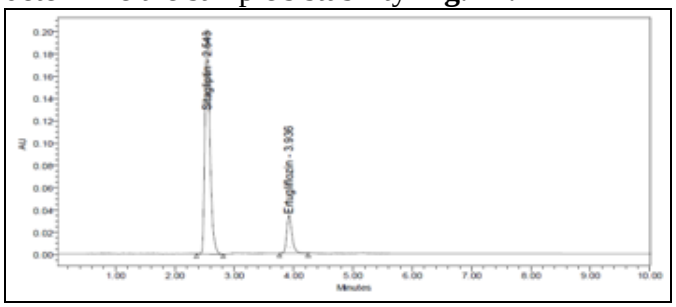


FIG. 21: THERMAL DEGRADATION CHROMATOGRAM

UV Degradation Studies: The photochemical stability of the drug was also studied by exposing the 75µg/ml & 500µg/ml solution to UV Light by keeping the beaker in UV Chamber for 7 days or 200-Watt hours/m^2 in photostability chamber.

For HPLC study, the resultant solution was diluted to obtain 7.5µg/ml & 50µg/ml solutions and 10µl were injected into the system and the chromatograms were recorded to assess the stability of sample **Fig. 22.**

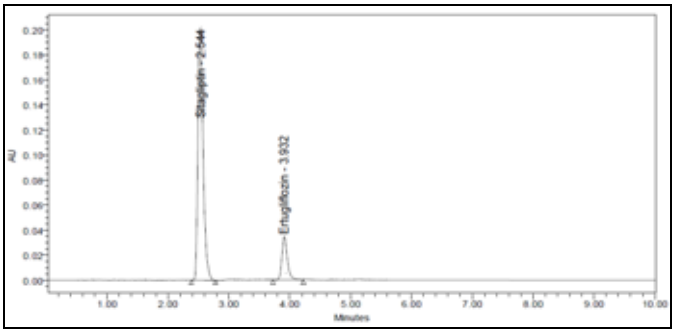


FIG. 22: UV DEGRADATION CHROMATOGRAM

Hydrolytic Degradation Studies: Stress testing under neutral conditions was studied by refluxing the drug in water for 6hrs at a temperature of 60º. For the HPLC study, the resultant solution was diluted to 7.5µg/ml & 50µg/ml solution, and 10µl were injected into the system. The chromatograms were recorded to assess the stability of the sample **Fig. 23.**

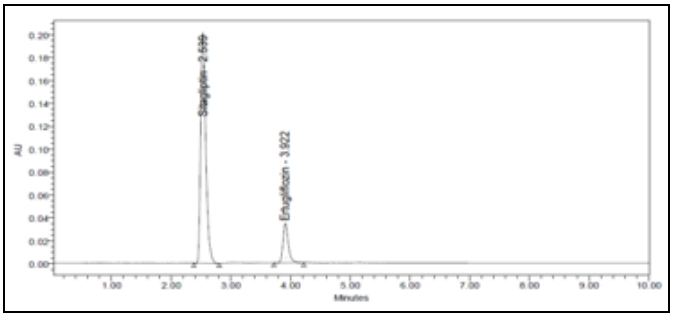


FIG. 23: HYDROLYTIC DEGRADATION CHROMATOGRAM

CONCLUSION: A Quality by Design approach to RP-HPLC method development has been described. The QbD approach to method development has helped to highly understand the method variables, leading to less chance of failure during method validation.

Optimized chromatographic conditions were performed, like the mobile phase composition by several trials. This has been done to achieve good resolution and the symmetric peak shapes of the analyte. All validated parameters were found to be within acceptable limits. This method is often wont to determine the purity of the drug available from various sources by detecting the related degradation peaks.

Stability indicating the nature of the method has been confirmed by forced degradation under different conditions viz. hydrolysis, thermal, and UV. The developed RP-HPLC stability indicating

method was simple, linear, precise, and robust for determining Ertugliflozin and Sitagliptin, so the method developed was simple and robust that can be adopted in regular Quality control tests in Industries.

ACKNOWLEDGEMENTS: It is with immense gratitude that I express my most cordial and humble thanks to my esteemed guide, Dr. Ram S. Sakhare Sir, M. Pharm PhD., for his valuable guidance, keen interest, perennial inspiration and everlasting encouragement. I am gratefully indebted to Dr. Amol A. Joshi sir, M. Pharm PhD., the honorable Principal of K, T. Patil College of Pharmacy, Osmanabad. And Mr. Sudhir K, Patil sir Chairman of ASPM's for providing the necessary facilities to carry out my work and for his constant support and encouragement. I would like to express my love and gratitude to my beloved parents and family. Their blessings always inspire me to work hard and overcome all my life's difficulties. I would express my special thanks to my admirable friends Pallavi, Anuja, and Dhanashri for their constant support and needful help.

CONFLICTS OF INTEREST: The authors declare that there is no conflict of interest regarding the publication of this paper.

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How to cite this article:
Kulkarni D, Sakhare R, Joshi A, Shendge S and Hangargekar P: Development and validation of stability indicating RP-HPLC method for ertugliflozin and sitagliptin by QBD approach. Int J Pharm Sci & Res 2023; 14(8): 3852-67. doi: 10.13040/IJPSR.0975-8232.14(8). 3852-67.

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