RESEARCH PAPER

Unveiling Analytical HPLC Method Development and Validation for Empagliflozin: A Paradigm of Quality by Design

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ABSTRACT
This research introduces a quality by design (QbD) strategy to develop a reversed-phase high-performance liquid chromatography (HPLC) technique for the analysis of Empagliflozine in both bulk drug and pharmaceutical formulations. The separation process utilized a Cosmosil C18 column (250mm x 4.6ID, particle size: 5 micron) with isocratic elution, employing a mobile phase composed of a mixture of methanol and water (80:20v/v). The flow rate was maintained at 1.0 mL/min, and detection was conducted using UV detection at 222 nm.To optimize the chromatographic method, a Box-Behnken design was utilized. The design considered factors such as the composition of the mobile phase (X1) and the flow rate (X2), while the measured responses included asymmetry (Y1), theoretical plates (Y2), retention time (Y3), and area (Y4). Statistical analysis of the experimental design was performed using analysis of variance (ANOVA), counter plots, and response surface plots.The method underwent further validation in accordance with the guidelines set by the International Council for Harmonisation (ICH). The validation process assessed accuracy, precision, ruggedness, limit of detection (LOD), and limit of quantitation (LOQ). The results of the validation demonstrated that the proposed method was straightforward, sensitive, and highly robust for the routine analysis of Empagliflozine.
To summarize, this study introduces a QbD-based approach for the development and optimization of a reversed-phase HPLC method intended for the routine analysis of Empagliflozine. The method underwent validation following ICH guidelines and exhibited outstanding performance in terms of accuracy, precision, sensitivity, and robustness.
Keywords: Empagliflozine, Box-Behnken design, ANOVA, HPLC, QBD approach, Validation.
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1. INTRODUCTION

Empagliflozin, marketed as Jardiance by Boehringer Ingelheim, is an oral medication belonging to the class of selective sodium glucose cotransporter-2 (SGLT-2) inhibitors. It is primarily used to lower blood glucose levels in individuals with type 2 diabetes. Empagliflozin achieves this by blocking the reabsorption of glucose in the kidneys and facilitating the excretion of excess glucose in the urine. [1][2] The sodium glucose cotransporter 2 (SGLT2), primarily found in the proximal tubules of the nephron, is responsible for approximately 90% of glucose reabsorption [3][4]. Empagliflozin, chemically known as empagliflozin; 1-chloro-4-[b-D-glucopyranos-1-yl]-2-[4-([S]-tetrahydrofuran – 3 – yl - oxy) benzyl]-benzene, acts as a potent and selective competitive inhibitor of the SGLT2 protein. [5] By inhibiting glucose reabsorption, SGLT2 inhibitors like empagliflozin offer an insulin-independent mechanism to improve blood glucose control. They promote urinary glucose excretion (UGE), leading to beneficial effects on glucose levels. Notably, SGLT2 inhibitors are associated with weight loss, reductions in blood pressure, and a minimal risk of hypoglycemia [6]

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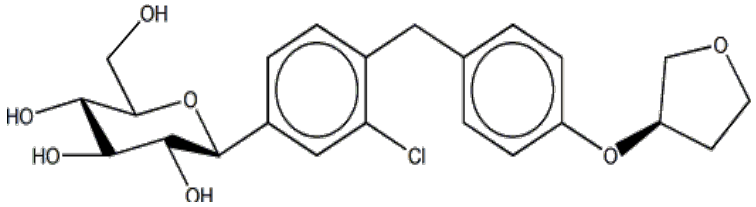


Figure 1: Chemical Structure of Empagliflozin

2. MATERIALS AND METHODS

Instrumentation:

The drug analysis was performed using an HPLC system, specifically the model no 3000 series. The system consisted of a P-3000M reciprocating (40MPa) pump and a UV 3000M Detector. The HPLC column used was Cosmosil C18 with dimensions of 250mm x 4.6ID and a particle size of 5 microns. The signal output was monitored and processed using HPLC workstation software.

Chemicals and reagents:

The working standard of Empagliflozin, a gift sample from Mylan Laboratories, Hyderabad, was utilized in the study. Commercially available Jardiance tablets containing 10 mg Empagliflozin were obtained for analysis. HPLC-grade methanol and HPLC-grade water were procured locally. The water was further purified using a Milli-Q water purification system involving double distillation. The mobile phase employed for chromatographic separation consisted of a binary mixture of methanol and water with a volumetric ratio of 80:20. Chromatographic separation was performed using a Cosmosil C18 column with dimensions of 250mm x 4.6ID and a particle size of 5 microns. The flow rate was set to 1.0 mL/min, and detection of analytes was achieved through UV detection at a wavelength of 222 nm. A sample injection volume of 20 µL was employed. Prior to sample injection, the column was appropriately equilibrated with the mobile phase. Data acquisition, storage, and analysis were carried out using specialized HPLC workstation software.

For method development and experimental design, a novel HPLC method was developed utilizing a mobile phase composition comprising methanol-water. While previous reports have recommended alternative solvents such as acetonitrile, dipotassium hydrogen phosphate, and 0.02M phosphate buffer, the adoption of methanol instead of other organic solvents was preferred due to its cost-effectiveness for routine analysis of pharmaceutical formulations.The experimental design and optimization were executed using Design-Expert 9.0 software, Full Version, employing the Box-Behnken Design (BBD) approach. This design strategy enables optimization of experiments using a 3k-factorial design, where k denotes the number of independent variables (equal to or greater than three) and incorporates multiple dependent variables and responses. The software was configured with various ratios of methanol to water in the mobile phase (ranging from 70-20% v/v) and flow rates between 0.8-1.0 mL/min. The resulting experimental design consisted of a total of 16 runs conducted in accordance with the BBD scheme.

The significance of the obtained model was evaluated through two approaches: analysis of variance (ANOVA) and assessment of good fit. ANOVA, employing the F-test, partitioned the total variation into components attributable to residual error, main effects, and interactions, enabling determination of model significance. Furthermore, the lack of fit, a component of the sum of squares in ANOVA, was examined to assess the adequacy of the proposed model in representing the experimental data. [7]

Preparation of standard stock solution:

To prepare the standard stock solution, an exact mass of 10 mg of Empagliflozin was measured and transferred into a standard volumetric flask with a capacity of 10 mL. A small volume of methanol was added to facilitate dissolution, and the flask was subsequently filled to the calibration mark with methanol. From this solution, a 1 mL aliquot was extracted and diluted to 10 mL using methanol, resulting in a concentration of 100 µg/mL of Empagliflozin. Following that, 1.0 mL of this diluted solution was further diluted to 10 mL using a diluent, producing a 10 µg/mL solution that was employed as the standard solution.

For the analysis of Jardiance tablets, a total of 10 units containing Empagliflozin were weighed and finely powdered. An accurately measured quantity corresponding to 50 mg of Empagliflozin powder was transferred to a 10 mL volumetric flask, which was then subjected to sonication for a duration of 20 minutes with 7 mL of distilled water (diluent). Subsequently, the resulting suspension was filtered through Whatman 1 filter paper, and the filtrate was further diluted with the diluent to a final volume of 10 mL. A suitable portion of this filtrate was then diluted with the diluent to obtain a concentration of 10 µg/mL. Finally, a 30 µL volume of the resulting solution was subjected to chromatographic analysis. [8]

Experimental design:

To investigate the influence of three independent factors, namely Mobile Phase (X1), Flow Rate (mL/min) (X2), and pH (X3), on three response variables, including Assymetry (Y1), Theoretical Plates (Y2), Retention Time (Y3), and Area (Y4) for method calculation, a 32-factorial design based on the Box-Behnken Design (BBD) was implemented. The specified factors and their corresponding ranges are provided in Table 1.

METHOD VALIDATION

The method employed in this study underwent validation in several aspects.

Linearity:
To assess the linearity of the method, the standard stock solution was diluted to obtain concentrations ranging from 5 µg/mL to 30 µg/mL.

Accuracy:
The accuracy of the method was evaluated by determining the recovery of Empagliflozin from a solution with a concentration of 20 µg/mL, which was spiked with additional quantities of Empagliflozin at levels of 50%, 100%, and 150%.

Limit of Detection (LOD) and Limit of Quantitation (LOQ):

The LOD and LOQ were determined using the slope (S) of the linearity plot and the standard deviation of the response to the blank sample. The formulas used for calculation were as follows. [9][10]

LOD = 3.3σ/S, where σ represents the standard deviation

LOQ = 10σ/S

RESULT AND DISCUSSION
The selection of the mobile phase combination, flow rate, and pH was based on various factors including linearity, assay, system suitability, retention time, peak parameters, and ease of preparation. Among the different combinations tested using the Box-Behnken Design (BBD), the mobile phase composition of Methanol-Phosphate Buffer was found to be suitable.

A 32-factorial design using the Box-Behnken Design (BBD) was implemented to observe the effects of three independent factors: mobile phase composition (% v/v of Methanol) (X1), flow rate (mL/min) (X2), and pH (X3) on three response variables: asymmetry (Y1), theoretical plates (Y2), and retention time (Y3). These parameters were considered for the calculation of the proposed method. The specific chromatographic conditions and ranges were predetermined and are provided in Table 1.

Table 1: Specific chromatographic conditions and ranges

Name	Goal	Lower Limit	Upper Limit	Lower Weight	Upper Weight	Importance
A:Compostion	is in range	70	90	1	1	3
B:Flowrate	is in range	0.8	1	1	1	3
C:Wavelength	is in range	222	226	1	1	3
Retention Time	is in range	3	4	1	1	3
Area	maximize	1759460	2169920	1	1	3
Theoretical Plates	maximize	4783	8878	1	1	3
Asymmetry Factor	is in range	1	1.42	1	1	3

A total of 17 runs were conducted for the fixed variables, including three center repetitions. These repetitions were performed to assess the experimental error variance and validate the predictive accuracy of the model. Each combination of mobile phase composition, flow rate, and pH, as recommended by the Box-Behnken Design (BBD), was executed on the system. The responses, such as peak area and retention time, were observed and recorded in Table 2.

To minimize the influence of uncontrolled factors that could introduce bias to the results, all experiments were carried out in a randomized order. Among the various models examined, the Quadratic model exhibited the highest least square regression values for response Y1, Y2, Y3, and Y4 compared to the other models.

Table 2: Box Behnken Experimental Design Using Factors and Their Responses

		Factor 1	Factor 2	Factor 3	Response 1	Response 2	Response 3	Response 4
Std	Run	A: Compostion	B: Flowrate	C: Wavelength	Retention Time	Area	Theoretical Plates	Asymmetry Factor
		%	ml/min	nm	min	AU	Unit	Unit
4	1	90	1	224	3.071	2055830.00	5236	0.92
10	2	80	1	222	3.814	2169920.00	8878	1.23
3	3	70	1	224	5.887	1799880.00	6582	1.25
8	4	90	0.9	226	3.393	2096230.00	5511	0.91
1	5	70	0.8	224	7.238	1945090.00	6173	1.42
6	6	90	0.9	222	3.395	2089380.00	5328	0.92
13	7	80	0.9	224	4.238	1846580.00	6556	1.39
12	8	80	1	226	3.391	1897350.00	5614	1.25
5	9	70	0.9	222	6.326	1826880.00	6719	1.37
7	10	70	0.9	226	6.434	1981040.00	6408	1.37
11	11	80	0.8	226	4.701	1759460.00	7550	1.27
15	12	80	0.9	224	4.238	1846580.00	6556	1.39
14	13	80	0.9	224	4.238	1846580.00	6556	1.39

9	14	80	0.8	222	4.678	2092500.00	7303	1.27
16	15	80	0.9	224	4.238	1846580.00	6556	1.39
2	16	90	0.8	224	3.796	1941490.00	4783	0.91
17	17	80	0.9	224	4.238	1846580.00	6556	1.39

The model underwent a Lack of Fit test, which revealed a lack of significant fit value with a higher p-value compared to the model F-value. Additionally, the model was validated through Analysis of Variance (ANOVA) applied to both the responses and variables to assess its significance. The ANOVA results demonstrated significant differences in the values of both responses. The Quadratic equations for all model responses, Y1, Y2, Y3, and Y4, are presented below,

Y1 (Assymetry) = +158.341+0.25075AX1+5.16500X2-46.71062X3 -0.0089 X1 X2-0.067143 X1 X3-0.0312 X2 X3-0.0051 X1 2 -2.06875 X2 2 +2.9000 X3 2

Y2(TheoreticalPlates)=626175.0+17716X1+71388.125X2-7518.125X3-64.28X1X2392.85 X1X3+831.25X2X3-105015X12-36465.625X22 +2359.375X3 2

Y3 (Retention Time) = +25.04804X1-0.305X1-6.483X2+1.389

Table 3: Anova Results For Response Y1 (Asymmetry)

Source	Sum of Squares	DF	Mean Square	F-value	p-value
Model	0.601331	9	0.066815	236.811	7.41E-08
A-Compostion	0.382813	1	0.382813	1356.804	2.82E-09
B-Flowrate	0.00605	1	0.00605	21.44304	0.002396
C-Wavelength	1.25E-05	1	1.25E-05	0.044304	0.839285
AB	0.0081	1	0.0081	28.70886	0.001055
AC	2.50E-05	1	2.50E-05	0.088608	0.774595
BC	1.00E-04	1	1.00E-04	0.35443	0.570354
AÂ²	0.150007	1	0.150007	531.6689	7.32E-08
BÂ²	0.02448	1	0.02448	86.76549	3.41E-05
CÂ²	0.014533	1	0.014533	51.50899	0.000181
Residual	0.001975	7	0.000282		
Lack of Fit	0.001975	3	0.000658		
Pure Error	0	4	0		
Cor Total	0.603306	16			

Table 4: Anova Results for Response Y2 (Theoretical Plates)

Source	Sum of Squares	DF	Mean Square	F-value	p-value
Model	13857844	9	1539760	8.58595	0.004876
A-Compostion	3155072	1	3155072	17.59319	0.004064
B-Flowrate	31375.13	1	31375.13	0.174953	0.688281
C-Wavelength	1236378	1	1236378	6.894242	0.034126
AB	484	1	484	0.002699	0.96002
AC	61009	1	61009	0.340196	0.578015
BC	3081780	1	3081780	17.1845	0.00432
AÂ²	5128371	1	5128371	28.59662	0.001067
BÂ²	244805.3	1	244805.3	1.365074	0.280907
CÂ²	1223814	1	1223814	6.824181	0.034796
Residual	1255344	7	179334.9		
Lack of Fit	1255344	3	418448.1		
Pure Error	0	4	0		
Cor Total	15113188	16			

Table 5: Anova Results For Response Y3 (Retention Time)

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	23.51255	9	2.612506	421.0857	9.97E-09	significant

A-Compostion	18.69661	1	18.69661	3013.534	1.75E-10	
B-Flowrate	2.257813	1	2.257813	363.9159	2.71E-07	
C-Wavelength	0.010805	1	0.010805	1.741478	0.228472	
AB	0.097969	1	0.097969	15.79072	0.005367	
AC	0.003025	1	0.003025	0.487572	0.507538	
BC	0.049729	1	0.049729	8.015358	0.025365	
AÂ²	2.37158	1	2.37158	382.2531	2.29E-07	
BÂ²	0.00038	1	0.00038	0.061249	0.811635	
CÂ²	0.043378	1	0.043378	6.991682	0.033221	
Residual	0.04343	7	0.006204			
Lack of Fit	0.04343	3	0.014477			
Pure Error	0	4	0			
Cor Total	23.55598	16				

Table 6: Anova Results For Response Y4 (Area):

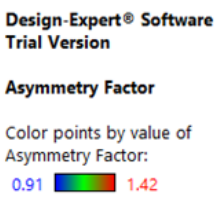
Source	Sum of Squares	Df	Mean Square	F-value	p-value
Model	1.19E+11	3	3.97E+10	4.013487	0.031754
A-Compostion	4.96E+10	1	4.96E+10	5.011016	0.043315
C-Wavelength	2.47E+10	1	2.47E+10	2.495387	0.138195
CÂ²	4.49E+10	1	4.49E+10	4.534059	0.052914
Residual	1.29E+11	13	9.9E+09		
Lack of Fit	1.29E+11	9	1.43E+10		
Pure Error	0	4	0		
Cor Total	2.48E+11	16			

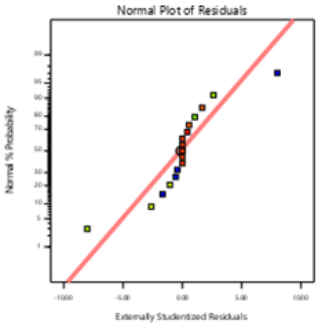
Table 3:
The Model F-value of 236.81 indicates that the model is highly significant. There is only a 0.01% probability that such a large F-value could occur due to random variation. P-values less than 0.0500 indicate that the model terms are significant, while values greater than 0.1000 indicate that the model terms are not significant.

Table 4:
The Model F-value of 8.59 suggests that the model is statistically significant. There is only a 0.49% probability that such a large F-value could occur due to random variation. P-values less than 0.0500 indicate that the model terms are significant, while values greater than 0.1000 indicate that the model terms are not significant.

Table 5:
The Model F-value of 421.09 indicates that the model is highly significant. There is only a 0.01% chance that such a large F-value could occur due to random variation. P-values less than 0.0500 indicate that the model terms are significant, while values greater than 0.1000 indicate that the model terms are not significant.

Table 6:
The Model F-value of 4.01 suggests that the model is statistically significant. There is only a 3.18% chance that such a large F-value could occur due to random variation. P-values less than 0.0500 indicate that the model terms are significant, and in this case, the term "A" is found to be significant. Values greater than 0.1000 indicate that the model terms are not significant.





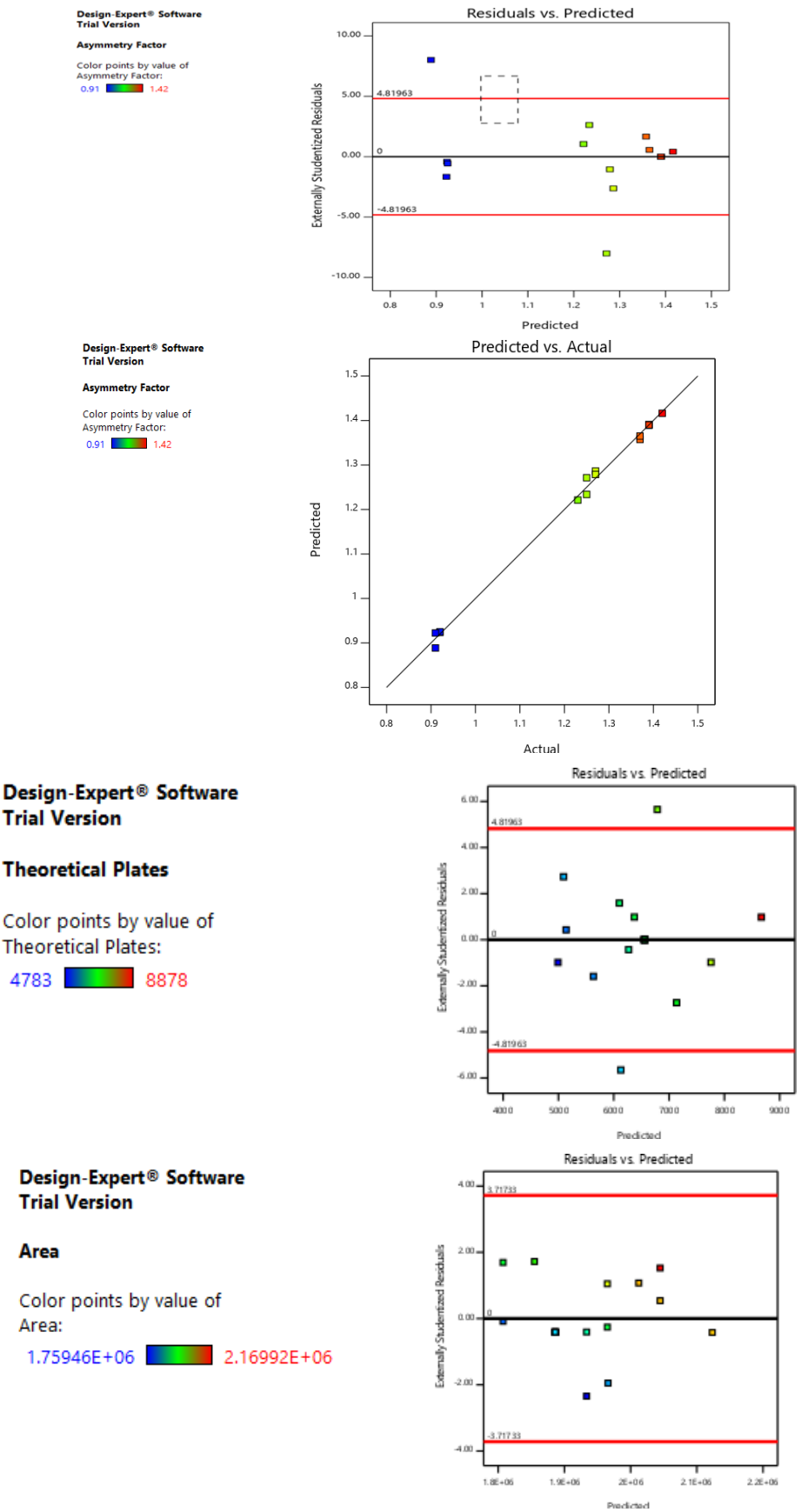


Figure 3: Counter plot and normal plot of residuals for y2 response (theoretical plates)

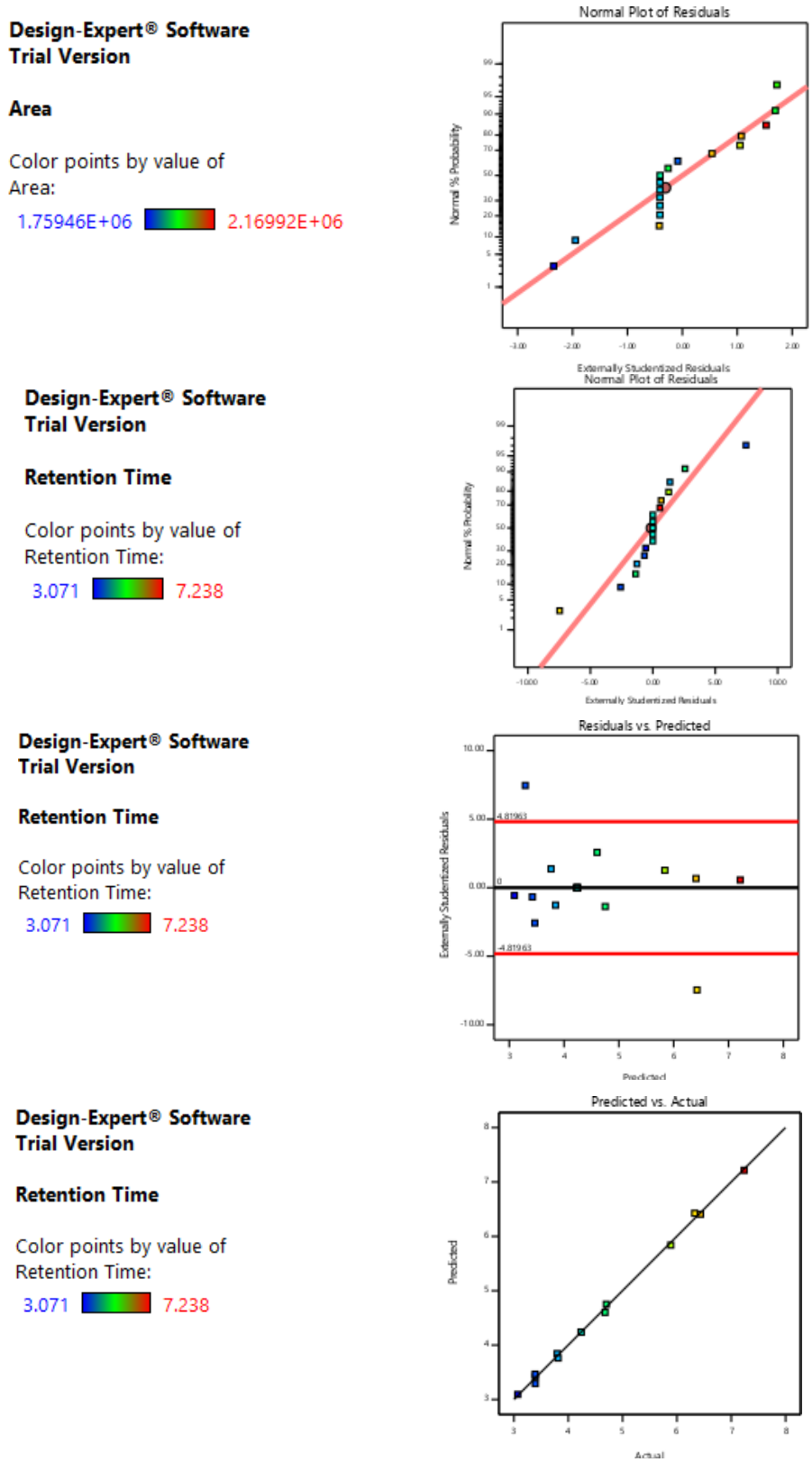


Figure 4: Counter plot and normal plot of residuals for y3 response (retention time)

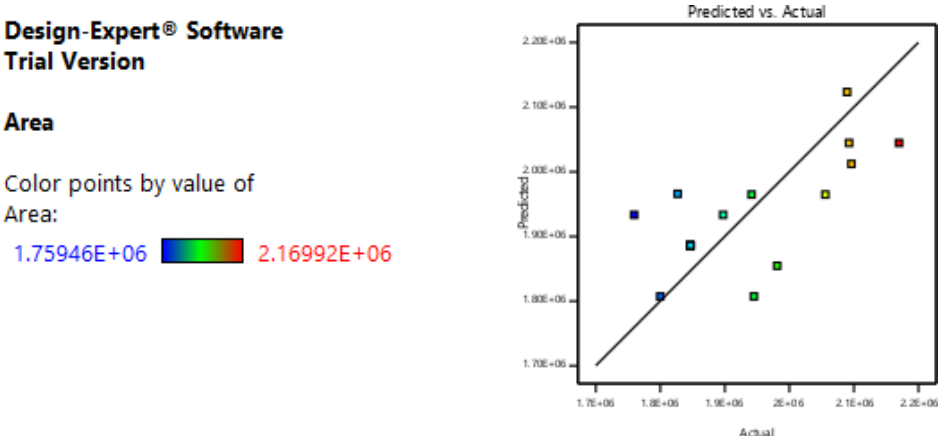


Figure 5: Counter plot and normal plot of residuals for y4 response (area)

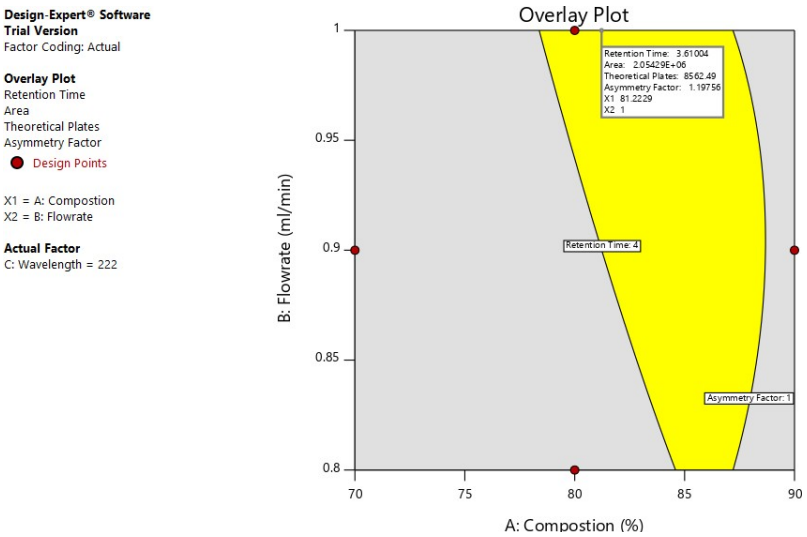


Figure 6: The overlay plot shows that retention time is less that is 3.61min and theoretical plates are 8562, asymmetry factor found to be 1.19.

Overlay plot showing optimize HPLC conditions as a flag within design space:

Assay:

The chromatogram displayed no additional peaks, indicating that the formulation excipient used in the tablet did not interfere with the analysis. The developed method exhibited satisfactory chromatographic separations, with an average percentage recovery from the tablet of 100.06%.

Linearity:

The obtained data was graphed by plotting peak area against concentration, resulting in a coefficient of correlation (r2) value of 0.999. This indicates a high degree of linearity in the relationship between the peak area and concentration.

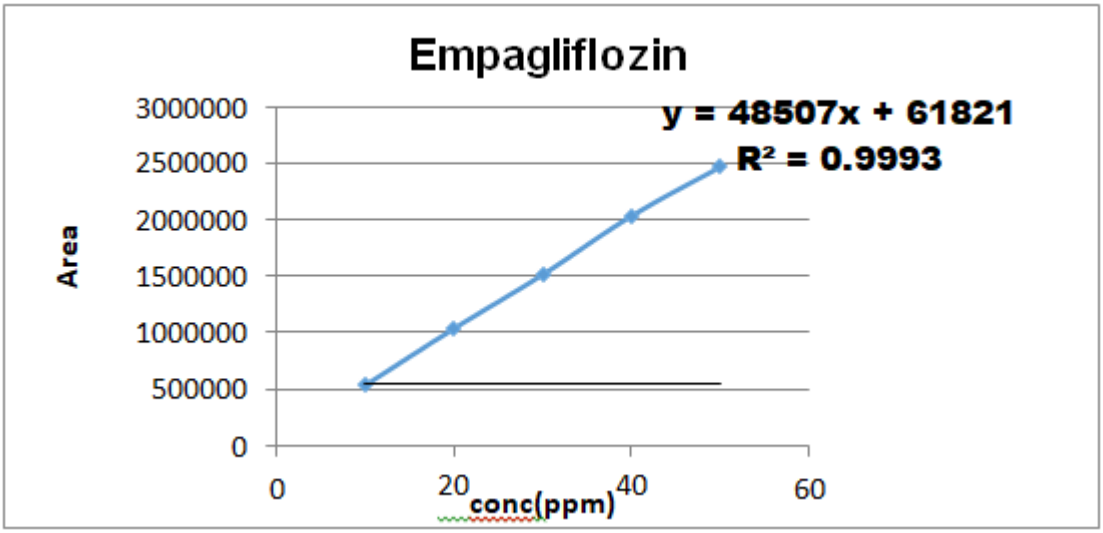


Figure: 7: UV-Spectrophotometric Analysis

Evaluation of system suitability:
Six replicate injections of the standard solution at a concentration of 30 µg/mL were performed. The %RSD (relative standard deviation) obtained from these injections was found to be below 2%. The tailing factor was less than 2, indicating symmetrical peaks. The number of theoretical plates exceeded 2000, indicating efficient chromatographic separation. Hence, all evaluated parameters for system suitability met the predefined acceptance criteria.

Limit of detection (LOD) and Limit of quantitation (LOQ):
The limit of detection was determined to be 0.52 µg/mL, indicating the lowest concentration of the analyte that can be reliably detected. The limit of quantitation was found to be 1.59 µg/mL, which represents the lowest concentration of the analyte that can be accurately quantified.

Precision and accuracy:
The precision of the method for empagliflozine was assessed through interday and intraday measurements. The relative standard deviation (RSD) for interday and intraday precision was found to be 0.38% and 0.33%, respectively, both falling within the acceptable limit of 2%. Additionally, the accuracy data revealed that the mean recovery of empagliflozine at each concentration level ranged from 98.02% to 102.0%, meeting the acceptance criteria.

Table 7: For precision:

	Day 1	**Day 2**	**Mean**	**% RSD**
	1514264	1519155		
Interday	1523137	1523078	1514800	0.38%
	1529673	1514800		
	Morning	**Evening**		
Intraday	1514264	1522348		
	1523137	1520512	1519533	0.33%
	1529673	1519264		

CONCLUSION
In conclusion, the developed HPLC method utilizing a Methanol: Water mobile phase has demonstrated excellent chromatographic separation for the analysis of empagliflozine. The method validation results have confirmed its suitability for quality control and routine analysis of empagliflozine in pharmaceutical dosage forms.The method exhibited desirable characteristics in terms of linearity, precision, accuracy, system suitability, ruggedness, limit of detection (LOD), limit of quantitation (LOQ), and solution stability. The measured signals showed high precision, accuracy, and linearity within the concentration range of 5-30 µg/mL, with a correlation coefficient of 0.998. Additionally, the retention time was less than 3.61 minutes, which contributes to lower solvent consumption and cost-effectiveness.The application of a 32-factorial design using Box-Behnken Design (BBD) highlighted the importance of closely monitoring two factors, namely asymmetry and theoretical plates, during chromatographic testing. This underscores the need for strict control and optimization of these factors to ensure accurate and reliable results.

Overall, the developed method offers a rapid, simple, and selective approach for the routine analysis of empagliflozine in both bulk samples and pharmaceutical formulations. Its effectiveness, coupled with its economic benefits due to reduced solvent consumption, makes it a

valuable tool for pharmaceutical quality control and research applications.

Disclosure statement

The authors of this study declare that they have no potential conflicts of interest.

Additional information

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