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QUALITY BY DESIGN (QBD) APPROACH TO DEVELOP STABILITY INDICATING RP-HPLC METHOD DEVELOPMENT FOR EMPAGLIFLOZIN AND METFORMINE HYDROCHLORIDE

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ABSTRACT

As per requisition of current regulatory requirements, simple, rapid and sensitive method by 3^3 factorial QbD approach was established and validated for Empagliflozin (EPZ) and Metformin Hydrochloride (MTF) by RP-HPLC. Equipped with Reverse Phase (Agilent) C_{18} column (4.6mm x 100mm; 2.5µm), a 20µl injection loop and UV730D Absorbance detector at 231nm wave length and running chemstation 10.1 software and drugs along with degradants were separated via Methanol: (0.1% OPA) Water (70:30) of pH 3.2 as mobile phase setting flow rate 0.7 ml/min at ambient temperature. The developed method was found linear over the concentration range of 2-10 µg/ml for EPZ and 20-100 µg/ml for MTF while detection limit and quantitation limit were found to be 0.629 µg/ml, 1.23 µg/ml and 1.03 µg/ml, 3.28 µg/ml respectively for EPZ and MTF. There are no interfering peaks underperformed degradation conditions. Therefore, a sensitive, robust, accurate and stability indicating method was developed with high degree of practical utility.

Keywords: Empagliflozin, Metformin Hydrochloride, QbD, RP-HPLC, Stability Study, Analytical method development

INTRODUCTION:

The concept of "Quality by Design" (QbD) was defined as an approach which covers a better scientific understanding of critical process and product qualities, designing controls and tests based on the scientific limits of understanding during the development phase and using the knowledge obtained during the life-cycle of the product to work on a constant improvement environment. Guidelines and mathematical models are used to ensure the establishment and use of the knowledge on the subject in an independent and integrated way **[1- 3]**.

Empagliflozin **Fig. 1** Empagliflozin is used along with diet and exercise, and sometimes with other medications, to lower blood sugar levels in people with type 2 diabetes as well as in stroke, heart attack associated with Diabetes.

Metformin hydrochloride is the salt of the biguanide having antihyperglycemic and antineoplastic. It inhibits complex-I (NADPH:ubiquinone oxidoreductase) of the mitochondrial respiratory chain, thereby increasing the cellular AMP to ATP ratio and leading to activation of AMP-activated protein kinase (AMPK) and regulating AMPK-mediated transcription of target genes. This eventually prevents hepatic gluconeogenesis, enhances insulin sensitivity and fatty acid oxidation and ultimately leads to a decrease in glucose levels **[4]**.

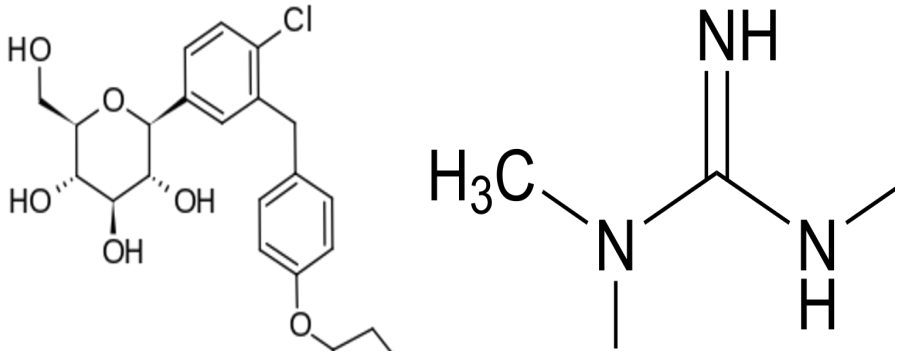

Figure 1: Structure of Empagliflozin and Metformin Hydrochloride

Literature surveys revealed that sensitive LC-MS methods are available for analysis of antidiabetic drugs and its metabolites in human plasma and urine **[6-11]**. Several HPLC methods have been developed individually and combined dosage forms in human plasma **[12-14]**. Even though various methods were reported in the literature for estimation of metformin, Metformin Hydrochloride and

Empagliflozin alone and in combination with other drugs **[15-20]** no method has been reported for simultaneous estimation of these drugs in combination using QbD based 3^3 factorial designing.

MATERIALS AND METHOD:

A simple RP-HPLC method has been developed and validated with different parameters such as linearity, precision, repeatability, LOD, LOQ, accuracy as per International Conference for Harmonisation guidelines (Q2R1). Statistical data analysis was done for data obtained from different aliquots Runs on Agilent Tech. Gradient System with Auto injector, UV (DAD) & Gradient Detector.

Chemicals and Reagents

Reference standards of Empagliflozin hydrochloride was obtained as gift sample from Dr. Reddy's Laboratories, Hyderabad, India while Metformin Hydrochloride was obtained as generous gift from Micro Labs Ltd., Bangalore, India. Pharmaceutical formulation was purchased from local market (Brand: Gibtulio Met tablet labelled claim Empagliflozin 12.5 mg and Metformin Hydrochloride 500 mg make Lupin Pharmaceuticals). The HPLC grade solvents used were of E-Merck (India) Ltd., Mumbai. HPLC grade Acetonitrile, Methanol and Ortho Phosphoric Acid (Merck, Mumbai, India) were used in the analysis. HPLC grade water was prepared using Millipore purification system.

Instruments

The analysis of the drug was carried out on Agilent Tech. Gradient System with Auto injector, UV (DAD) & Gradient Detector. Equipped with Reverse Phase (Agilent) C_{18} column (4.6mm x 100mm; 2.5µm), a 20µl injection loop and UV730D Absorbance detector and running chemstation 10.1 software.

RP-HPLC Optimised Chromatographic Condition using QbD

Column C_{18} (100 mm× 4.6mm); particle size packing 5µm; detection wavelength 231 nm; flow rate 0.7 ml/min; temperature 26^0C ambient; sample size 20 µl; mobile phase methanol: water (OPA 0.1% PH 3.2) (70:30); run time 15 min. The retention time for Empagliflozin and Metformin Hydrochloride were found at 2.9333 min and 6.9667 min respectively **Figure 2**. The RP-HPLC method developed for estimation of Empagliflozin and Metformin Hydrochloride was validated as per ICH Q2 (R1) guidelines using various parameters **Table 1**.

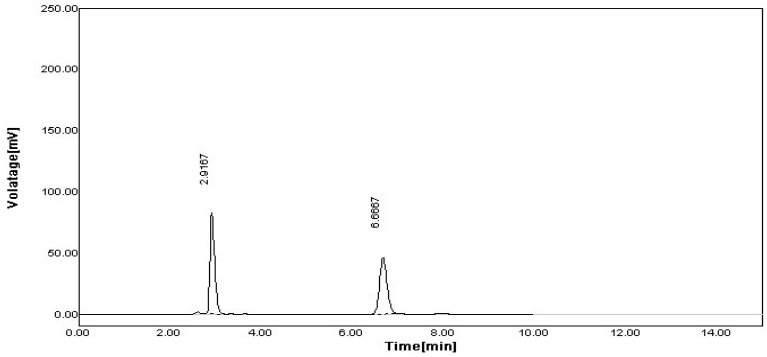

Figure 2: Chromatogram of Standard EPZ and MTF at 231 nm

Preparation of standard solution:

All solutions were prepared on a weight basis to avoid striving in quantification thus proportion was kept within practical range as 1:10. Internal standard addition method adopted for pharmaceutical formulation which was available in the proportion of 1:40.

Stock preparations:

Standard stock solution was prepared by dissolving 10 mg of each API in 10 ml clean dry volumetric flask and dilution was upto the mark with Methanol to obtain final concentration of 1000 µg/ml of each API. All the stock solutions were filtered through 0.45 µm membrane filter.

Detection of λ_{max}: The sample solution has been prepared and scanned in the UV region of 200-400 nm and the spectrum showed the maximum absorbance at 231 nm **Figure 3**.

QbD approach to analysis:

The application of QbD in HPLC method development commences with establishing analytical objectives based on sound science to ensure consistent method performance characteristics are achieved **[21]**.

Thus the objective of this work was to perform experimental design by using Design Expert Software leading to develop simple, rapid and sensitive method by QbD approach and validated as per ICH Guidelines $Q_2(R_1)$ for Empagliflozin and Metformin Hydrochloride and its stability indicating method by RP-HPLC. Further statistical data analysis was done along with numerical and graphical optimization to develop Analytical Design Space (ADS).

METHOD VALIDATION:

Calibration Curve:

A calibration curve was constructed succeeding replicate (n=5) analysis of five standards of 2, 4, 6, 8, 10 µg/ml of EPZ and 20, 40, 60, 80, 100 µg/ml of MTF. The peak height ratio of drugs was calculated and plotted AUC versus concentration after which least squares linear regression analysis of data was undertaken to establish the equation for the best fit line and the

correlation coefficient (R_2) to authorise linearity. Samples were injected and peaks were recorded at 231 nm and the graph plotted as concentration of drug verses peak area as shown in **Table 1**.

Table 1: Linearity Study

EPZ			MTF		
Conc. [µg/ml]	Mean peak area ± SD [n=5]	%RSD	Conc. [µg/ml]	Mean peak area ± SD [n=5]	%RSD
2	173.6±3.05	1.76	20	208.8±3.70	1.77
4	311.4±5.98	1.92	40	412.2±6.87	1.67
6	453.0±7.28	1.61	60	619.4±5.81	0.94
8	571.6±4.83	0.84	80	823.2±7.12	0.86
10	729.6±5.90	0.81	100	1055.4±9.34	0.89

Precision:

Intra-day (repeatability) precision was established following analysis of replicate samples (n=5) at three concentrations indicative of low, medium and high levels within the linear range *viz*., 4, 6, 8, 10 µg/ml of EPZ and 40,60,80 µg/ml of MTF. Analysis was performed over a short period of time on the same day. Inter-day precision or reproducibility was assessed at low, medium and high concentration on three consecutive days and the percent relative standard deviation (% RSD) was used to assess intra- and inter-day precision. An upper limit of 2% was used to confirm precision in our laboratory. **Table 02-03** describes the Intraday, Interday and Repeatability of method.

Table 2: Results of Precision Studies (Intra-Day and Inter-Day)

Drug	Conc. [µg/ml]	Intraday Amount Found [µg/ml]		Inter day Amount Found [µg/ml]	
		Mean±S.D.	% RSD [n= 3]	Mean ±S.D.	% RSD [n= 3]
EPZ	4	3.77±2.00	0.29	4.13±2.00	0.28
	6	6.25±3.06	0.27	6.09±5.57	0.50
	8	8.39±5.51	0.35	7.65±5.03	0.34
MTF	40	29.87 ±4.16	0.34	29.51 ± 8.50	0.69
	60	44.37±10.21	0.55	44.77±7.64	0.41
	80	59.50±6.66	0.27	59.50±9.45	0.38

Table 3: Results of Repeatability Study

Drug	Concentration [µg/ml] [n=1]	Peak Area	Mean[µg/ml] ± SD	% RSD
EPZ	6	377.677	6.25 ± 0.255	1.677
MTF	60	622.833	45.15 ± 0.94	1.26

Accuracy:

Recovery studies were performed to validate the accuracy of developed method. To pre-analysed tablet solution, a definite concentration of standard drug (80%, 100%, and 120%) was added and then its recovery was analyzed. Statistical validation of recovery studies are shown in **Table 4**.

Table 4: Results of Recovery Studies

Drug	Initial amount [µg/ml]	Amount added [µg/ml]	Amt. recovered ± S.D. [µg/ml, n =4]	% Recovery	% RSD
EPZ	6	0	6.15 ±0.27	100.99	1.83
	6	4.8	10.87 ±0.20	100.57	1.67
	6	6	12.08 ±0.24	100.51	1.58
	6	7.2	12.84 ±0.18	99.11	1.02
MTF	60	0	45.29 ±0.67	100.39	0.89
	60	36	80.89 ±0.89	99.81	1.49
	60	45	89.69 ±1.09	99.58	1.45
	60	99	144.49 ±1.28	100.55	1.42

Limits of detection (LOD) and quantitation (LOQ):

By convention, the LOD is estimated as one third of the LOQ. A series of samples of 2, 2.7, 3.4 and 4 µg/ml of EPZ and 15, 20, 25 and 30 µg/ml of MTF were prepared and analysed using the optimized RP-HPLC method and the peak height ratio calculated. The LOQ was determined by establishing the lowest concentration of drugs that resulted in a % RSD value for precision of <2 %.

Specificity:

The specificity of an analytical method is defined as the ability of a method to ensure that the peak(s) of interest elute as distinct responses in the presence of excipients, impurities or degradation compounds.

Robustness:

To evaluate robustness few parameters were deliberately varied. The parameters include variation of flow rate, percentage of methanol as described in **Table 5**.

Table 5: Robustness Evaluation of the HPLC Method

Chromatographic conditions	EPZ			MTF		
	Tailing (T')	Capacity Factor (K')	Theoretical Plate (N)	Tailing (T')	Capacity Factor (K')	Theoretical Plate (N)
A: Mobile phase pH						
3.0	1.26	1.23	2683.9	1.28	0.99	7591.4
3.2	1.22	1.27	2683.5	1.23	1.09	7632.5
3.	1.21	1.33	2625.5	1.25	1.15	7414.7
Mean ±SD	1.23±0.02	1.27±0.05	2687.63±36.80	1.25±0.02	1.07±0.02	7546.2±115.7
B: Flow rate (ml/min.)						
0.5 ml	1.23	0.98	2723.8	1.26	0.76	7587.3
0.7 ml	1.16	1.08	2818.9	1.29	1.10	7668.8
1.0 ml	1.15	1.09	2768.7	1.22	0.88	7423.5
Mean ±SD	1.18±0.04	1.05±0.06	2770.47±47.50	1.25±0.03	0.91±0.17	7593.2±75.82
C: Percentage methanol in mobile phase (v/v)						
60	1.09	1.22	2646.2	1.18	0.87	7623.8
70	1.06	1.13	2687.4	0.94	0.95	7667.3
80	1.19	1.18	2638.3	1.23	0.87	7433.2
Mean ±SD	1.11±0.06	1.17±0.04	2657.3±26.36	1.11±0.15	0.89±0.04	7574±124.51

Study of system suitability parameters

The system suitability is used to verify, whether the resolution and reproducibility of the chromatographic system are adequate for analysis to be done. The test was performed by collecting data from five replicate injections of standard solution as shown in **Table 6**.

Table 6: System Suitability Test

EPZ		MTF	
System suitability parameters	**Proposed method**	**System suitability parameters**	**Proposed method**
Retention time (Rt)	**2.9333**	**Retention time (Rt)**	**6.9167**
Capacity factor (K')	**1.18**	**Capacity factor (K')**	**0.98**
Theoretical plate (N)	**2838.7**	**Theoretical plate (N)**	**7465.8**
Tailing factor (T)	**1.16**	**Tailing factor (T)**	**0.95**

FORCED DEGRADATION STUDIES:

Forced degradation study was performed to evaluate the stability of the developed method using the stress conditions like exposure of sample solution to acid, base, Hydrogen peroxides (H_2O_2) and Neutral. Investigations were done for the degradation products in different conditions and are shown in **Table 7**.

Procedure for Empagliflozin and Metformin Hydrochloride degradation

Acid hydrolysis

The acid hydrolysis performed using 0.1N HCl at 70 ºC for 1st hr and 2nd hr for both Metformin Hydrochloride and Empagliflozin indicated degradation. The major degradation products for Metformin Hydrochloride and Empagliflozin were observed at relative retention time (RRT) for 1st and 2nd Hours.

Alkaline hydrolysis

The alkaline hydrolysis condition was performed using 0.1N NaOH at 70 ºC for 1st hr and 2nd hr both Metformin Hydrochloride and Empagliflozin. The major degradation products for Metformin Hydrochloride and Empagliflozin were observed at relative retention time (RRT) for 1st and 2nd Hours

Oxidation

In the oxidation condition with 3% H_2O_2 for 1st hr and 2nd hr both Metformin Hydrochloride and Empagliflozin show oxidative stress degradation peak in the chromatogram.

Neutral

There was no major degradation observed for both Metformin Hydrochloride and Empagliflozin and hence they were not sensitive to light at 70 ºC for 1st hr and 2nd hr.

Table 7: Forced Degradation

Sample Exposure condition	Total Number of products with their Rt	EPZ		MTF	
		Degradation remained (150 µg/ml)	Recovery (%)	Degradation remained (30 µg/ml)	Recovery (%)
Acidic, 1N, 1 Hr	5 (2.95, 4.80,6.05,7.08,7.65)	136.224	90.81	28.25	94.18
Basic, 1N, 1 Hr	6 (2.61, 2.80, 2.95, 3.38, 4.51, 7.20)	122.22	81.48	13.28	44.29
Per oxide, 30%, 1 Hr	4 (2.63, 2.83, 4.76, 7.03)	128.50	85.67	20.92	69.73
Heat, 50°C, 1 Hr	3 (2.61,2.81,6.766)	136.58	91.05	22.20	74.01

Application of analytical method:

To determine the content of EPZ and MTF in marketed tablets (label claim 15mg of Empagliflozin and 2 mg Metformin Hydrochloride), 20 tablets powder weighed as 5.96 gm and average weight of powder was calculated in 0.298 gm. Tablets were triturated and powder equivalent to weighed in 298 mg. The drug was extracted from the tablet powder with 10 ml Methanol. To ensure complete extraction it was sonicated for 15 min. 0.1 ml of supernatant was then diluted up to 10 mL with mobile phase. The resulting solution was injected in HPLC and drug peak area was noted**.**

RESULTS AND DISCUSSION

Such analytical methods are, in fact, an indicator of a quality product and the robustness of that product for the duration on the lifecycle of that product. The main goal of any HPLC method is to separate and quantitate analyte(s) of interest from any impurity and/or excipients. Initially it is important to establish the critical quality attributes (CQA) of a system that may impact the quality of the analytical method. Development of Analytical RP-HPLC Method with Design Space and Control Strategy determination by optimization study all the computations for the current optimization study and statistical analysis were performed using Design Expert® software (Design Expert trial version) State-Ease Inc., Minneapolis, MN, USA).

Application of design of experiments for method optimization Design of experiments (DOE-1)

Thus, 3 randomized response surface designs with a full fraction design were used with 17 trial runs to study the impact of three factors on the three key response variables. In this design 3 factors were evaluated, each at 3 levels, and experimental trials were performed at all 3 possible combinations. The mobile phase composition (X1), Wavelength (X2) and flow rate (X3), were selected as independent variables and retention time (RT) and Resolution were selected as dependent variables. The resulting data were fitted into

Design Expert 10 Software and analyzed statistically using analysis of variance (ANOVA) and F-Test. **Figure 3** indicates the normal plot of residuals for retention time with other chromatographic parameters. The data were also subjected to 3-D response surface methodology to determine the influence of flow rate, Wavelength and mobile phase composition on dependent variables as shown in **Figure 4**. The probable trial runs using 3^3 full fraction designs are as shown in **Table 4**. Further ANOVA and F-test with variables are shown in **Table 8-12**. More over degradation peaks of API were shown in **Figure 5-8** from acidic, alkaline, peroxide and Heat.

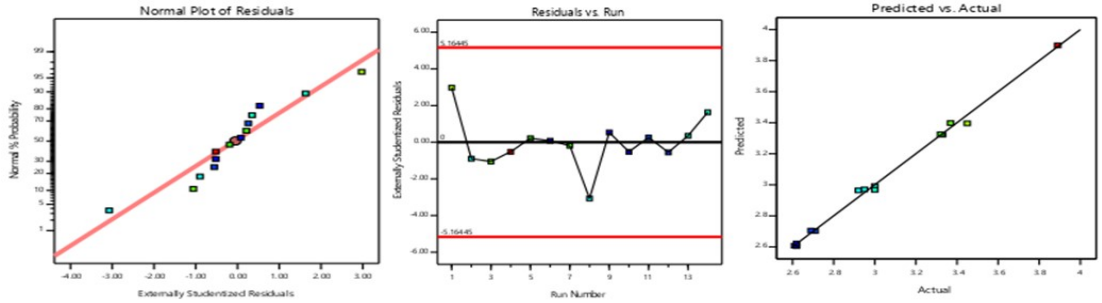

Figure 3A: Normal Plot of Residuals for Retention Time and Plot of Predicted Vs. Actual Data For Retention Time of CP

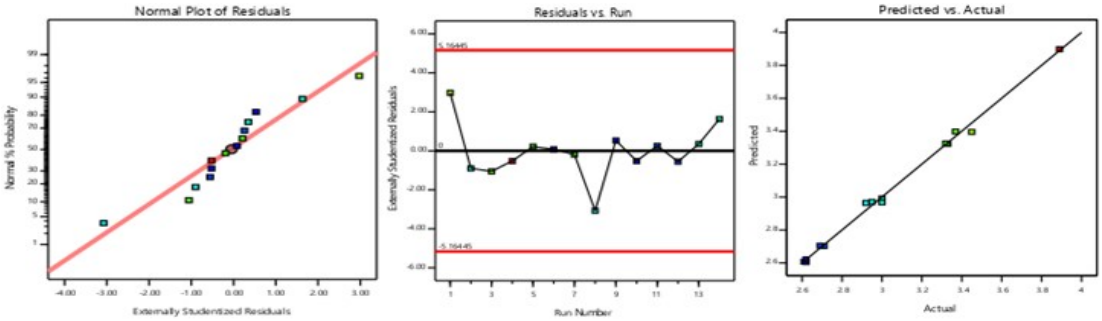

Figure 3b: Normal Plot of Residuals for Retention Time and Plot of Predicted Vs. Actual Data For Retention Time By the Value Of 2.61 To 3.89

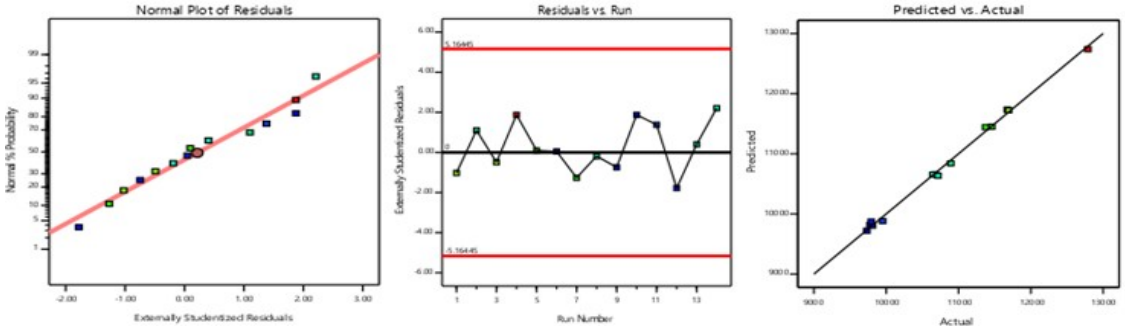

Figure 3C: Normal Plot of Residuals for Retention Time and Plot of Predicted Vs. Actual Data for Retention Time by the Value of 9733 To 12789

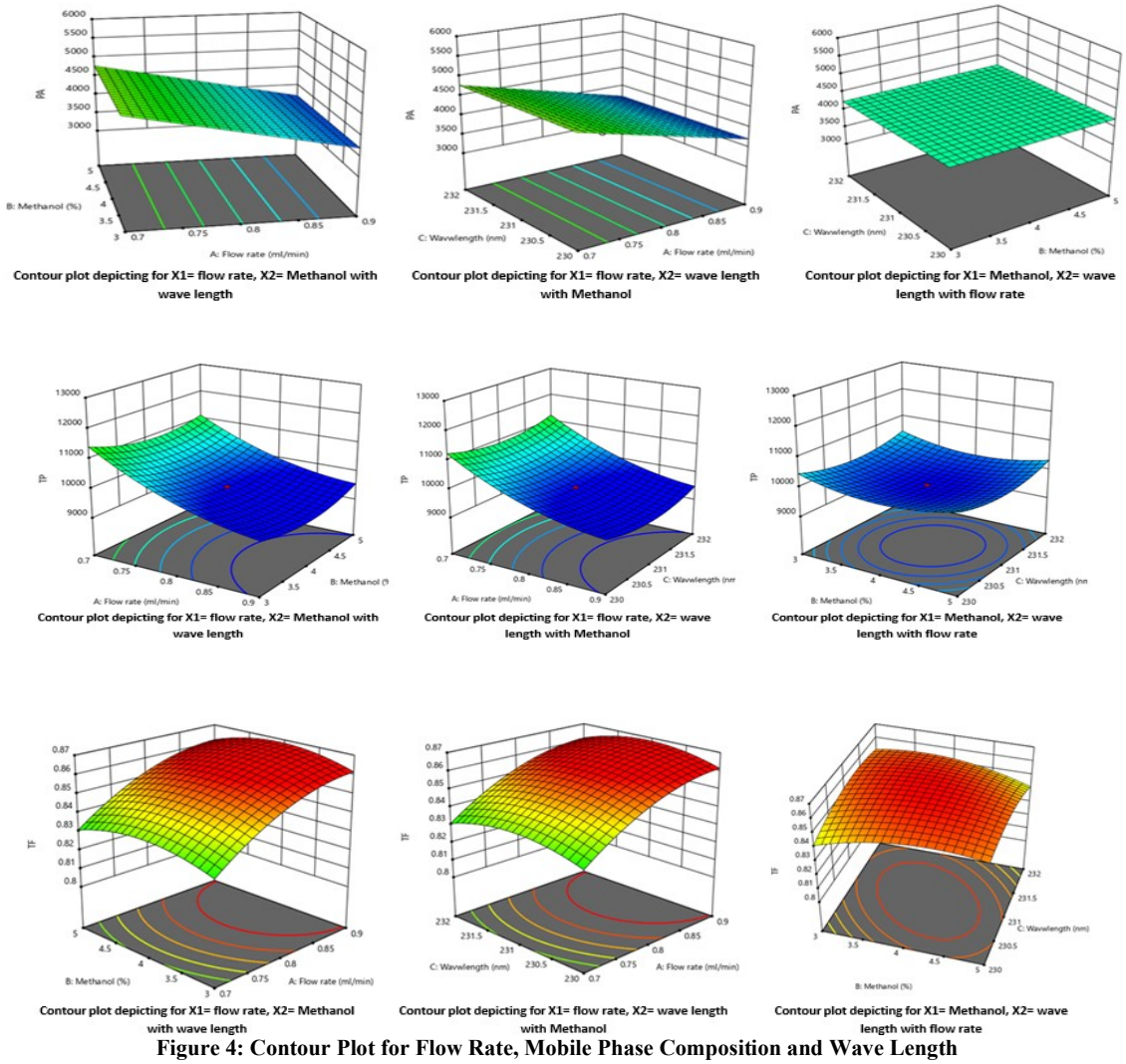

Figure 4: Contour Plot for Flow Rate, Mobile Phase Composition and Wave Length

Table 8: Probable trial runs using 3^3 full fraction designs

Std	Run	Factor 1	Factor 2	Factor 3	Response 1	Response 2	Response 3	Response 4
		A:Flow rate	B:Methanol	C:Wave length	RT	PA	TP	TF
		ml/min	%	nm				
1	1	0.7	3	230	3.45	4850.37	11675	0.82
11	2	0.8	2.3	231	2.95	4019.71	10892	0.84
5	3	0.7	3	232	3.369	4521.28	11693	0.83
9	4	0.6	4	231	3.89	5516.24	12789	0.8
3	5	0.7	5	230	3.33	4896.5	11458	0.83
6	6	0.9	3	232	2.61	3555.04	9810	0.86
7	7	0.7	5	232	3.32	4665.06	11373	0.82
13	8	0.8	4	229.3	2.92	4373.36	10645	0.84
2	9	0.9	3	230	2.62	3755.37	9777	0.85
10	10	0.8	4	231	2.62	3707.75	9733	0.86
4	11	0.9	5	230	2.71	4018.88	9950	0.86
8	12	0.9	5	232	2.69	3785.56	9793	0.85
12	13	0.8	5.7	231	3	4326.7	10679	0.84
14	14	0.8	4	232.7	3	4484.22	10716	0.84

Table 9: ANOVA for Reduced Quadratic Model (Response 1: RT)

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	1.95	7	0.2783	211.69	< 0.0001	significant
A-Flow rate	1.10	1	1.10	833.33	< 0.0001	
B-Methanol	0.0005	1	0.0005	0.4083	0.5464	
C-Wavelength	0.0000	1	0.0000	0.0124	0.9149	
AB	0.0144	1	0.0144	10.93	0.0163	
A^2	0.1479	1	0.1479	112.53	< 0.0001	
B^2	0.0882	1	0.0882	67.09	0.0002	
C^2	0.0810	1	0.0810	61.60	0.0002	
Residual	0.0079	6	0.0013			
Cor Total	1.96	13				

Table 10: ANOVA for Reduced Linear model (Response 2: PA)

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	3.294E+06	1	3.294E+06	57.63	< 0.0001	significant
A-Flow rate	3.294E+06	1	3.294E+06	57.63	< 0.0001	
Residual	6.858E+05	12	57151.71			
Cor Total	3.979E+06	13				

Table 11: ANOVA for Reduced Quadratic model (Response 3: TP)

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	1.120E+07	7	1.600E+06	288.01	< 0.0001	significant
A-Flow rate	6.682E+06	1	6.682E+06	1202.92	< 0.0001	
B-Methanol	40072.40	1	40072.40	7.21	0.0363	
C-Wavwlength	358.64	1	358.64	0.0646	0.8079	
AB	60031.13	1	60031.13	10.81	0.0167	
A^2	7.371E+05	1	7.371E+05	132.68	< 0.0001	
B^2	7.252E+05	1	7.252E+05	130.54	< 0.0001	
C^2	5.848E+05	1	5.848E+05	105.27	< 0.0001	
Residual	33330.78	6	5555.13			
Cor Total	1.123E+07	13				

Table 12: ANOVA for Reduced Quadratic model (Response 4: TF)

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	0.0040	7	0.0006	646.40	< 0.0001	Significant
A-Flow rate	0.0020	1	0.0020	2334.61	< 0.0001	
B-Methanol	0.0000	1	0.0000	0.0000	1.0000	
C-Wavelength	0.0000	1	0.0000	0.0000	1.0000	
BC	0.0002	1	0.0002	228.17	< 0.0001	
A^2	0.0004	1	0.0004	427.66	< 0.0001	
B^2	0.0003	1	0.0003	298.35	< 0.0001	
C^2	0.0003	1	0.0003	298.35	< 0.0001	
Residual	5.259E-06	6	8.765E-07			
Cor Total	0.0040	13				

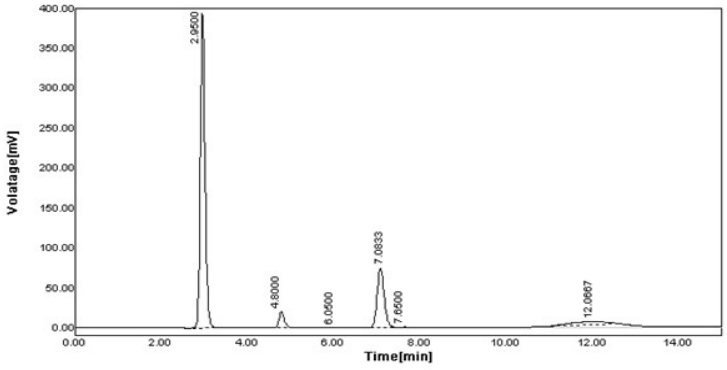

Figure 5: Acidic Degradation

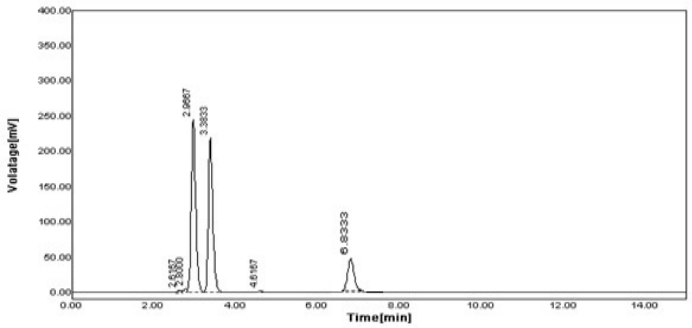

Figure 6: Alkaline Degradation

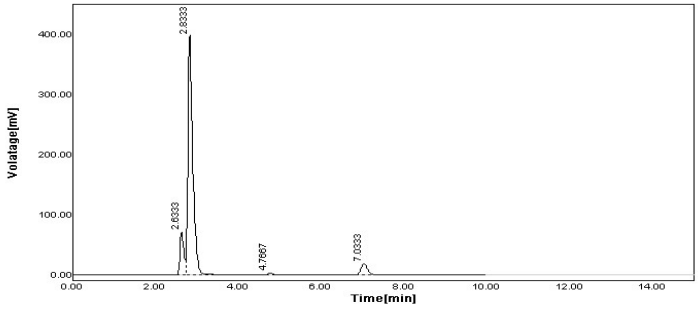

Figure 7: Per Oxide Degradation

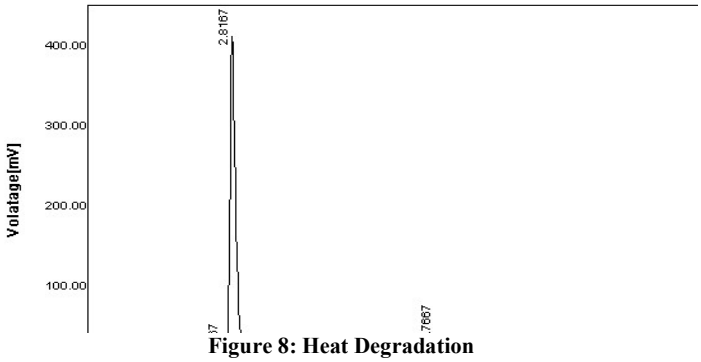

Figure 8: Heat Degradation

CONCLUSION:

A simple, rapid, reliable, robust and optimized reversed phase high performance liquid chromatographic method for estimation of Empagliflozin and Metformin Hydrochloride was successfully developed and validated as per International Conference on Harmonization guidelines. Percentage of mobile phase, flow rate and wave length were optimised by using QbD approach *i.e.* 3^3 factorial design. There are no interfering peaks observed in performed degradation conditions. Therefore, a sensitive, accurate and stability indicating method was developed with high degree of practical utility.

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