DOE APPROACH: A VALIDATED RP -HPLC METHOD FOR THE DETERMINATION OF DAPAGLIFLOZIN

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ABSTRACT

Background: Dapagliflozin is a competitive inhibitor of sodium / glucose co transporter 2 (SGLT2). It will Inhibiting the reabsorption of filtered glucose in the kidney leads to elevated urinary glucose excretion, thereby lowering blood

Method: A new, simple, accurate, rapid, precise, reproducible and cost-effective RP-HPLC method for the quantitative estimation of Dapagliflozin in bulk and pharmaceutical dosage form. The developed RP-HPLC method for the quantitative estimation of Dapagliflozin is based on measurement of absorption at maximum wavelength 254 nm using 0.1% urea: methanol (35:65% v/v) as a solvent. The stock solution for Dapagliflozin was prepared, and subsequent suitable dilution was prepared in mobile phase to obtained standard curve. The standard solution of Dapagliflozin shows absorption maxima at 254 nm.

Results: Dapagliflozin will obeys Beers -Lamberts law in the concentration range of 20 - 100µg/ml with regression 0.999 at 254nm.The overall % recovery was found to be 101.59% for Dapagliflozin which reflects that the method was free from the interference of impurities and other impurities, used in bulk and marketed dosage forms. The low value of % RSD was indicative of accuracy and reproducibility of the method. The % RSD for inter-day and intra-day precision was found to be 0.1 for Dapagliflozin respectively which is & it <2% hence proved that method is precise.

Conclusion: The results of analysis have been validated as per International Conference on Harmonization (ICH) guidelines. The developed method can be adopted in routine analysis of Dapagliflozin in bulk and tablet dosage form. The Proposed method was found to be rapid, accurate, precise, specific, robust, rugged and economical.

Keywords: Dapagliflozin, SGLT, Diabetes, Urea, Methanol, RP-HPLC,

Introduction

Pharmaceutical research is constantly striving to discover novel approaches that enhance drug development, formulation, and analysis. Dapagliflozin is a potent drug and includes in class of anti hyper glycaemic agents (AHAs) for the treatment of type 2 diabetes (T2D). Ensuring the effectiveness and safety of this drug heavily reliable on precise and depends on determination of its concentration. High-Performance Liquid Chromatography (HPLC) is a widely employed analytical technique for drug quantification due to its high sensitivity, specificity and reproducibility.

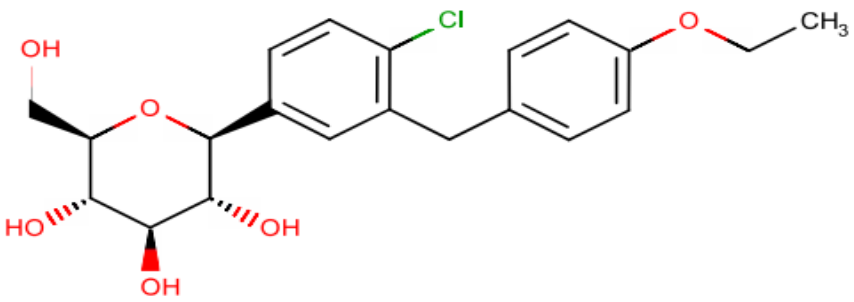

Fig:1 Structure of Dapagliflozin

This research presents a novel approach to estimate Dapagliflozin using a Design of Experiment (DOE). RP-HPLC method in conjunction with hydrotropic solubilization. Design of Experiment is a statistical methodology that allows researchers to optimize experimental parameters systematically, leading to more robust and efficient analytical methods. The objectives of this study are as follows:

1. To develop HPLC method utilizing design expert Optimization of the method by DOE applying Box-Been statistical software.
2. The analytical method for the estimation of Dapagliflozin will be developed by RP-HPLC method by optimizing the chromatographic conditions.
3. The developed method is validated according to ICH guidelines for various parameters specified in ICH guidelines, Q2 (R1).

The significance of this research lies in its potential to provide a more sensitive and efficient HPLC method for the estimation of Dapagliflozin, thus contributing to the quality control of this important antiretroviral drug. Additionally, the utilization of Design of Experiment principles ensures a systematic and thorough exploration of the experimental space, leading to robust and transferable analytical methods.

The remainder of this paper will develop into the methodology, results, and discussion of the experimental findings. Furthermore, the validation and application of the optimized RP-HPLC method for the estimation of Dapagliflozin in real pharmaceutical samples will be presented, along with a comparison to existing methods. Ultimately, this research aims to offer a valuable contribution to the field of analytical chemistry and pharmaceutical analysis.

EXPERIMENTAL METHODS

Chemicals and reagents:

Dapagliflozin (Forxiga, **Manufacturer** – Dr.Reddy's Laboratories.), 0.1% urea and Methanol

METHOD DEVELOPMENT BY RP- HPLC:

HPLC system (Shimadzu) with PDA detector was used. The software LC-Solution can be used and a Rheodyne injection with a 20µL loop was used for injection of the sample. Thermosil C_{18} Column (100mm x 4.6mm) 5µm was used. The mobile phase was composed of 0.1% Urea and methanol (35:65 v/v) with flow rate of 1ml/min. HPLC system was operated at ambient temperature.

1. Preparation of Standard Stock Solution

10mg of Dapagliflozin standard was transferred into 10 ml volumetric flask and 7ml of diluent was added slowly and made up to the mark with diluent to obtain a concentration of 1000µg/ml.

2. Preparation of Working stock solution:

0.6ml of the standard stock solution was pipetted out and transferred into 10ml of volumetric flask and diluted up to the mark with diluent to obtain a concentration of 60µg/ml.

3. Preparation of Mobile phase:

Mobile phase consists of 0.1% urea : Methanol of P^H 2.5 (35:65) was taken sonicated and degassed for 10min and filtered through 0.45 µm nylon membrane filter.

4. Design of Experiment: The standard drug sample of Dapagliflozin was subjected to the design of experiment process. Box-Behnken response surface design was employed to identify the underlying facts of effects of factors and their interaction effects on selected method responses. A total of 17 runs were conducted.

Statistical analysis:

- By using ANOVA, the statistical calculations were processed for variables screening and optimization of the method.
- The statistical tools provide the numerical verification of variables and its effect on responses.

Method operable design region:

The different amalgamation and reciprocity of input factors produces the space referred as Design space. The establishment of design space was made by utilizing the contour graphs of Sigma tech software.

Method Verification:

The optimized method conditions were proposed by the software in order to reach the desired method goals. The method was verified to check the predictability of the proposed model.

Table: 1 Box - Behnken design experimental runs

	Factor 1	Factor 2	Factor 3	Response 1	Response 2
Run	A: BUFFER	B: Flow Rate	C: Temperature	Rt of Dapagliflozin	Tailing factor of DAPA
				min	
1	3.5	1	15	2.075	0.99
2	3	0.9	25	2.524	1.05
3	3	0.9	15	2.413	1.07
4	3	0.8	20	2.524	1.05
5	3.5	0.9	20	2.225	1.05
6	4	0.8	20	2.581	1.36
7	3.5	0.8	15	2.538	1.4
8	4	0.9	25	2.255	1.38
9	3.5	0.9	20	2.369	1.41
10	3.5	0.9	20	2.396	1.41
11	3.5	0.9	20	2.396	1.41
12	3	1	20	2.5	1.06
13	4	0.9	15	2.23	1.31
14	3.5	0.9	20	2.52	1.41
15	3.5	1	25	2.12	0.96
16	4	1	20	2.22	1.25
17	3.5	0.8	25	2.54	0.94

Method Validation

1. Linearity:

From the above standard stock solution pipetted out 0.2, 0.4, 0.6, 0.8 and 1ml into a five 10ml volumetric flask and made up to the volume 10ml with diluent to get 2, 4, 6, 8 and 10µg/ml concentrated solutions of was filtered and injected into HPLC system and peak area was measured. Plotted a graph between peak area and concentration. Correlation coefficient was determined by regression analysis.

2. Precision:

From the standard stock solution an aliquot of 0.6ml was added into a six 10ml volumetric flasks, made up to 10ml with diluent. Later it was filtered and six replicates were injected into HPLC system and measured the area for all six injections.

3. Accuracy:

Preparation of standard stock solution:

1000µg/ml of standard stock solution was prepared. Further pipetted out 0.6ml of Standard stock solution into 10ml volumetric flask and was diluted up to the mark with diluent.

Preparation of sample solution:

Accuracy solutions at 50% level:

5mg of sample is weighed and transferred into a 10ml volumetric flask added about 7ml of diluent and sonicated to dissolve it completely and made volume up to the mark with diluent. Further pipetted out0.6ml of above stock solution into a 10ml volumetric flask and made volume up to mark with diluent and injected sample into HPLC injector.

Accuracy solutions at 100% level:

10mg of sample is weighed and transferred into a 10ml volumetric flask added about 2ml of diluent and sonicated to dissolve it completely and made volume up to the mark with diluent. Further pipetted out 0.6ml of above stock solution into a 10ml volumetric flask and made volume up to mark with diluent and injected sample into HPLC injector.

Accuracy solutions at 150% level:

15mg of sample is weighed and transferred into a 10ml volumetric flask added about 2ml of diluent and sonicated to dissolve it completely and made volume up to the mark with diluent. Further pipetted out 0.6ml of above stock solution into a 10ml volumetric flask and made volume up to mark with diluent and injected sample into HPLC injector.

4. LOD and LOQ:

The limit of detection and limit of quantification was calculated based on the standard deviation of the response and slope of calibration curve.

5. **Robustness:**

It is the capacity of the method to remain unaffected by small deliberate variations like change in the flow rate and mobile phase composition was made to evaluate the impact on the method.

Flow rate variations:
60ppm of Dapagliflozin was prepared and injected into HPLC system using the variation in flow rates along with method flow rate, i.e., 0.8ml/min, 1.0ml/min and 1.2ml

Variation in organic composition in mobile phase:
60ppm of Salmeterol was prepared and injected into HPLC system using the varied organic composition in mobile phase along with method mobile phase composition i.e., 10% less, Actual and 10% more.

RETENTION TIME

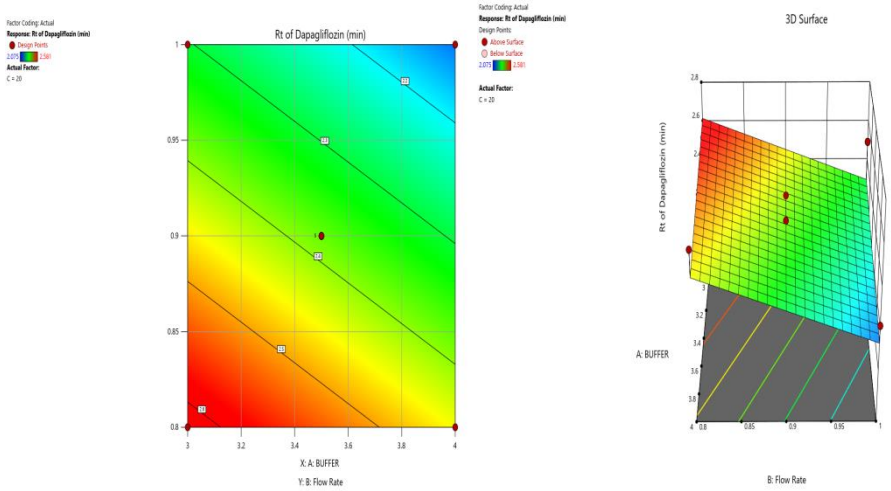

Figure 2: 3D RSM plots for retention time

TAILING FACTOR

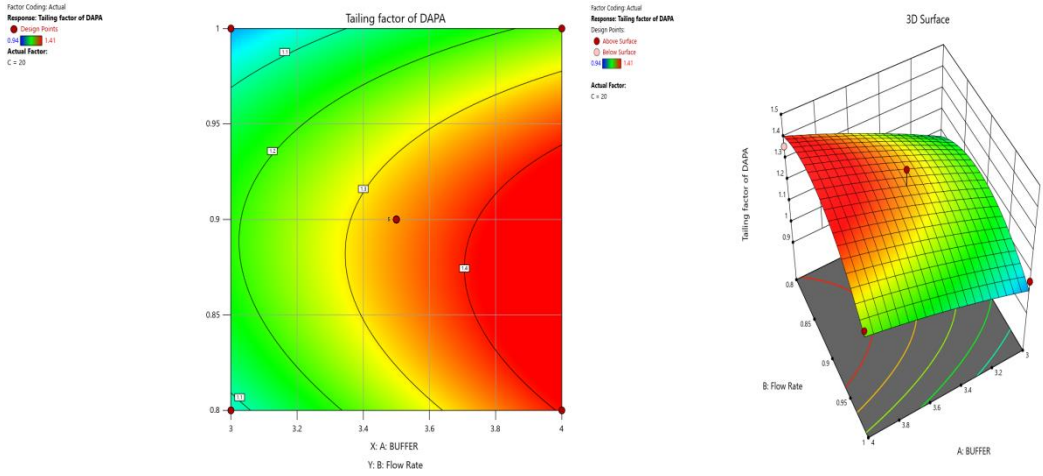

Figure 3: 3D RSM plots for Tailing factor

Optimization and development of RP-HPLC-PDA method using Box-Behnken design

- In the proposed investigation, 17 experimental runs were performed and analyzed for obtained results of retention time and tailing factors in accordance with the Box-Behnken design.
- Further investigation was performed using response surface methodology (RSM) to evaluate the relationship between the dependent responses and independent variables (Factors) using obtained data was reported in Table 1.
- The model was also validated by analysis of variance (ANOVA) using design expert software, and the results are as presented in Table 2. Based on value, a quadratic model was selected for responses such as retention time and tailing factor.
- The significant effects showed p value less than 0.05, while the low standard deviation (% C.V) and a high adjusted R-square value indicated a good relationship between the experimental data and those of the fitted model.
- The predicated R-square value was in acceptance concordance with the adjusted R-square value for all responses.
- The final equation in terms of actual components and factors which can be used to make predictions about the response for given levels of each factor.

S. NO	Response	±S. D	Mean	%CV	R^2	Adjusted R^2	Predicated R^2	Adequate precision	P value
1	Retention time	0.1613	2.38	3.60	0.8824	0.5439	0.3268	9.7377	0.0039
2	Tailing factor	0.1873	1.21	9.76	0.4566	0.3844	-0.6439	6.6143	0.01448

Table: 2 ANOVA Table

The chromatographic conditions were validated by evaluating linearity, accuracy, method precision, limit of detection (LOD), limit of quantization (LOQ), ruggedness and robustness in accordance with ICH guidelines.

Specificity

Preparation of solutions

a) Placebo interference

Amount of 352.6 mg of the capsule powder was taken in to 100ml standard flask. A volume of 70ml of mobile phase was added and sonicate for 30min.Then the solution was cooled and diluted to volume with mobile phase and filtered through 0.45µm membrane filter. (Stock solution)Further pipette 0.25ml of Dapagliflozin of the above stock solution in to a 10ml volumetric flask and dilute up to the mark with diluent.

Acceptance criteria

Chromatogram of placebo should not show any peak at the retention time of analyte peak.

Standard preparation

Weigh accurately 10mg Dapagliflozin Working Reference Standard is taken in to 100ml volumetric flask and then it was dissolved and diluted to volume with mobile phase up to the mark. After that 50ml of the above solution was taken into 100ml standard flask and made up with mobile phase. (Stock solution) Further pipette 0.5ml of the above stock solution in to a 10ml volumetric flask and dilute up to the mark with diluent.

Sample preparation

Amount of 352.6 mg of the tablet powder was taken in to 100ml standard flask. A volume of 70ml of mobile phase was added and sonicate for 30min.Then the solution was cooled and diluted to volume with mobile phase and filtered through 0.45µm membrane filter.

(stock solution)

Further pipette 0.25ml of Dapagliflozin of the above stock solution in to a 10ml volumetric flask and dilute up to the mark with diluent.

Linearity and Range

Procedure

Linearity was performed over the concentration range of 20 – 60 µg/ml by withdrawing 0.2ml to 0.6ml from standard stock solution. The prepared dilutions were filtered and injected into Hplc. Peak responses were noted, the calibration curve was plotted across concentration on X-axis and peak area on Y- axis.

Accuracy

Accuracy was carried by standard addition method.the optimized standard solution spiked to sample solution at three different concentration levels of 50%,100% and 150% respectively. The prepared sample solution was injected into HPLC system and mean recovery of the sample was calculated.

$$\%\boldsymbol{Recovery} = \frac{\text{Sample peak area x weight of standard}}{\text{Standard peak area x weight of sample}}\ \boldsymbol{x\ 100}$$

Precision

Intermediate Precision (Ruggedness)

Intermediate precision was performed on differentdays. The optimized concentration was performedfrom the stock solution ad six replicates were injected into HPLC

Robustness

As part of the robustness, deliberate change in the flow rate and mobile phase composition was made to evaluate the impact on the method.

a) The flow rate was varied at 0.8ml/min to 1.2ml/min.

b) The organic composition in the mobile phase was varied from 65% to75 % standard solution 10µg/ml of prepared and analysed using the varied mobile phase composition along with the actual mobile phase composition in the method.

Limit of detection & Quantification (LOD)

The solutions for limit of detection & quantification were prepared based on the signal tonoise ratio (S/N) obtained from standard deviationand slope, filtered and injected.

LOD= 3.3 x standard deviation/ slope

LOQ= 10 x standard deviation/ slope

System Suitability:

In system suitability, six replicates of concentrations were prepared from standard stock solution, filtered, injected. The peak areas, Retention time, tailing factor, theoretical plates of all the prepared concentrations were noted andcompared with limits.

Assay

From the sample stock solution, 2ml was drawn &transferred into 10ml volumetric flask and made upto the mark with mobile phase, filtered, injected into HPLC in triplicate, and the chromatograms were recorded. The peak areas were determined and the amount of Dapagliflozin calculated. The values obtained for Assay

Results and discussion

Method Optimization

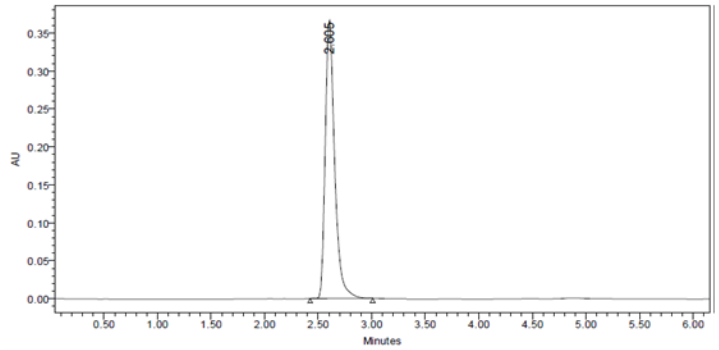

Fig:4 Chromatogram of Dapagliflozin

Analytical method validation:

Validation parameters and their acceptance criteria were mentioned in Table no.

System Suitability

S.No	Peak Name	R_t	Area	Height	USP Plate Count	USP Tailing
1	Dapagliflozin	2.589	2004682	342227	5167	1.3

Table. No:3 System Suitability results of Dapagliflozin

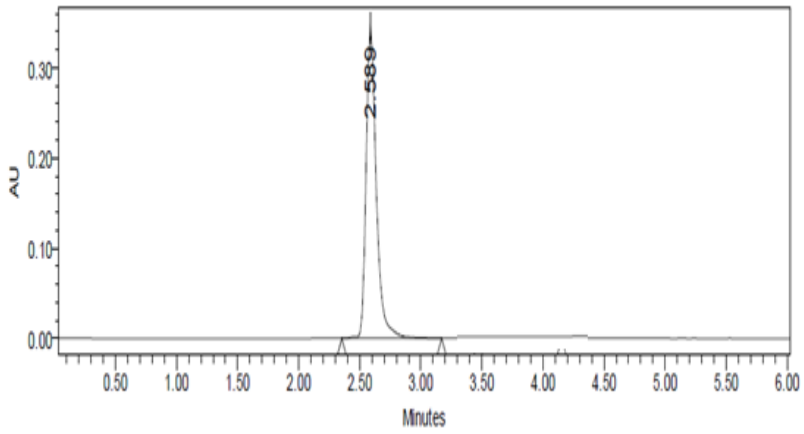

Fig-5 Chromatograms of System Suitability

Remarks: Standard deviation and % RSD were calculated and the values were found to be within the limits.

Linearity:

S.NO	Concentration (µg/mL)	Peak area
1	20	1224140
2	30	1595681
3	40	**1992966**
4	50	2356546
5	60	2797214

Table. No:4 Linearity results of Dapagliflozin:

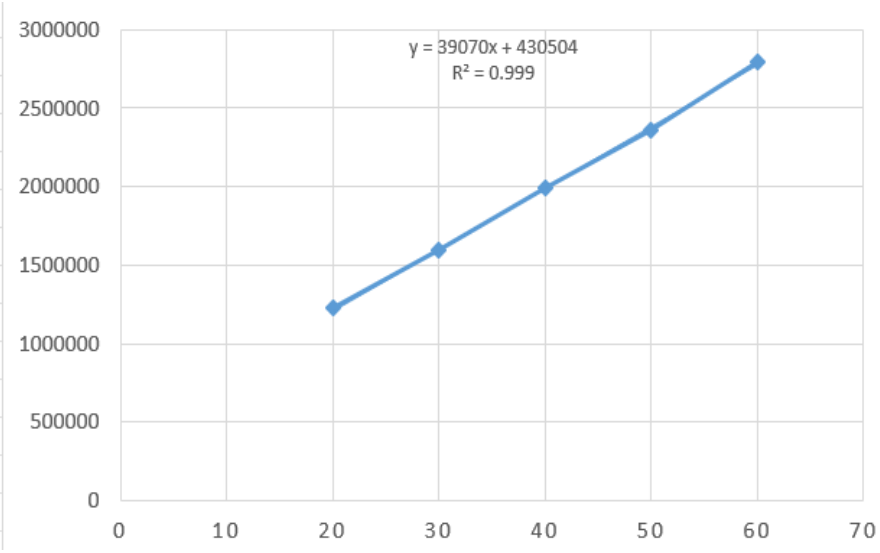

Fig:6 Linearity Curve of Dapagliflozin

The Chromatogram of linearity concentrations from 20- 60 µg/mL were represented.

Accuracy

The accuracy was performed at 3 levels the values obtained for Accuracy were mentioned below

Average % Recovery	Amount taken (mcg/ml)	Area	Average area	Amount recovered (mcg/ml)	Percentage Recovery	Average % Recovery
50%	5	1011326	1017498.5	101.3927	101.3927	100.599%
	5	1015029				
	5	1026141				
100%	10	1986534	1987384.8	100.0106	100.0106	
	10	1987425				
	10	1988195				
150%	15	2989367	2992493.4	100.3936	100.3936	
	15	2991556				
	15	2996557				

Table No.5 Results of accuracy of Dapagliflozin

Precision

Repeatability

The values obtained for inter day Repeatability were mentioned below in Table.no:6

S.No	**Injection**	**Peak Name**	**R_t**	**Area**	**Height**
1	Injection-1	**Dapagliflozin**	**2.586**	**2010800**	346322
2	Injection-2	**Dapagliflozin**	**2.588**	**2002956**	340800
3	Injection-3	**Dapagliflozin**	**2.590**	**2012800**	**346911**
4	Injection-4	**Dapagliflozin**	**2.590**	**2005243**	**344089**
5	Injection-5	**Dapagliflozin**	**2.591**	**2011092**	**345720**
Average				2008578.1	
Standard Deviation				4237	
%RSD				0.2	

Table. No.6 Results of Precision (Repeatability)

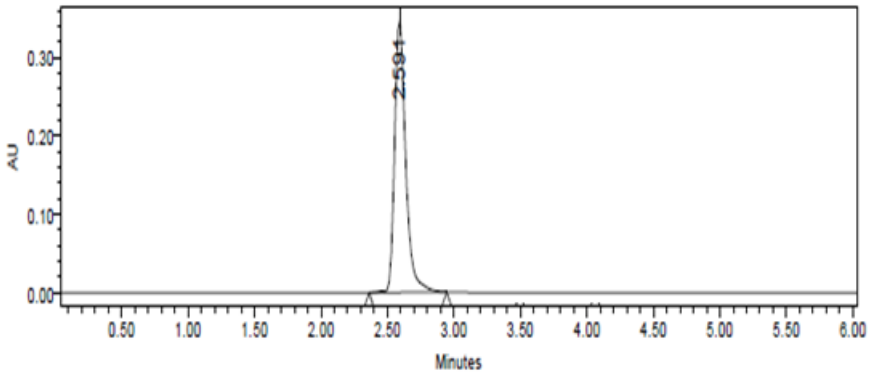

Fig:7 Chromatogram of precision (Repeatability)

Remarks: the % RSD values were 0.2 and these values are within the limits.

Intermediate precision

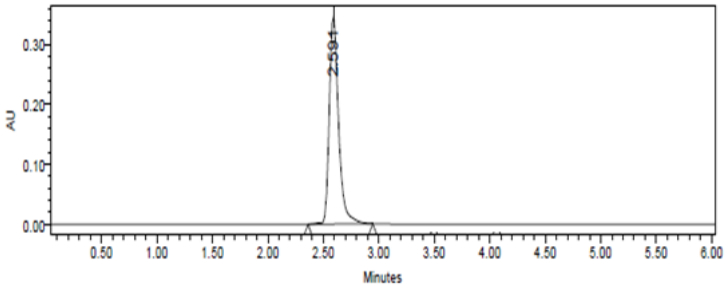

Fig.No.8. Chromatogram showing intermediate precision injection

Injection	**Area**
Injection-1	2005053
Injection-2	2007362
Injection-3	2007473
Injection-4	2009153
Injection-5	2012800
Average	2008368.1
Standard Deviation	2874.8
%RSD	**0.1**

Table. No.7 Results of Intermediate Precision

Remarks: the % RSD value was 0.1 and these values are within the limits.

Robustness:

The values obtained for Robustness, when flowrate was changed ±0.2 ml. was mentioned below

S.No	**Flow Rate (ml/min)**	**System Suitability Results**	
		USP Plate Count	**USP Tailing**
1	0.8	5752	1.4
2	1.0	5026.5	1.3
3	1.2	4476	

Table No.8: Results of Flow rate variation

S. No	**Change in organic composition in the mobile phase**	**System suitability results**	
		USP Plate Count	**USP Tailing**
1	5 % less	6498	1.2
2	***Actual**	5026.5	1.3
3	5 % more	6471	1.2

Table.No.9. Organic phase results for Dapagliflozin

Discussion: from different conditions and the values were within the limit, sorobustness was passed

LIMIT OF DETECTION (LOD) AND LIMIT OF QUANTIFICATION (LOQ)

LOD concentration	LOQ concentration
0.001 µg/ml	0.004 µg/ml

Table. No 10: Concentrations of LOD&LOQ

Remarks: The concentration of LOD and LOQ were calculated by using standard deviation and slope.

Assay

Assay of Dapagliflozin

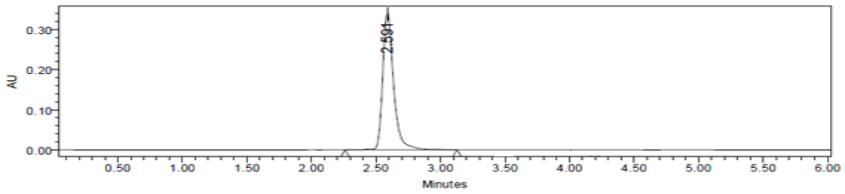

Fig.9: Chromatogram of Assay

Discussion: The percentage assay of Dapagliflozin was found to be 99.77%

S. No	Parameter	Requirement	Acceptance criteria	
			Dapagliflozin	
1	**System suitability**	R_t	2.589	
2		Tailing factor	1.3	NMT 2
3		Resolution		NLT 2
4		Plate count	5167	NLT 2000
5		Assay value	99.77%	100 ± 2.0%
6	**Accuracy**	% recovery	100.59%	100 ± 2.0%
7	**Precision**	%RSD	0.2	NMT 2%
8	**Intermediate precision**	%RSD	0.1	NMT 2%
9	**Linearity**	Correlation coefficient	0.999	NLT 0.999
10..	**LOD**		0.001	LOQ is three times more than LOQ
11	**LOQ**		0.004	
12	**Robustness**	More flow	R_t=2.168	Robust even by change in the flow rate ±0.2ml/min
		Less flow	R_t=3.215	
		More organic	R_t=2.572	Robust even by change in the mobile phase ±5%.
		Less organic	R_t=2.618	

Table:11 Summary of developed and validated method results

Conclusion

The proposed RP-HPLC method avoids the requirement for organic solvents by using a hydrotropic solution as the mobile phase, avoiding difficulties like volatility, pollution & cost. Furthermore, by using hydrotropic solvents as the mobile phase, the majority of the organic solvents in the analysis can be reduced. The Design of Experiment was carried out by using the Box-Behnken design & the assessment of independent variables. A rapid, novel, precise cost effective & robust RP-HPLC method for estimation of Dapagliflozin in bulk and dosage form was developed. Hydrotropic solvent i.e., 0.1% urea: Methanol of P^H 2.5 (35:65) was found to be a more deliberate factor for method optimization, according to response surface plots. The use of DOE approach is a flexible strategy for reducing the no. of trial experimental runs required for a method to be created in a short period of time. The proposed method was found to be rapid, accurate, precise, specific, robust, rugged and economical.

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