A New Analytical Method Development and Validation for the Estimation of Dapagliflozin by Using Reverse Phase-High Performance Liquid Chromatography

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ABSTRACT : *A new, simple, rapid, precise, accurate and reproducible RP-HPLC method for estimation of Dapagliflozin in bulk form and marketed formulation. Separation of Dapagliflozin was successfully achieved on a Develosil ODS HG-5 RP C18, 5µm, 15cmx4.6mm i.d. column in an isocratic mode of separation utilizing Methanol : Phosphate buffer (0.02M, pH-3.6) in the ratio of 45:55% v/v at a flow rate of 1.0 mL/min and the detection was carried out at 255nm. The method was validated according to ICH guidelines for linearity, sensitivity, accuracy, precision, specificity and robustness. The response was found to be linear in the drug concentration range of 12-28mcg/mL for Dapagliflozin. The correlation coefficient was found to be 0.9995 for Dapagliflozin. The LOD and LOQ for Dapagliflozin were found to be 5.004µg/mL and 15.164µg/mL respectively. The proposed method was found to be good percentage recovery for Dapagliflozin, which indicates that the proposed method is highly accurate. The specificity of the method shows good correlation between retention times of standard solution with the sample solution. Therefore, the proposed method specifically determines the analyte in the sample without interference from excipients of pharmaceutical dosage forms.*

Keywords: Dapagliflozin, RP-HPLC, Accuracy, Precision, Robustness, ICH Guidelines.

I.INTRODUCTION

Dapagliflozin[1] is a sodium-glucose cotransporter 2 inhibitor indicated for managing diabetes mellitus type 2. When combined with diet and exercise in adults, Dapagliflozin helps to improve glycemic control by inhibiting glucose resorption in the proximal tubule of the nephron and causing glycosuria. Dapagliflozin was approved by the FDA on Jan 08, 2014. Dapagliflozin is a C-glycosyl comprising beta-D-glucose in which the anomeric hydroxy group is replaced by a 4-chloro-3-(4-ethoxybenzyl) phenyl group. Used (in the form of its propanediol monohydrate) to improve glycemic control, along with diet and exercise, in adults with type 2 diabetes. It has a role as a hypoglycemic agent and a sodium-glucose transport protein subtype 2 inhibitor. It is a C-glycosyl compound, an organochlorine compound and aromatic ether. Dapagliflozin[2] is a selective sodium-glucose co-transporter subtype 2 (SGLT2) inhibitor with antihyperglycemic activity. Dapagliflozin selectively and potently inhibits SGLT2 compared to SGLT1, which is the cotransporter of glucose in the gut. Dapagliflozin inhibits the sodium-glucose contransporter 2(SGLT2) which is primarily located in the proximal tubule of the nephron. SGLT2 facilitates 90% of glucose resorption in the kidneys and so its inhibition allows for glucose to be excreted in the urine. This excretion allows for better glycemic control and potentially weight loss in patients with type 2 diabetes mellitus. Dapagliflozin[3] inhibits the sodium-glucose contransporter 2(SGLT2) which is primarily located in the proximal tubule of the nephron1. SGLT2 facilitates 90% of glucose resorption in the kidneys and so its inhibition allows for glucose to be excreted in the urine. This excretion allows for better glycemic control and potentially weight loss in patients with type 2 diabetes mellitus. The IUPAC Name of Dapagliflozin is (2S, 3R, 4R, 5S, 6R)-2-[4-chloro-3-[(4-ethoxyphenyl) methyl] phenyl]-6-(hydroxy methyl) oxane-3, 4, 5-triol. The Chemical Structure of Dapagliflozin is as follows

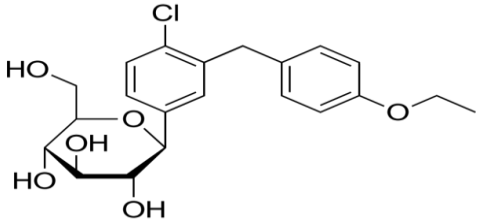

Fig.1.Chemical Structure of Dapagliflozin

II. MATERIALS AND METHODS

Instruments: HPLC with Empower2 Software with Isocratic with UV-Visible Detector (Waters), lambda max can be determined by T60-LAB INDIA UV – Vis spectrophotometer, Electronic Balance (SHIMADZU ATY224), Ultra Sonicator (Wensar wuc-2L), Thermal Oven, Symmetry ODS RP C18,5µm, 15mm x 4.6mm i.d. Column, PH Analyzer (ELICO) and Vacuum filtration kit (BOROSIL).

Chemicals/Reagents: Doubled distilled water, HPLC Grade Water, Acetonitrile, Methanol and Hydrochloric Acid, Sodium Hydroxide, Ethanol and Octanol all are 99.9% obtained from Sd fine-Chem ltd; Mumbai and Dapagliflozin was provided as a gift sample by Syncorp Clincare Technologies Pvt Ltd. Hyderabad.

Method Development

Wavelength Detection (Or) Selection of Wavelength:

The detection wavelength[4] was selected by dissolving the drug in mobile phase to get a concentration of 10µg/ml for individual and mixed standards. The resulting solution was scanned in U.V range[5] from 200-400nm.

Preparation of phosphate buffer

6.8 grams of Potassium dihydrogen orthophosphate was weighed and transferred into a 1000 ml beaker, dissolved and diluted to 1000 ml with HPLC grade water and pH was adjusted to 3.6 with Orthophosphoric acid.

Preparation of mobile phase

Mix a mixture of above buffer[5] 550 mL (55%) and 450 mL of methanol HPLC grade (45%) and de gas in ultrasonic water bath for 15 minutes. Filter through 0.45 µ filter under vacuum filtration[6].

Standard solution preparation

Twenty tablets were taken and the average weight was calculated as per the method prescribed in I.P. The weighed tablets were finally powdered and triturated[7] well. A quantity of powder of Dapagliflozin equivalent to 10mg were transferred to clean and dry 10 ml volumetric flask and 7 ml of HPLC grade methanol was added and the resulting solution was sonicated[8] for 15 minutes. Make up the volume up to 10 ml with same solvent. Then 1 ml of the above solution was diluted to 10 ml with HPLC grade methanol. One ml (0.1 ml) of the prepared stock solution diluted to 10 ml and was filtered through membrane filter (0.45µm) and finally sonicated to degas[9].

Sample solution preparation

10 mg of Dapagliflozin working standard was accurately weighed and transferred into a 10 ml clean dry volumetric flask. Add about 7 ml of diluents and sonicate to dissolve it completely and volume was made up to the mark with the same solvent which gave stock solution of 1000 ppm. Further pipette 1 ml of the above stock solution into a 10 ml volumetric flask was diluted up to the mark with diluents (100 ppm solution). Further 1 ml of prepared 100 ppm solution was pipetted into a 10 ml volumetric flask and was diluted up to the mark with diluents which gave 10 ppm Dapagliflozin working standard[10] solution. The solution was mixed well and filtered through 0.45µm filter.

Method Validation:

The validation[11,12] of an analytical method confirms the characteristics of the method to fulfill the requirements of the application domain. The method was validated according to the ICH guidelines[13,14] for specificity, linearity, precision, recovery, and stability.

System Suitability: A standard solution of Dapagliflozin working standard was prepared as per procedure and injected 6 times into the HPLC system. Then, the system suitability parameters were evaluated from standard chromatograms obtained. The % relative standard deviations (RSD) of retention time, tailing factor, theoretical plates, and peak areas from six replicate injections was within range and results were shown in Table 2 and 3, and the chromatogram was shown in Fig. 3.

Linearity: To demonstrate linearity of the assay method, five standard solutions with concentrations of about 12-28 ppm (Table-4) of Dapagliflozin was injected. Then, a graph was plotted between concentrations and peak area. Linearity[15] plot was shown in Fig. 4.

Accuracy: Three concentrations of 80%, 100%, and 120% were injected in a triplicate manner then % recovery[16] and % RSD were calculated and shown in Table 5.

Precision: Precision[17] was estimated by studying repeatability, intra- and interday tests by injecting 10 ppm concentration of Dapagliflozin. The results were calculated as standard deviation, relative standard deviation[18] and shown in Table 6.

Limit of detection (LOD): LOD[19] is the lowest level of concentration of analyte in the sample that can be detected, though not necessarily quantitated. It can be calculated from the below formula.

$$LOD = 3.3\ \sigma/S$$

Where, σ = Standard deviation of the response,

S = Slope of calibration curve.

Limit of quantitation (LOQ): LOQ[20] is the lowest concentration of analyte in a sample that may be determined with acceptable accuracy and precision when the required procedure is applied. It can be calculated from the below formula.

$$LOQ = 10\ \sigma/S$$

Where, σ = Standard deviation of the response,
S = Slope of calibration curve.

Robustness[21]: It is the capacity of the method to remain unaffected by small but deliberate variations in method parameters. The analysis was performed by slightly changing the temperature, mobile phase composition and flow rate. The results were calculated as % RSD and were given in Table 8.

Assay: – Assay[22,23] refers to chromatography based purity assay where a compound of unknown activity or purity is compared to a reference standard with precisely determined bioactivity or purity. The Assay results were shown in table-9.

$$\text{Assay} = \frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \text{Average weight} = \text{mg/tab}$$

Where:

AT = Test Preparation Peak Area
AS = Standard preparation Peak Area
WS = Working standard weight taken in mg
WT = Sample weight taken in mg
DS = Standard solution dilution
DT = Sample solution dilution
P = Working standard percentage purity

III. RESULTS AND DISCUSSION

Wavelength Detection (Or) Selection of Wavelength: The UV spectrum[24] of Dapagliflozin was obtained and the Dapagliflozin showed absorbance's maxima[25] at 255nm. The UV spectra of drug are follows:

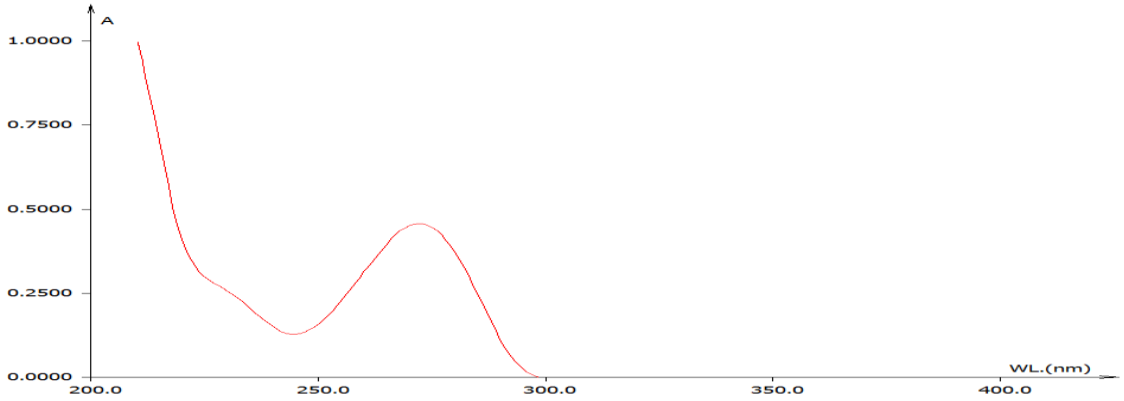

Fig.2.UV Spectrum of Dapagliflozin

Method Development:

Optimized Chromatographic Method:

Table-1: Optimized Chromatographic Conditions

Mobile phase	Methanol : Phosphate buffer (0.02M, pH-3.6) = 45:55
Column	Develosil ODS HG-5 RP C_{18}, 5µm, 15cmx4.6mm i.d.
Column Temperature	Ambient
Detection Wavelength	255 nm
Flow rate	1.0 ml/ min.
Run time	07 min.

Temperature of Auto sampler	Ambient
Diluent	Mobile Phase
Injection Volume	20µl
Type of Elution	Isocratic

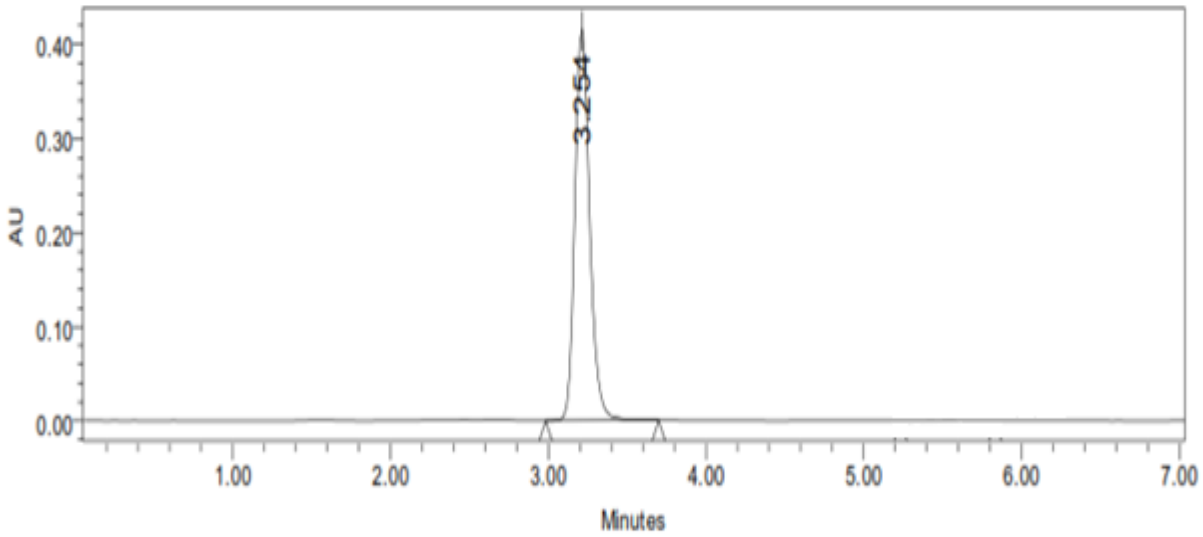

Fig.3.Chromatogram of Optimized Chromatographic Condition

Method Validation:

System Suitability: System suitability testing is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as such. Following system suitability test parameters were established. The data are shown in Table-2 and 3.

Table-2: Data of System Suitability Test

S.No.	Injection No.	RT	Area	USP Plate Count	USP Tailing
1	Injection 1	3.253	284568	7368	1.26
2	Injection 2	3.254	285684	7295	1.25
3	Injection 3	3.215	283659	7346	1.27
4	Injection 4	3.297	284754	7394	1.29
5	Injection 5	3.253	283695	7425	1.25
6	Injection 6	3.213	284578	7385	1.27
Mean			**284489.7**	**7368.833**	**1.265**
S.D			**752.5617**		
%RSD			**0.26453**		

Table-3: System suitability results for Dapagliflozin (Flow rate)

S.No.	Parameter	Limit	Result
1	Asymmetry	T ≤ 2	Dapagliflozin = 0.12
2	Theoretical plate	N > 2000	Dapagliflozin = 7258
3	Tailing Factor	(Tf) < 2	Dapagliflozin = 1.25

Linearity: To evaluate the linearity, serial dilution of analyte were prepared from the stock solution was diluted with mobile phase to get a series of concentration ranging from 0-28µg/ml for Dapagliflozin. The prepared solutions were filtered through Whatman filter paper (No.41). From these solutions, 20µl injections of each concentration were injected into the HPLC system and chromatographed under the optimized conditions. Calibration curve was constructed by plotting the mean peak area (Y-axis) against the concentration (X-axis).

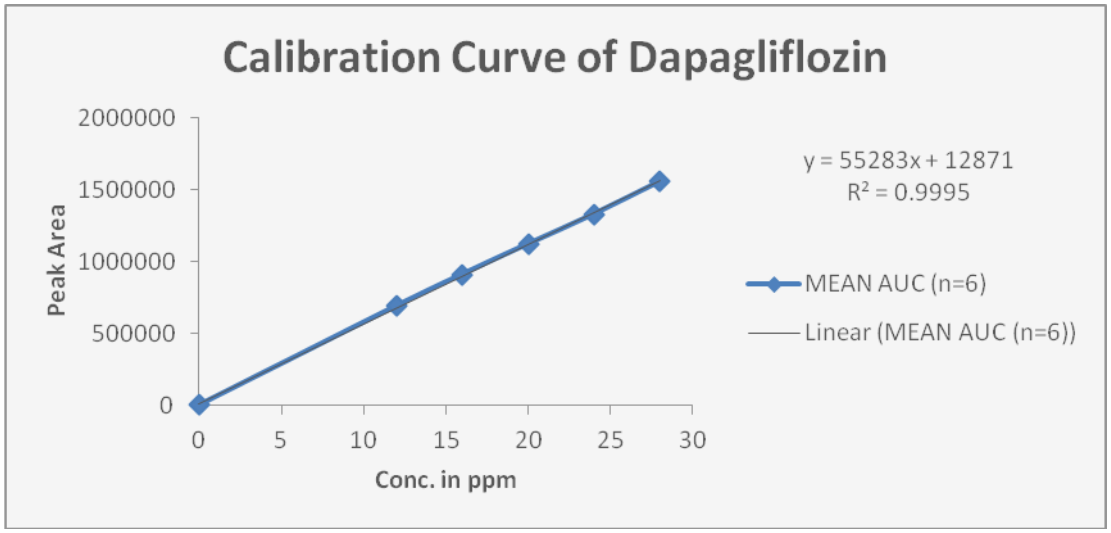

Fig.4. Standard curve for Dapagliflozin

Observation: Linearity range was found to be 0-28µg/ml for Dapagliflozin. The correlation coefficient was found to be 0.9995, the slope was found to be 55283 and intercept was found to be 12871 for Dapagliflozin.

Table-4: Linearity Range of Dapagliflozin

CONC.(µg/ml)	MEAN AUC (n=6)
0	0
12	690316
16	910621
20	1121057
24	1328903
28	1554666

Accuracy:

Table-5: Accuracy results of Dapagliflozin

Sample ID	Concentration (µg/ml)			%Recovery of Pure drug	Statistical Analysis
	Conc. Found	Conc. Recovered	Peak Area		
S_1 : 80 %	8	8.064107	458679	99.867	Mean= 100.4113% S.D. = 0.473694346 % R.S.D.= 0.471753
S_2 : 80 %	8	7.843532	446485	100.637	
S_3 : 80 %	8	8.19449	465887	100.73	
S_4 : 100 %	10	9.892661	559767	99.41	Mean= 100.6646667% S.D. = 1.166369295 R.S.D.= 1.158667
S_5 : 100 %	10	9.978655	564521	100.868	
S_6 : 100 %	10	10.19623	576549	101.716	
S_7 : 120 %	12	11.85907	668476	99.878	Mean= 100.4637% S.D. = 0.51154309 % R.S.D. = 0.509181
S_8 : 120 %	12	12.16785	685546	100.69	
S_9 : 120 %	12	12.18644	686574	100.823	

Observation: The mean recoveries were found to be 100.411, 100.664 and 100.463% for Dapagliflozin. The limit for mean % recovery is 98-102% and as both the values are within the limit, hence it can be said that the proposed method was accurate.

Precision: The precision of each method was ascertained separately from the peak areas obtained by actual determination of six replicates of a fixed amount of drug Dapagliflozin. The percent relative standard deviations were calculated for Dapagliflozin are presented in the Table-6.

i) Repeatability

Table-6: Repeatability Results of Dapagliflozin

HPLC Injection Replicates	AUC for Dapagliflozin
Replicate – 1	285479
Replicate – 2	284571
Replicate – 3	286954
Replicate – 4	283261
Replicate – 5	285964
Replicate – 6	284259
Average	**285081.3**
Standard Deviation	**1318.666**
% RSD	**0.462558**

Observation: The repeatability study which was conducted on the solution having the concentration of about 20µg/ml for Dapagliflozin (n =6) showed a RSD of 0.462558% for Dapagliflozin. It was concluded that the analytical technique showed good repeatability.

ii) Intermediate Precision / Ruggedness

Table-7: Ruggedness Results for Dapagliflozin

Conc. of Dapagliflozin (API) (µg/ml)	Observed Conc. of Dapagliflozin (µg/ml) by the proposed method			
	Intra-Day		Inter-Day	
	Mean (n=3)	% RSD	Mean (n=3)	% RSD
8	8.21	0.76	8.23	0.46
10	10.37	0.33	10.36	0.57
12	12.56	0.23	12.56	0.75

Observation: Intraday and interday studies show that the mean RSD (%) was found to be within acceptance limit (≤2%), so it was concluded that there was no significant difference for the assay, which was tested within day and between days. Hence, method at selected wavelength was found to be precise.

Robustness: Robustness is defined as the capacity of that method to be unaffected by even small deliberate changes that occur in the method parameters. The evaluation of robustness of a method is done by varying the

chromatographic parameters such as pH, temperature, flow rate, mobile phase proportions change, ionic strength etc., and determining any possible effect on the results obtained by that method.

Table-8: Result of Method Robustness Test for Dapagliflozin

Change in parameter	% RSD
Flow (0.8 ml/min)	0.554
Flow (1.2 ml/min)	0.867
More Organic	0.886
Less Organic	0.817
Wavelength of Detection (257 nm)	0.813
Wavelength of detection (253 nm)	0.794

Observation: Influence of small changes in chromatographic conditions such as change in flow rate (± 0.1ml/min), Temperature (±2^{0}C), Wavelength of detection (±2nm) & organic phase (±5%) studied to determine the robustness of the method are also in favour of (Table-38, % RSD < 2%) the developed RP-HPLC method for the analysis of Dapagliflozin (API).

LOD: The limit of detection (LOD) is the lowest concentration of analyte in a sample which can be detected, but not quantitated. LOD is a limit test that specifies whether an analyte is above or below a certain value.

L.O.D. = 3.3 (SD/S).

Where, SD = Standard deviation of the response

S = Slope of the calibration curve

Observation: The LOD was found to be 5.004µg/ml for Dapagliflozin.

LOQ: The Limit of Quantitation (LOQ) is defined as the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated operational conditions of the method.

L.O.Q. = 10 (SD/S)

Where, SD = Standard deviation of the response

S = Slope of the calibration curve

Observation: The LOQ was found to be 15.164µg/ml for Dapagliflozin.

Assay: – Assay refers to chromatography based purity assay where a compound of unknown activity or purity is compared to a reference standard with precisely determined bioactivity or purity.

Table-9: Recovery Data for estimation Dapagliflozin in Oxra

Brand name of Dapagliflozin	Labelled amount of Drug (mg)	Mean (± SD) amount (mg) found by the proposed method (n=6)	Assay % (± SD)
Oxra 10mg Tablet (UCB India Pvt Ltd)	10mg	9.893 (± 0.368)	99.698 (± 0.476)

Result & Discussion: The amount of drug in Armotraz Tablet was found to be 9.893 (±0.368) mg/tab for Dapagliflozin & % Purity was 99.698 (± 0.476) %.

IV. SUMMARY AND CONCLUSION

Isocratic elution is simple, requires only one pump & flat baseline separation for easy and reproducible results. So, it was preferred for the current study over gradient elution.

In case of RP-HPLC various columns are available, but here Develosil ODS HG-5 RP C_{18}, 5µm, 15cmx4.6mm i.d. column was preferred because using this column peak shape, resolution and absorbance were good.

Detection wavelength was selected after scanning the standard solution of drug over 200 to 400nm. From the U.V spectrum of Dapagliflozin it is evident that most of the HPLC work can be accomplished in the wavelength range of 255 nm conveniently. Further, a flow rate of 1 ml/min & an injection volume of 20µl were found to be the best analysis.

A sensitive & selective RP-HPLC method has been developed & validated for the analysis of Dapagliflozin API.

Further the proposed RP-HPLC method has excellent sensitivity, precision and reproducibility.
The result shows the developed method is yet another suitable method for assay, purity which can help in the analysis of Dapagliflozin in different formulations.

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