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ANALYTICAL METHOD DEVELOPMENT AND VERIFICATION OF DAPAGLIFLOZIN RELATED SUBSTANCES ESTIMATION IN DAPAGLIFLOZIN TABLETS BY RP-HPLC

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ABSTRACT

The objective of the research is to develop a simple, sensitive, accurate, precise, and linear Reverse Phase High Performance Liquid Chromatographic (RP-HPLC) Related Substances (RS) method for the quantification of Dapagliflozin related substances (degradants) and verify in Dapagliflozin tablets. The optimized method uses a reverse phase Agilent poroshell 120 EC- C18 column, (250 X 4.6 mm; 2.7µm), part number 690975-902, mobile phase-A consisting of 0.05% OPA buffer: Methanol in the proportion of 90:10 v/v, mobile phase-B consisting of water: acetonitrile in the proportion of 30:70 v/v, flow rate of 0.9 ml/min, injection volume of 5 µL, and detection wavelength of 225 nm. The developed method eluted Dapagliflozin at about 10 min by gradient elution mode. Dapagliflozin (unknown impurity) exhibited linearity in the range LOQ of 0.05% (0.25 µg/ml) to 150% (1.6µg/ml) considering the unknown impurity specification limit as 0.2%. The precision is exemplified by relative standard deviation of 2.3% and 2.5% for single maximum known impurity and total impurities. Percentage individual recovery was found to be in the range of 98.86 and 101.96 during accuracy studies.

Specificity established non-interference of blank, placebo, known impurities with the degradants and Dapagliflozin peak along with the resolution between the known impurities and the degradants. Peak purity passed for Dapagliflozin peak in diluted standard, as such sample, spiked sample and forced degradation sample, indicating that method developed is a stability-indicating and hence this method can be explored for the routine analysis of Dapagliflozin related substances quantification in Dapagliflozin tablets in various pharmaceutical industries.

KEYWORDS: HPLC, Dapagliflozin Tablets, Related substances, Impurities, Degradants.

1. INTRODUCTION

Dapagliflozin propanediol mono hydrate (**Figure 1**) is an anti-diabetic drug that prevents glucose reabsorption in the kidney. IUPAC name of Dapagliflozin propanediol monohydrate is(2S,3R,4R,5S,6R)-2-[4-Chloro-3-(4-ethoxybenzyl)phenyl)phenyl]-6-(hydroxymethyl) tetra hydro-2*H*-pyran-3,4,5-triol, (2*S*)-1,2-propanediol, monohydrate, having the molecular formula of $C_{21}H_{25}ClO_6$. $C_3H_8O_2$. H_2O and molecular weight of 502.98 g/mol. Dapagliflozin acts by inhibiting selectively human Sodium-Glucose Co-Transporter (SGLT2), the major transporter responsible for glucose reabsorption. Literature survey indicates that Dapagliflozin has been estimated quantitatively from bulk API and pharmaceutical dosage forms.[1-2] Related substances methods by HPLC are reported for bulk API and very few in tablet dosage forms.[3-7] Here, this article talks about the development and verification of a simple, sensitive, specific, accurate, precise and linear RP-HPLC method for the quantification of Dapagliflozin related substances (**Figures 2-6**) in Dapagliflozin tablets. Impurities mentioned in figures 2-5 are reported as process related and not degradants and hence, specificity is proved for all the process related impurities, while accuracy, precision, linearity is proved for the unknown impurity.

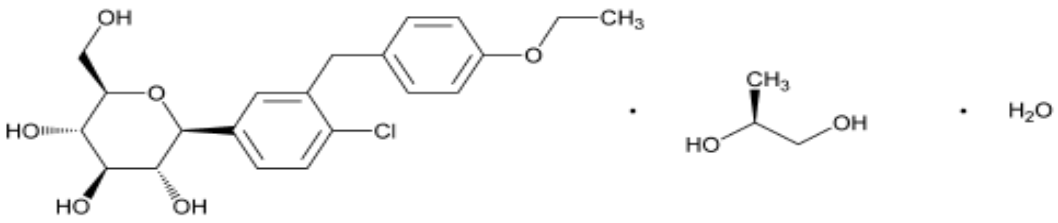

Figure 1: Structure of Dapagliflozin Propanediol monohydrate.

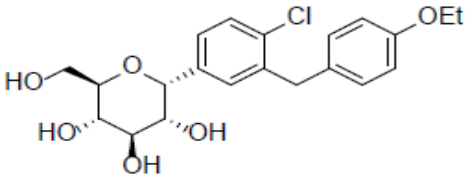

Figure 2: Structure of Dapagliflozin Impurity 1.

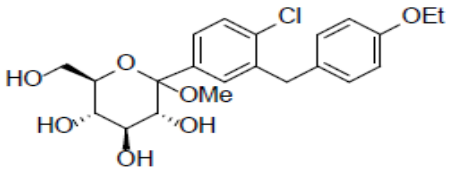

Figure 3: Structure of Dapagliflozin Impurity 2.

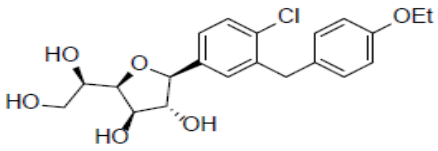

Figure 4: Structure of Dapagliflozin Impurity 3.

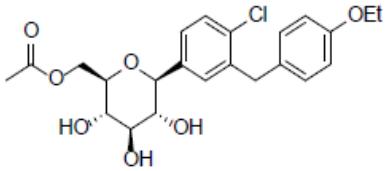

Figure 5: Structure of Dapagliflozin Impurity 4.

2. MATERIALS AND METHODS

2.1 Chemicals and reagents

An analytically pure sample of Dapagliflozin propanediol monohydrate and their related impurities with purities greater than 90% were used for the study, and the tablet formulation was prepared in our Formulation R&D laboratory, with a label claim of 10mg of Dapagliflozin. Acetonitrile (HPLC grade of Standard make), Methanol (HPLC grade of Standard make), orthophosphoric acid (OPA) (Finar HPLC grade) and water (Milli Q) were used for the analysis.

2.2 Instrument

HPLC analysis was performed on Agilent and Waters makes HPLC's having UV/PDA detector capable of setting detection wavelength of 225 nm. A reverse phase Agilent

Poroshell 120 EC- C18 column, (250 X 4.6 mm; 2.7µm), part number 690975-902 is finalized for the method. The HPLC system was controlled with "EMPOWER" software. An electronic analytical weighing balance (0.1mg sensitivity, Sartorius make, ME5 model), Sonicator (Hwashin Make, Power sonic 420 model), Water purification system (Merck make, Milli-Q IQ 7000 model) and Centrifuge (Eppendorf make, 5810 model) were used for the analysis.

2.3 Selection of wavelength

Suitable wavelength for the HPLC analysis for Dapagliflozin and their impurities was determined by recording UV spectrum in the range of 200-400 nm for Dapagliflozin and the related impurities. Suitable wavelength selected was 225 nm considering the maximum absorbance for Dapagliflozin and their impurities (**Figure 6-10**).

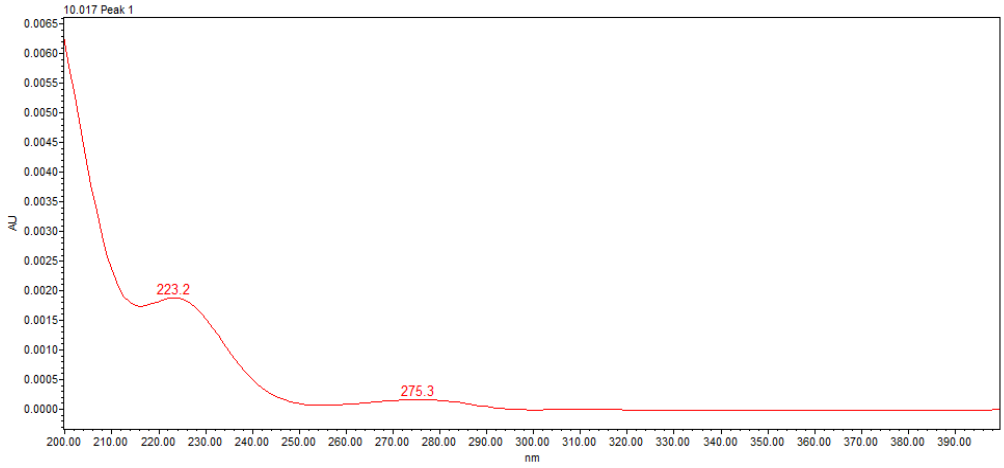

Figure 6: UV spectrum of Dapagliflozin.

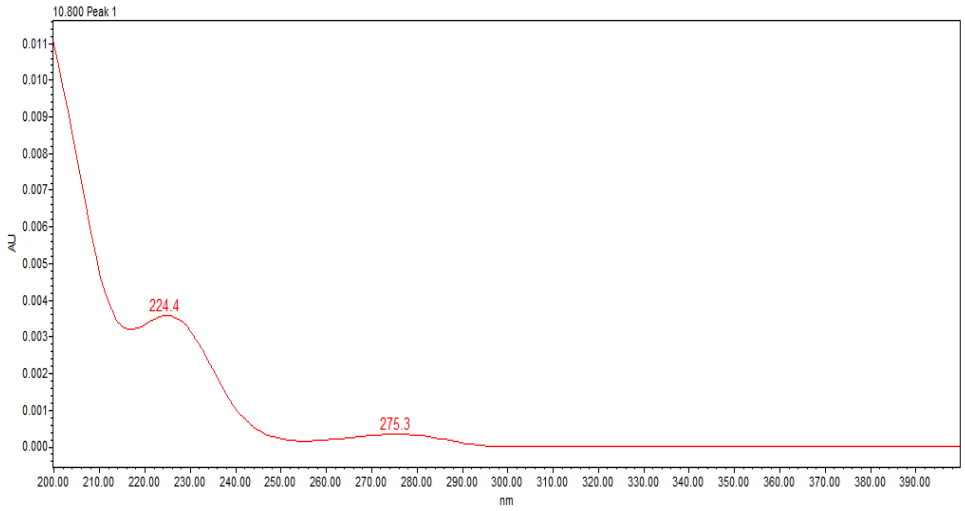

Figure 7: UV spectrum of Dapagliflozin Impurity 1.

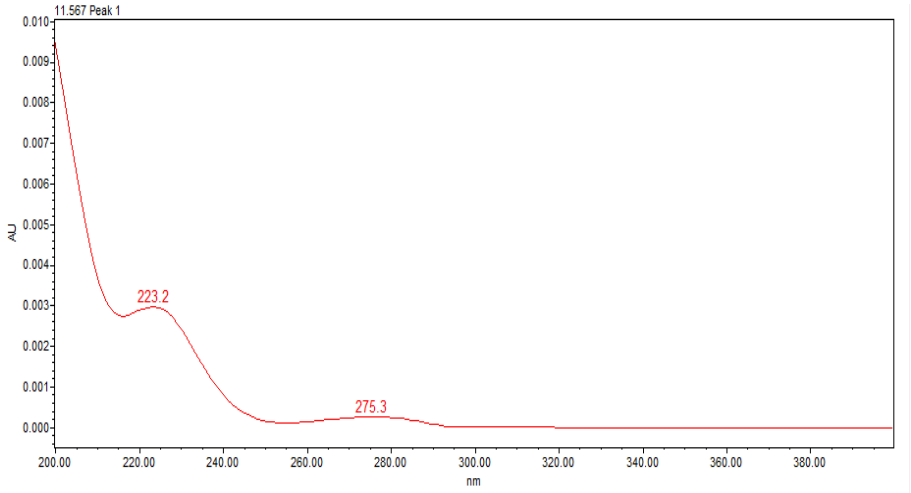

Figure 8: UV spectrum of Dapagliflozin Impurity 2.

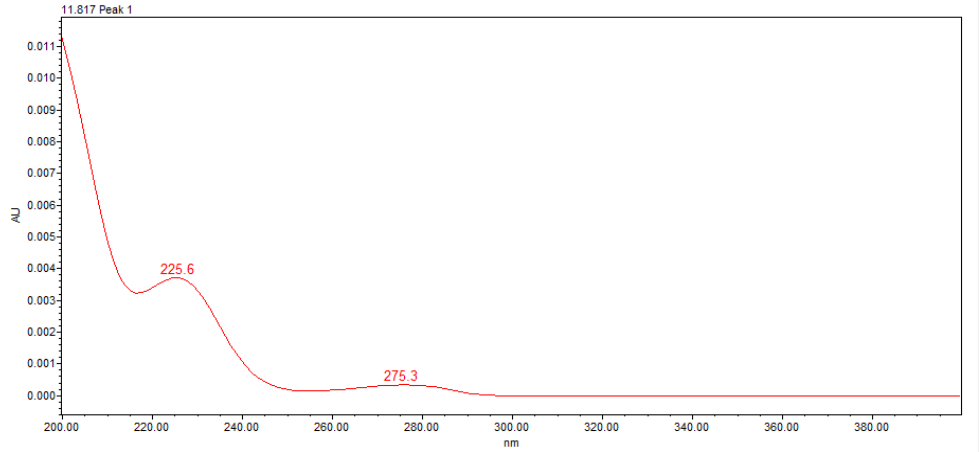

Figure 9: UV spectrum of Dapagliflozin Impurity 3.

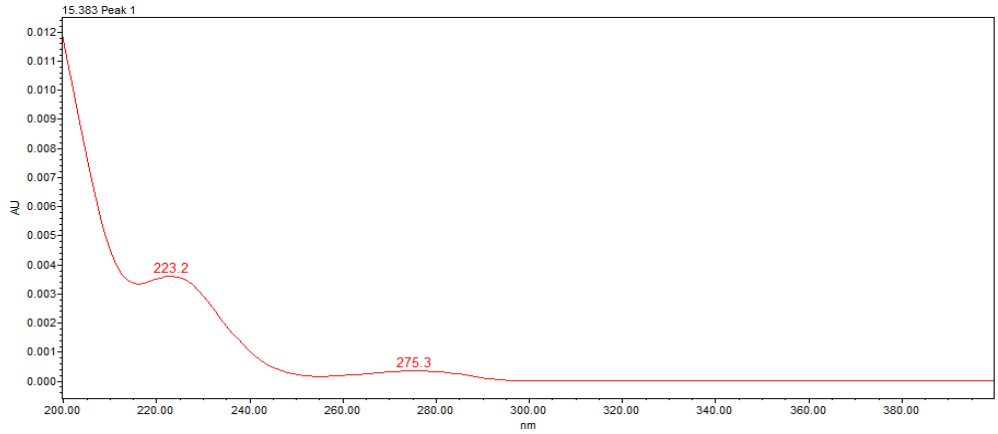

Figure 10: UV spectrum of Dapagliflozin impurity 4.

2.4 Chromatographic conditions

The developed method uses a reverse phase Agilent poroshell 120 EC- C18 column, (250 X 4.6 mm; 2.7µm), part number 690975-902, mobile phase-A consisting of 0.05% OPA:

Methanol in the proportion of 90:10 v/v, mobile phase-B consisting of Water : Acetonitrile in the proportion of 30:70 v/v, flow rate of 0.9 ml/min, injection volume of 5 µL, detection wavelength of 225 nm using a UV/PDA detector, setting column temperature and sample compartment temperature of 40°C and 5°C respectively and run time as 35 minutes for gradient program.

Time	Mobile phase-A	Mobile phase-B
0.0	50	50
1.0	50	50
10.0	35	65
20.0	10	90
25.0	10	90
25.10	50	50
35.0	50	50

2.5 Reagents solution preparation

Mobile phase Buffer (0.05%-OPA) Preparation

Mixed 0.5 ml of Ortho-phosphoric acid in 1000 ml of water and degassed in a sonicator for 10 minutes.

Mobile phase A

Mixed 0.05% Ortho-phosphoric acid buffer and methanol in the ratio of 90:10 v/v respectively followed by degassing in a sonicator for 10 minutes.

Mobile phase B

Mixed water and acetonitrile in the ratio of 30:70 v/v respectively followed by degassing in a sonicator for 10 minutes.

Diluent Preparation

The diluent solution was prepared by mixing water and Acetonitrile in the proportion of 20:80 v/v respectively followed by degassing in a sonicator for 10 minutes.

Preparation of stock and diluted standard solution

Weigh accurately about 50 mg of Dapagliflozin propanediol standard and transfer into a clean and dried 25 ml volumetric flask. Later add 15 mL of the diluent, sonicate to dissolve. Dilute to volume with the diluent and mix well to get a standard stock concentration of about 2000µg/mL.

Further diluted 4 ml of above standard stock solution into a clean and dried 200 ml of volumetric flask, make up to the mark with diluent and mix well mix well to get a working standard concentration of about 40µg/mL.

Further diluted 6 ml of above standard stock solution (concentration of about 40µg/mL) into a clean and dried 200 ml of volumetric flask, make up to the mark with diluent and mix well to get a diluted standard concentration of about 1µg/mL, inject into HPLC.

Preparation of stock and sample solution

Drop 10 doses of tablets into a clean and dry 200 mL volumetric flask. Add 40 mL of the water and then sonicate for 10 minutes (maintain the temperature of water in sonicator between 20-25ºC) with intermittent shaking. Later add 100 ml of acetonitrile, sonicate for 20 minutes (maintain temperature of water in sonicator at 25ºC) with intermittent shaking and make up to the mark with acetonitrile and mix well to get a sample concentration of about 500µg/mL. Centrifuge a portion of the sample solution at 3000 rpm for 10 minutes. Inject the supernatant into HPLC.

3. RESULTS AND DISCUSSION

3.1 Method development and Method Verification

A Reverse phase Related Substances (RS) method by HPLC-UV/PDA detector was developed keeping in mind the system suitability parameters of the diluted standard solution *i.e.*, Tailing factor (T), % RSD from six replicate injections, Blank and Placebo interference, resolution between the adjacent peaks due to blank, placebo, impurities and Dapagliflozin along with simple extraction procedure, mobile phase gradient program optimization, diluent and run time. The optimized method developed resulted in the elution of Dapagliflozin at about 10.0 min, impurities (1-4) eluting at about 10.78, 11.56, 11.82 and 15.37 min. **Figures 11-15** represent specimen chromatograms of blank, placebo, diluted standard, sample, and spiked sample solutions. The total run time is 35 minutes. The optimized method was verified for below parameters to ensure repeatability and reproducibility of the method. Below parameters were executed to ensure method verification.

- System suitability
- Specificity
- Forced degradation studies
- Method precision

- Accuracy and
- Linearity

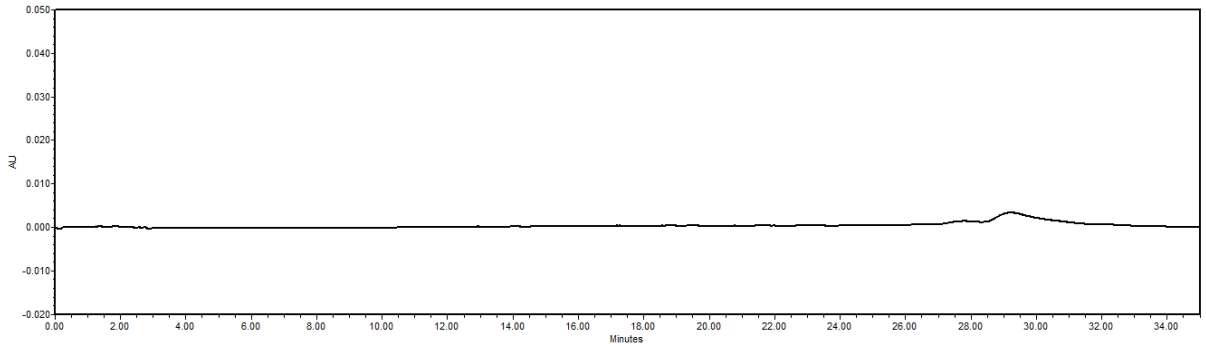

Figure 11: Typical Chromatogram of Blank Solution.

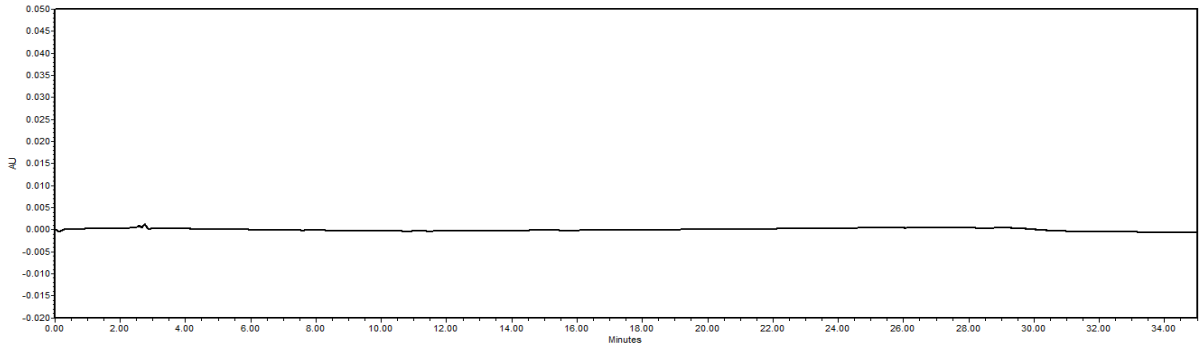

Figure 12: Typical chromatogram of the Placebo solution.

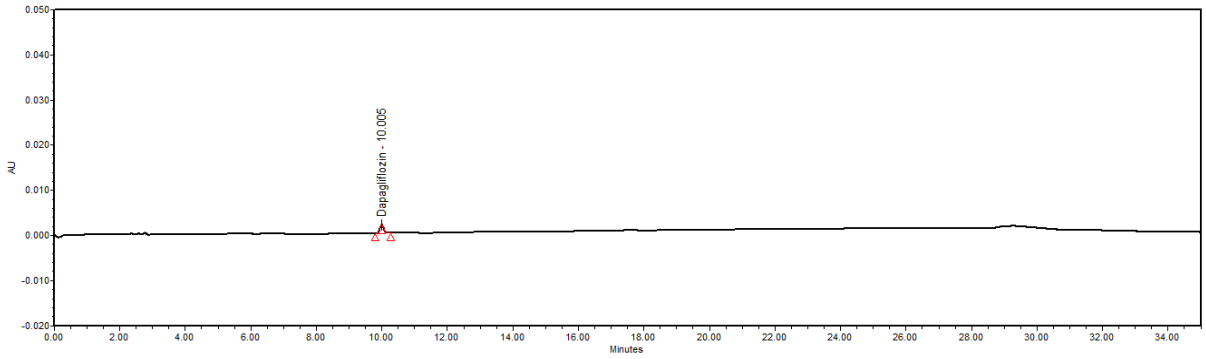

Figure 13: Typical chromatogram of the Diluted Standard solution.

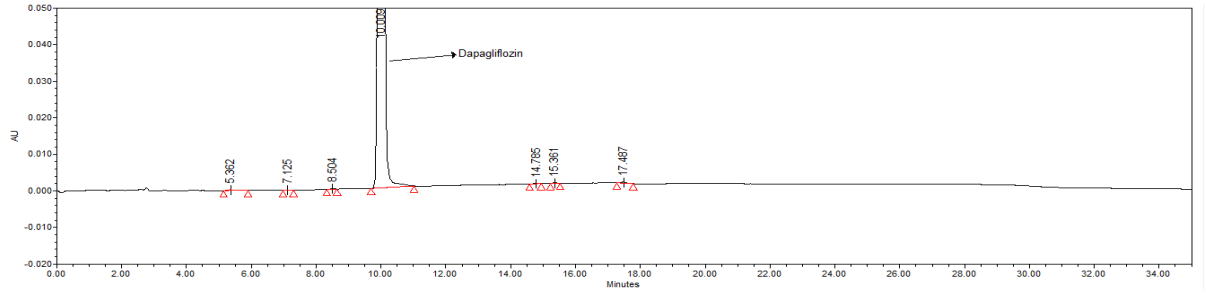

Figure 14: Typical chromatogram of the Sample solution.

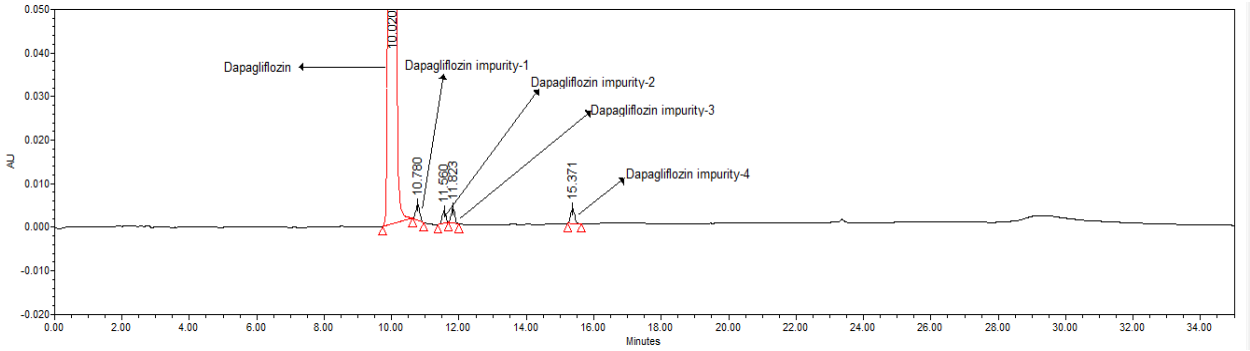

Figure 15: Typical chromatogram of the Spiked Sample solution.

3.2 System Suitability

Injected diluent as blank and diluted standard in six replicates into the chromatograph as per the finalized chromatographic conditions. Acceptance criteria are "% RSD from six replicate injections of standard solution is not more than 10.0" and "Tailing Factor of the first standard injection is not more than 2.0." Results of system suitability summarized in Table 1, met the proposed acceptance criteria, and hence can be concluded that the finalized method meets the system suitability requirements.

Table 1: Results of System Suitability.

Parameter, Acceptance criteria	Result
% RSD from six replicate injections of standard solution is not more than 10.0	0.54
Tailing Factor of the first standard injection is not more than 2.0	1.1

3.3 Specificity

Blank and Placebo Interference

To establish non-interference of the blank and placebo, injected Dapagliflozin tablets of 10mg strength along with blank, placebo, standard, and sample into HPLC. Acceptance Criteria is "No peak shall be observed in the blank and placebo chromatogram at not more than LOQ% of the average standard area of Dapagliflozin diluted standard under system suitability at the retention time of Dapagliflozin API." Results (Table 2) met the acceptance criteria and hence, can be concluded that the method meets the requirements of blank and placebo interference.

Table 2: Results of Placebo, Blank Interference.

Sample Type	% Interference
Placebo for Dapagliflozin Tablets 10 mg	Nil
Blank	Nil

Interference from Known Impurities

A study was conducted to establish the non-interference of impurities. Prepared un-spiked sample preparation, spiked sample preparation related to 10mg strength and individual known impurity and injected into HPLC. Spiked sample was prepared by spiking all the known impurities on to the sample preparation at 0.2% level. Acceptance criteria are "No interference from the known impurities should be obtained for the main analyte" and "Purity angle should be less than Purity threshold for the API peak and the peak should not have any flag in purity results table (For Waters Empower software)" in sample and spiked sample solution. Chromatogram of spiked sample had shown that the individual impurities were well separated from each other and there was no interference of these impurities with the Dapagliflozin API, along with peak purity for Dapagliflozin peak was passing in the sample and spiked sample solutions as summarized in Tables 3-4, and hence can be concluded that the method meets the requirements of interference from known Impurities.

Table 3: Results of Interference from Known Impurities.

S. No.	Name of the Analyte / Impurity Peak	RT	RRT	Resolution
1	Dapagliflozin	10.020	1.000	NA
2	Dapagliflozin Impurity-1	10.780	1.076	3.6
3	Dapagliflozin Impurity-2	11.560	1.154	3.7
4	Dapagliflozin Impurity-3	11.823	1.180	1.3
5	Dapagliflozin Impurity-4	15.371	1.534	16.8

Table 4: Peak Purity Table for Dapagliflozin.

Batch No.	Purity Angle	Purity Threshold	Peak purity	Purity flag
Standard	0.68	1.38	Pass	No
Sample (10mg)	1.03	1.65	Pass	No
Spiked Sample (10 mg)	1.75	3.35	Pass	No

Forced Degradation study

Forced degradation study was conducted to obtain degradants of about 5% to 20% in at least one stress condition from degraded samples. Sample and placebo were exposed to following stress condition to induce degradation. Degradation studies was done only in the below condition as the API was proved to degrade in the below condition.

Table 5: Forced degradation condition (Stress condition)

S.No.	Stress	Forced degradation conditions
1	Acid-Catalysis stress	5mL of 30% Peroxide and 5mL of 5N HCl;5 mins; RT

Stressed placebo and sample were injected into the HPLC system with photodiode array detector (PDA) in the test method. Acceptance criteria is " Purity angle should be less than Purity threshold for the API peak and the peak should not have any flag in purity results table (For Waters Empower software)." The chromatogram of the stressed sample was evaluated for peak purity of Dapagliflozin peak using Waters Empower 3 software. For forced degradation sample, the Purity angle was found less than Purity threshold and no purity flag was observed in stressed condition for Dapagliflozin peak. Hence, can be concluded that the method meets the requirements of Forced degradation study, and the method developed is a stability-indicating method.

Table 6: Stress Conditions and Peak Purity of Dapagliflozin in Stressed Sample.

Sample	RT	Purity Angle	Purity Threshold	Peak Purity	Purity Flag
Control sample	10.026	1.03	1.65	Pass	No
Acid Catalysis Stressed 30% Peroxide; 5 ml; 5ml of 5N HCl;5 mins; RT	10.059	0.40	0.84	Pass	No

3.4 Linearity

Linearity was established only for unknown impurity (Dapagliflozin) and not for the known impurities as the known impurities are process related and not degradants. Linearity was established for the unknown impurity (Dapagliflozin) by preparing serial dilutions ranging in the concentration from 0.2650 µg/mL to 1.6565 µg/mL (LOQ to 150% of the sample concentration). A graph was plotted using concentration in mg/mL on X-axis versus peak area on Y-axis. Calculated the correlation coefficient and Y-intercept value at 100 % response and found within the limits. Intercept, Slope value and Residual sum of squares were calculated and reported. Acceptance Criteria are "The correlation coefficient should be not less than 0.997, Y-Intercept should not be more than ±5%". Results have met the acceptance criteria, provided in below Table 7 and Figure 16. Hence, can be concluded the developed method meets the requirements of Linearity.

Table 7: Results of Linearity of unknown impurity (Dapagliflozin).

Level	Concentration (µg/mL)	Area
LOQ (0.05)	0.2650	4641
50% Level	0.6626	10870
100% Level	0.9939	16081
130% Level	1.3252	21897
150% Level	1.6565	27129
Coefficient of correlation		0.9998

Slope	16245395.8253
Intercept	192.03781
Bias at 100% response	1.19
Residual sum of squares	126334.00780

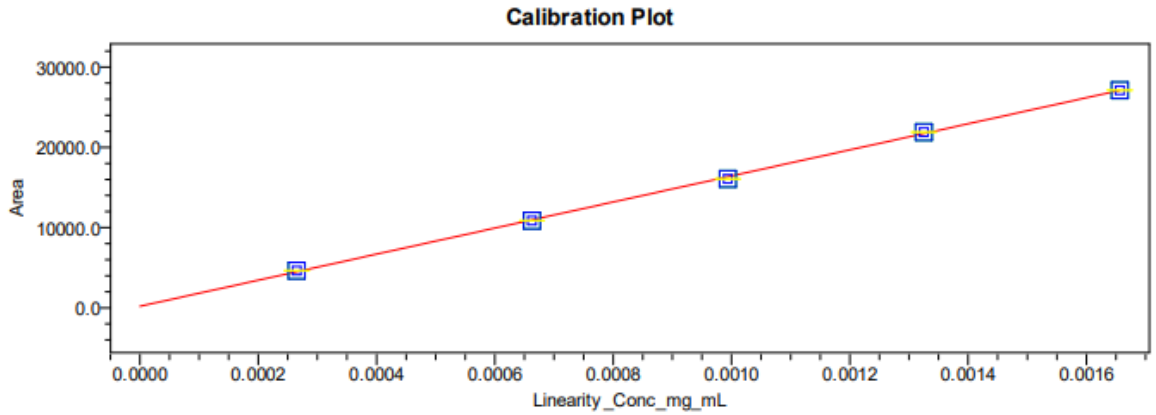

Figure 16: Linearity Plot for Dapagliflozin.

3.5 Method Precision

The precision of test method for related substances method was performed on the drug product. Prepared six sample preparations for the highest strength (10 mg) as per the test method and injected into the HPLC system. Calculated the % of single maximum unknown impurity and total impurities of each sample and it is % RSD. Acceptance Criteria are "% RSD for the single maximum unknown impurity and total impurities from the six sample preparations should be Not more than 15.0." Results met the acceptance criteria and hence, can be concluded that the method meets the precision requirements.

Table 8: Results of Method Precision for unknown Impurities and total impurities.

Sample No.	Impurity (%)	
	% Single maximum Unknown Impurity	% Total Impurities
1	0.023	0.060
2	0.023	0.062
3	0.023	0.061
4	0.022	0.059
5	0.023	0.058
6	0.022	0.059
Average	0.023	0.060
%RSD	2.3	2.5

3.6 Accuracy

For Related substances test method, accuracy studies are not applicable for the known impurities as all the known impurities are process-related and not degradants. For unknown

impurity, performed recovery study on not less than three levels, for concentration at 50% to 150% of target concentration related to Dapagliflozin unknown impurity specification (0.3%). Calculated the % recovery and mean recovery. Acceptance Criteria is "The individual % recovery for Dapagliflozin (unknown impurity) should be not less than 85.0 and not more than 115.0." The results obtained at 50% to 150% level have met the acceptance criteria. Hence, can be concluded that the method conditions are proven to be effective and accurate.

Table 9: Results of Accuracy of Dapagliflozin for Assay

S. No.	Accuracy Level	% Recovery	Mean % Recovery
1	50%	101.96	101.1
2		100.09	
3		101.37	
1	150%	99.97	99.4
2		99.27	
3		98.86	

3.7 Limit of Detection (LOD) and Limit of Quantification (LOQ)

Establishment of LOD and LOQ

LOD and LOQ were established by signal-to-noise ratio method by spiking Dapagliflozin on the diluent. Stock solutions of the Dapagliflozin was prepared in diluent and serial dilutions were done till the desired S/N ratio for the peak is achieved. Acceptance Criteria are "The signal-to-noise ratio should be more than 10 for LOQ for Dapagliflozin" and "The signal-to-noise ratio should be more than 3 for LOD for Dapagliflozin". The method conditions are proven to be effective and accurate in LOQ.

Table 10: Establishment of LOD and LOQ for Dapagliflozin (Unknown Impurity)

Name	USP S/N Ratio for LOQ	LOQ Concentration		USP S/N Ratio for LOD	LOD Concentration	
		Percent	µg/mL		Percent	µg/mL
Dapagliflozin	101.06	0.050	0.25	33.73	0.017	0.08

4. CONCLUSION

A reverse phase HPLC gradient method was developed and verified for the estimation of Dapagliflozin Related substances in dapagliflozin tablets. Related substances test method has been verified for specificity, accuracy, precision, linearity, and sensitivity and the results met the pre-determined acceptance criteria. Accordingly, the developed RS method can be

explored for the routine analysis of Dapagliflozin related substances estimation in Dapagliflozin tablets in various pharmaceutical industries.

5. ACKNOWLEDGEMENT

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