AMERICAN JOURNAL OF PHARMTECH RESEARCH

Journal home page: http://www.ajptr.com/

Validation of Stability-Indicating Reverse Phase HPLC Method for the Determination of Related Substances in Dapagliflozin Drug Substance

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ABSTRACT

A gradient reversed phase high performance liquid chromatography (RP-HPLC) method has been developed and validated for the determination for related substances of Dapagliflozin drug substance. Chromatographic separation of Dapagliflozin from its process and degradation related substances was achieved on YMC Pack Pro C18, 250mm × 4.6mm 5µ i.e A stainless steel column 250 mm long, 4.6 mm internal diameter filled with Octadecyl silane chemically bonded to porous silica particles of 5 µm diameter maintained column oven temperature at 25°C. Orthophosphoric acid buffer is mobile phase A and acetonitrile is mobile phase B. Wavelength for UV detection: 225nm, flow rate: 0.8 ml/min and Injection volume: 20µl. The developed method suitability was checked and validated as per ICH guidelines for specificity, linearity, accuracy, precision, limit of quantification, limit of detection robustness and ruggedness experiments. Dapagliflozin drug substance was subjected to stress conditions of thermal, hydrolysis, humidity, peroxide and photolytic to observe the degradation products. Limit of detection of each RS is less than 0.008%w/w indicating that the developed method is highly sensitive. The experiment results are given in detailed in this research article.

Keywords: Dapagliflozin, Related substances, HPLC, Validation

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Received 20 September 2018, Accepted 30 September 2018

Please cite this article as: Sen G *et al.*, Validation of Stability-Indicating Reverse Phase HPLC Method for the Determination of Related Substances in Dapagliflozin Drug Substance. American Journal of PharmTech Research 2018.

INTRODUCTION

Dapagliflozin is chemically known as (2*S*,3*R*,4*R*,5*S*,6*R*)-2-[3-(4-ethoxybenzyl)-4-chlorophenyl]-6-(hydroxymethyl)tetrahydro-2*H*-pyran-3,4,5-triol, molecular formula is $C_{21}H_{25}ClO_6$ and molecular weight is 408.88. Dapagliflozin was the first drug in a class of therapies that took a new approach to glycemic control in adults with type 2 diabetes (T2D). Dapagliflozin is an inhibitor of the sodium glucose cotransporter, resident in the proximal nephron, which is responsible for the recovery of filtered glucose back into circulation. Inhibiting this cotransporter reduces glucose recovery, increases glucose excretion, and reduces hyperglycemia. It works in the kidneys to prevent absorption of glucose (blood sugar). This helps lower the blood sugar level. The literature reviews discussed, the clinical evidence and rationale for the use of dapagliflozin as add-on therapy in T2DM. The clinical research results suggest that dapagliflozin add-on therapy is a promising new treatment option for a wide range of patients with T2DM. Results from an ongoing cardiovascular outcomes trial are needed to establish the long-term safety of dapagliflozin [1-3]. The recommended maximum daily dose is 10 mg orally once a day in patients tolerating therapy with lower dose and requiring additional glycemic control [4] and this dosage is approved by USFDA. Dapagliflozin is marketed under trade name FARXIGA [4], it was developed by Bristol-Myers Squibb in partnership with AstraZeneca and supplied as 5 mg and 10mg tablets for oral administration. The chemical structure of Dapagliflozin is shown in Figure 1.

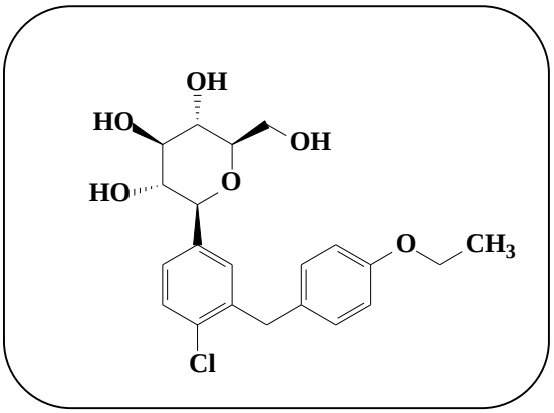
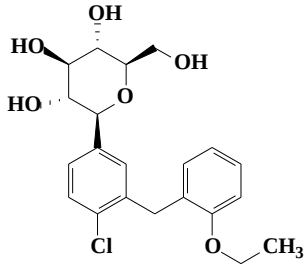
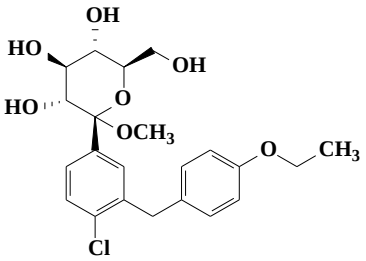
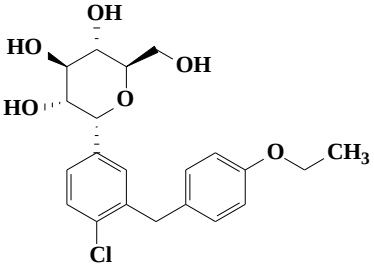


Figure 1: Chemical structure of Dapagliflozin

As per literature search, there is no pharmacopeial monograph available for this drug substance or drug product and no HPLC method is available in literature for quantification of Dapagliflozin related substances. However, some of methods have been reported in literature for the determination of Dapagliflozin in formulations and clinical studies related. In this year 2018, N. Singh and et al reported HPLC method for the estimation of dapagliflozin and sitagliptin in fixed dose combination formulations [5]. Stability indicating HPLC method has been published for API

and formulation products by Mitali V Verma and etal in 2017 [6]. Sanagapati Manasa and etal, estimated dapagliflozin by validated HPLC [7] apart from HPLC methods, some of UV methods also reported in literature [8-9]. The chemical structures of five related substances [Impurity-1 to 5] are given in Figure 2. Forced degradation studies were carried out to establish stability indicating nature of the method according with ICH stability guidelines [10]. Limit of detection (LOD), limit of quantification (LOQ) and linearity were established as per ICH guidelines. The limit of these impurities has been considered as 0.15% in accordance with ICH guideline based on maximum daily dose [4 &11]. This HPLC method well separated all of five impurities with good resolution to attain better chromatographic conditions and the optimized method has been validated in accordance with ICH guidelines [12].

Impurity	**RRT**	**Chemical Structure**
Impurity-1	1.08	
Impurity-2	1.14	
Impurity-3	1.22	

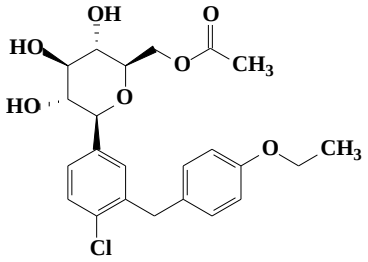
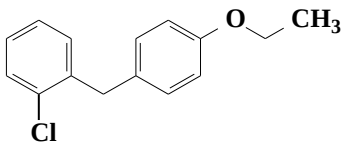
Impurity-4	1.66	
Impurity-5	2.37	

Figure 2: Chemical structures of Dapagliflozin related substances

MATERILAS AND METHOD

Chemicals, Reagents, Standards and Samples

The investigated samples of Dapagliflozin drug substance and reference sample, its related substances and Dapagliflozin for system suitability (Dapagliflozin enriched with Impurity-1) were gifted from APL Research Centre-II Laboratories (A division of Aurobindo Pharma Ltd., Hyderabad). GR grade of Orthophosphoric acid, Acetonitrile (HPLC grade) and pure milli-Q water was used with the help of millipore purification system (Millipore®, Milford, MA, USA). Hydrochloric acid (~35%), Sodium hydroxide (GR grade) and Hydrogen peroxide (~30 %) are used for forced degradation experiments under method validation.

Instrumentation, Chromatographic Conditions and Methodology

The HPLC system used for method development, method validations as well as for forced degradation studies were Waters Alliance e2695 separation module equipped with 2489 UV detector, waters 2695 separation module with 2996 PDA detector with Empower data handling system i.e Empower 3 software, [Waters Corporation, MILFORD, MA 01757, USA]. HPLC column: A stainless steel column 250 mm long, 4.6 mm internal diameter filled with Octadecyl silane chemically bonded to porous silica particles of 5 µm diameter [YMC Pack Pro C18, 5µm (250mm x 4.6mm) (Make: YMC)], column oven temperature: 25°C, mobile phase A is buffer (1 ml of Orthophosphoric acid in 1000 ml of Water. Filter through 0.45 µ or finer porosity membrane filter), mobile phase B is Acetonitrile, diluent: degassed mixture of water and acetonitrile in the ratio of 50:50 v/v. Flow rate: 0.8 ml/min, injection volume: 20µl, UV detection: 225nm, data

acquisition time: 65 min. Retention time of Dapagliflozin is at about 21 min. The pump is in gradient mode and the program is as follows:

Time (min)	***Buffer (% v/v)***	***Acetonitrile (% v/v)***
$T_{0.01}$	80	20
T_{10}	60	40
T_{25}	60	40
T_{35}	40	60
T_{50}	2	98
T_{65}	2	98
T_{67}	80	20
T_{75}	80	20

Preparation of solutions

System suitability solution

0.4 mg/ml concentration of Dapagliflozin for system suitability (Dapagliflozin enriched with Impurity -1) in diluent.

Standard solution

0.0006 mg /ml concentration of solution using Dapagliflozin standard in diluent [i.e 30 mg of Dapagliflozin working standard into a 100 ml clean, dry volumetric flask, add 70 ml of diluent and sonicate to dissolve, make up to volume with diluent. Dilute 5 ml of this solution to 100 ml with diluent. Further dilute 4 ml of this solution to 100 ml with diluent.

Sample solution

0.4 mg/ml concentration of solution using Dapagliflozin sample in diluent [40 mg of sample into a 100 ml clean, dry volumetric flask, add 70 ml of diluent and sonicate to dissolve, make up to volume with diluent.

System suitability evaluation

The column efficiency as determined from the USP resolution between Dapagliflozin and Impurity-1 is not less than 2.5 from system suitability solution and the USP plate count for Dapagliflozin peak is not less than 10000 and USP tailing for the same peak is not more than 1.5 from first injection of standard.

[**Recommendations:** Before starting the experiment, it is recommended to condition the column with complete gradient programme and it is recommended "in case of carryover is observed at the retention time of Dapagliflozin in diluent chromatogram (after injection of system suitability solution or sample solution), inject 1 or 2 diluents before standard solution.]

RESULTS AND DISCUSSION

Method validation

The developed HPLC method was established through the method validation experiments in accordance with ICH guidelines individually in terms of specificity or selectivity, forced degradation studies, LOD/LOQ, linearity, precision, accuracy, robustness and stability of solutions.

Specificity

Specificity experiment is the ability of assess unequivocally of analytic in the presence of components which may be expected to be present and prove the capability of method performance. For determination of specificity, blank, all individual related substances solutions were prepared and injected to confirm the individual retention times. The solutions of Dapagliflozin drug substance (Control Sample) and Dapagliflozin spiked with known related substances at specification level (Spiked Sample) were prepared and injected into HPLC. Peak purity was established by using Empower Software. A typical representative HPLC chromatogram of Dapagliflozin drug substance spiked with all related substances is shown in Figure 3. The specificity results are tabulated in Table 1.

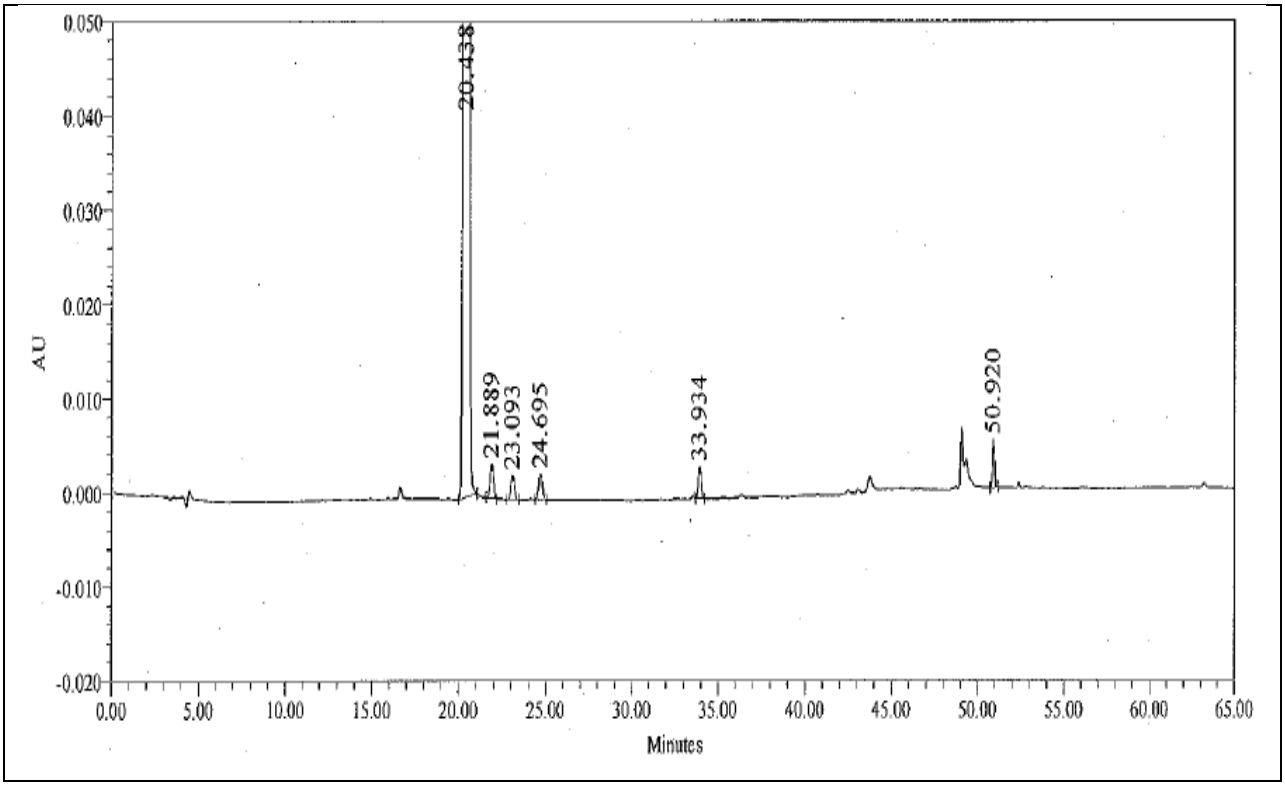


Figure 3: A typical representative HPLC chromatogram of Dapagliflozin drug substance spiked with related substances

Table 1 Specificity experiments results

Name	Retention time (min)	RRT	Peak purity	
			Purity angle	Purity threshold
Dapagliflozin	20.438	1.00	0.449	2.958
Impurity-1	21.889	1.07	0.427	0.754
Impurity-2	23.093	1.13	0.771	1.245
Impurity-3	24.695	1.21	0.800	1.192
Impurity-4	33.934	1.66	0.570	1.008
Impurity-5	50.920	2.49	0.283	0.649

Specificity experiments established that no peak was observed at the retention time of Dapagliflozin and its known related substances in the diluent chromatogram. From the specificity chromatograms of all known related substances and spiked sample indicates that the related substances are well separated from closely eluting peaks of other known related substances and from Dapagliflozin. The peak purity data of Dapagliflozin peak in control sample and spiked sample, known related substances peaks in spiked sample show that the peaks are homogeneous and have no co-eluting peaks. Based on this information, it can be concluded that the test method is specific for the determination of related substances in Dapagliflozin drug substance.

Forced degradation

The stability indicating nature of Dapagliflozin drug substance has been established by conducting forced degradation study experiments. Dapagliflozin was subjected to different stress conditions i.e acid/base hydrolysis [2.5M HCl/85°C/6 hours & 2.5M NaoH/85°C/6 hours], peroxide degradation under oxidative stress [15% H_2O_2/85°C/6 hours], thermal degradation [50°C/120 Hours], humidity degradation study [92.5% RH/25°C /120 hours)] and photolytic degradation [White fluorescent light, 1.2 million Lux hours and UV light, 200 watt hours / meter square] w.r.t ICH option 2 of Q1B [13]. Peak purity of Dapagliflozin peak was established by using PDA detector in these stress samples along with unstressed sample. The forced degradation results are tabulated in Table 2.

There was no significant change observed w.r.t all known impurities in forced degradation studies. During the evaluation of forced degradation studies, it was observed that, no unknown impurities found in experiments except peroxide degradations and Photolytic conditions. In peroxide degradation experiments, many of unknown impurities detected below 0.1%, but at RRTs 0.59, 0.73 and 0.74 higher impurity levels were found 0.35%, 0.19% and 0.20% respectively. Further, in photolytic conditions three unknown impurities were detected below 0.1%.

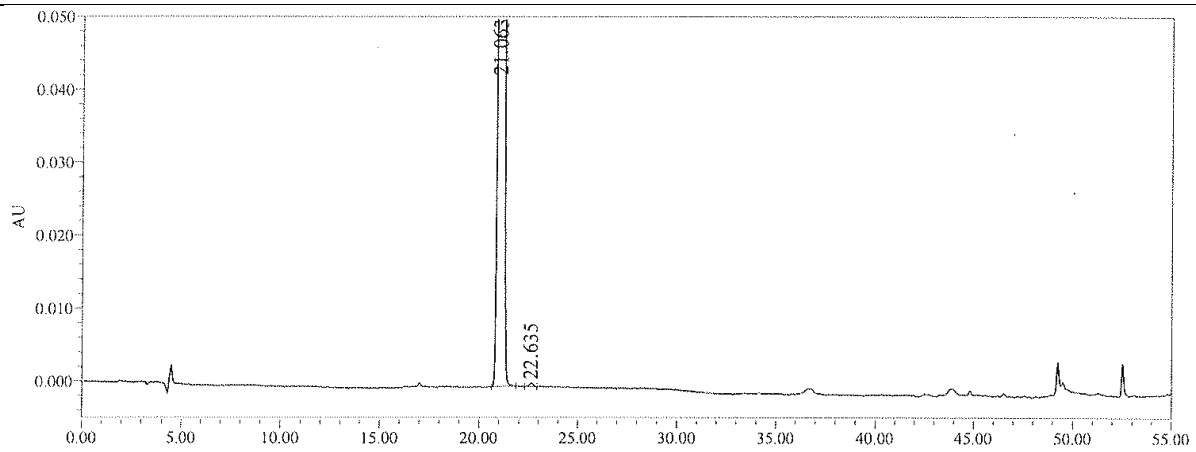
The forced degradation results of various stress conditions employed to degrade Dapagliflozin drug substance indicate that the drug substance is slightly sensitive in oxidative stress condition,

where as it is found to be stable in acidic, base hydrolysis, thermal, photolytic and humidity stress conditions.

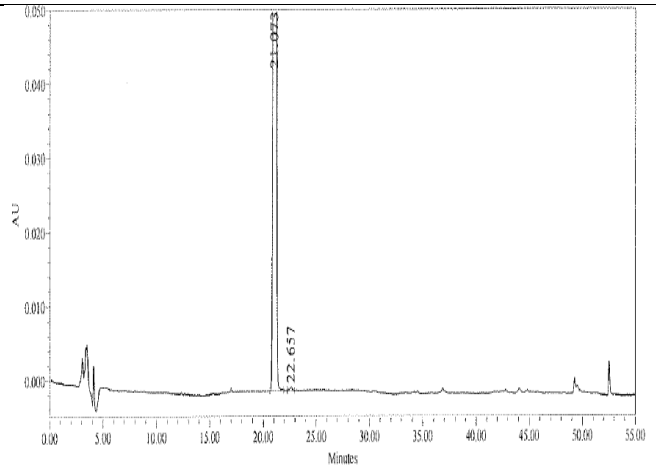
Further, the evaluation of peak purity Dapagliflozin peak from the analysis of every degradation sample showed that it is homogeneous, and there are no co-eluting peaks. Based on this information, the test method is declared to be specific and stability indicating for the specified / unspecified related substances in Dapagliflozin drug substance. The forced degradation experiments results are shown in Table 2 and typical HPLC chromatograms are shown in Fig.4.

Table 2 Dapagliflozin Forced degradation experiments data

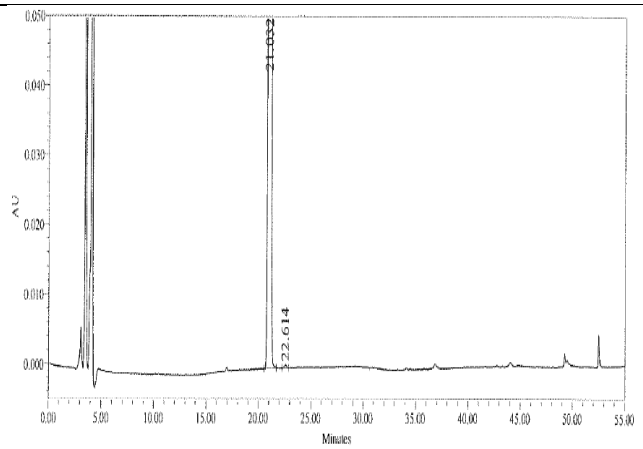
Degradation mechanism	Degradation condition	Area	Degradation(%)	Peak purity of Dapagliflozin	
				Purity angle	Purity threshold
	Undegraded sample	11262853	-	0.044	0.411
Acid	2.5M HCl/85°C/6 hours	11202554	0.5	0.052	0.426
Base	2.5M NaOH/85°C/6 hours	11145229	1.0	0.047	0.492
Peroxide	15% H_2O_2/85°C/6 hours	10817536	3.9	0.054	0.421
Thermal	50°C/120 hours	11442239	No degradation	0.052	0.463
Photolytic	White fluorescent light, 1.2 million Lux hours and UV light, 200 watt hours / meter square	11371067	No degradation	0.044	0.495
Humidity	92.5% RH/25°C /120 hours	11385152	No degradation	0.064	0.490



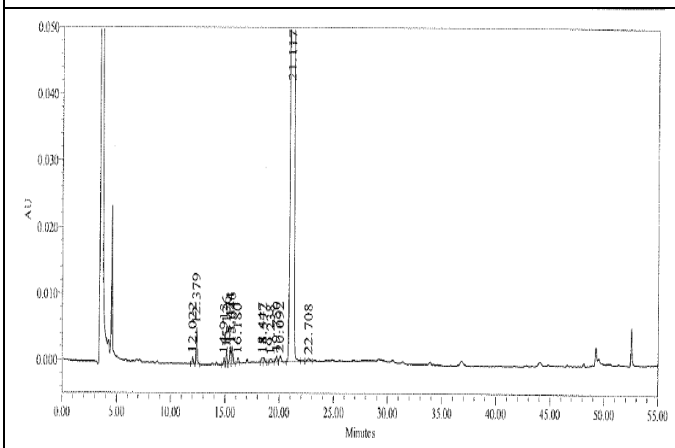
Undegraded



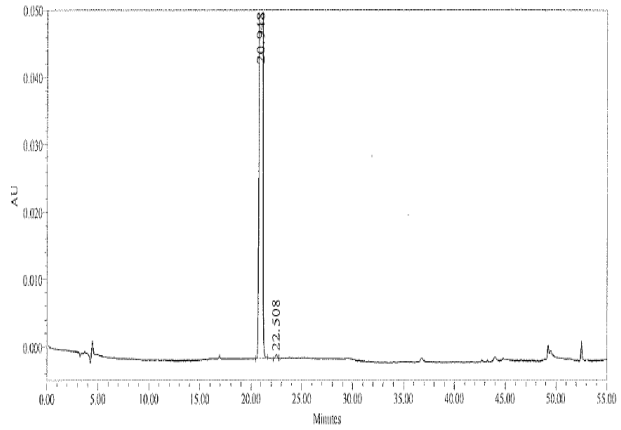
Acid



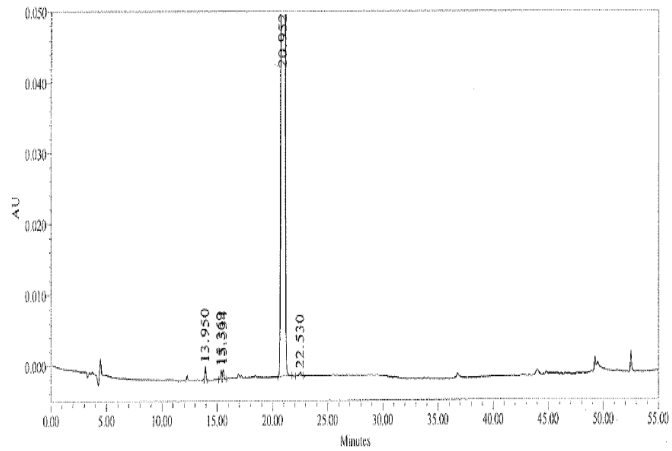
Base



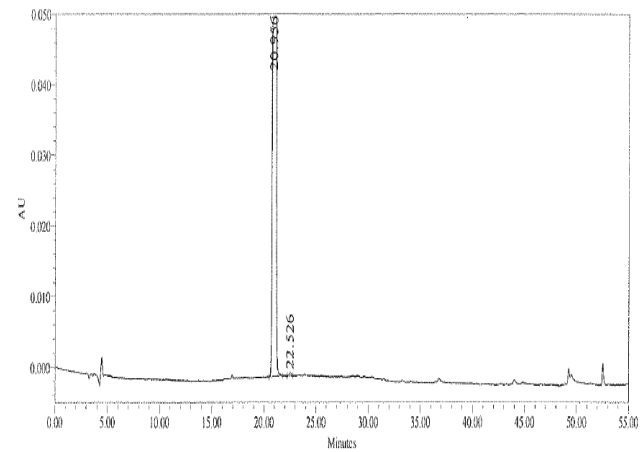
Peroxide



Thermal



Photolytic



Humidity

Figure 4: Typical representative HPLC chromatograms of Dapagliflozin drug substance - Forced degradation experiment

Limit of Detection (LOD) / Limit of Quantification (LOQ)

LOD and LOQ values were predicted on the basis of response and slope of the regression equation. These values are calculated from the formula 3.3δ/S and 10 δ /S respectively where 'δ is standard deviation of the y-intercept of the regression line and 'S' is slope of the calibration curve which were obtained from linearity experiments carried out from 5% to 150% specification levels for each RS . The precision study was carried out at about predicted LOD and LOQ levels by injecting six replicates and calculating the % RSD of the area of each impurity LOD and LOQ values are presented in Table 3.

Linearity

A series of solutions were prepared by using Dapagliflozin and its related substances at concentration levels from 5 to 150% of specification level and each solution was injected and calculated the statistical values like slope, intercept, STEYX and correlation coefficient from linearity plot drawn for concentration versus area. From this data after establishing LOQ level for all the related substances including drug substance, the linearity has been deduced from LOQ level to 150% specification level and statistical values are presented in Table 3.

Table 3: Linearity and LOD/LOQ experiments

	Impurity-1	**Impurity-2**	**Impurity-3**	**Impurity-4**	**Impurity-5**	**Dapagliflozin**
Concentration range (µg/mL)	0.081-0.918	0.064-0.897	0.062-0.911	0.070-0.908	0.058-0.922	0.062-0.922
Slope	54931	63432	74271	62458	77930	72176
Intercept	-220	350	-400	1161	-552	-339
STEYX	419	394	491	442	506	459
Response factor	1.31	1.14	0.97	1.15	0.93	1.00
Correlation Coefficient	0.9997	0.9998	0.9998	0.9997	0.9998	0.9998
LOD(%w/w)	0.007	0.005	0.005	0.006	0.005	0.005
%RSD	1.7	2.3	2.1	2.5	1.1	1.9
LOQ(%w/w)	0.020	0.016	0.016	0.018	0.015	0.016
%RSD	0.9	1.0	0.9	1.6	0.3	0.8

Precision

The system precision was evaluated by injecting six injections of Dapagliflozin standard solution at a concentration of 0.0006 mg /ml and calculating the % relative standard deviation (% RSD). The method precision was checked by injecting six individual preparations of Dapagliflozin drug substance spiked with 0.15% level for each impurity, % RSD of content of each related substance was calculated. The intermediate precision of the method was also evaluated using different analyst, different instrument, and different lot of column on different day. The inter day variations were calculated. The precision experiments results are given in Table 4. The results (method

precision and intermediate precision) indicated that the method is rugged for the determination of related substances in Dapagliflozin drug substance w.r.t analyst-to-analyst, system-to-system, column-to-column and day-to-day variations.

Table 4a System Precision experiment results

Injection ID	Dapagliflozin peak area
Injection-1	42691
Injection-2	42605
Injection-3	42655
Injection-4	42665
Injection-5	42686
Injection-6	42659
Mean	42660
SD	31
%RSD	0.1
95% Confidence Interval (±)	33

Table 4b Method Precision experiment results

	Inj-1	Inj-2	Inj-3	Inj-4	Inj-5	Inj-6	Mean(%w/w) *n=6*	SD	% RSD	95% CI*(±)
Impurity1	0.228	0.229	0.227	0.229	0.228	0.229	0.228	0.001	0.4	0.001
Impurity2	0.151	0.151	0.149	0.151	0.150	0.150	0.150	0.001	0.7	0.001
Impurity3	0.154	0.154	0.153	0.154	0.153	0.154	0.154	0.001	0.6	0.001
Impurity4	0.140	0.142	0.141	0.142	0.142	0.142	0.142	0.001	0.7	0.001
Impurity5	0.146	0.146	0.144	0.146	0.145	0.145	0.145	0.001	0.7	0.001

**Confidence Interval*

Table 4c Intermediate Precision experiment results

	Inj-1	Inj-2	Inj-3	Inj-4	Inj-5	Inj-6	Mean(%w/w) *n=6*	SD	% RSD	95% CI*(±)
Impurity1	0.211	0.212	0.213	0.213	0.215	0.214	0.213	0.001	0.5	0.001
Impurity2	0.138	0.139	0.140	0.140	0.141	0.140	0.140	0.001	0.7	0.001
Impurity3	0.140	0.140	0.141	0.141	0.142	0.141	0.141	0.001	0.7	0.001
Impurity4	0.151	0.152	0.150	0.151	0.150	0.151	0.151	0.001	0.7	0.001
Impurity5	0.129	0.129	0.130	0.130	0.131	0.131	0.130	0.001	0.8	0.001

**Confidence Interval*

Table 4d Cumulative results of method precision & intermediate precision experiments

		Inj-1	Inj-2	Inj-3	Inj-4	Inj-5	Inj-6
Set-1(for method precision)	Impurity1	0.228	0.229	0.227	0.229	0.228	0.229
	Impurity2	0.151	0.151	0.149	0.151	0.150	0.150
	Impurity3	0.154	0.154	0.153	0.154	0.153	0.154
	Impurity4	0.140	0.142	0.141	0.142	0.142	0.142
	Impurity5	0.146	0.146	0.144	0.146	0.145	0.145
Set-2	Impurity1	0.211	0.212	0.213	0.213	0.215	0.214
(for intermediate precision)	Impurity2	0.138	0.139	0.14	0.140	0.141	0.140

	Impurity3	0.140	0.140	0.141	0.141	0.142	0.141
	Impurity4	0.151	0.152	0.150	0.151	0.150	0.151
	Impurity5	0.129	0.129	0.130	0.130	0.131	0.131

Overall		**Mean**	**SD**	**% RSD**	**95% CI*(±)**
	Impurity1	0.221	0.008	3.6	0.005
	Impurity2	0.145	0.006	4.1	0.004
	Impurity3	0.147	0.007	4.8	0.004
	Impurity4	0.146	0.005	3.4	0.003
	Impurity5	0.138	0.008	5.8	0.005

**Confidence Interval*

Accuracy

The accuracy of the method was determined by analyzing Dapagliflozin (n=3) samples spiked with five related substances at different levels (LOQ, 50, 100 and 150% of specification levels). The percentage recovery values for all the impurities are calculated and tabulated in Table.5. The recovery results indicated that the test method has an acceptable level of accuracy for the determination of related substances in Dapagliflozin drug substance from LOQ to 150% of specification.

Table 5 Accuracy experiment results

Recovery details (average 3 replicates)		Impurity-1	Impurity-2	Impurity-3	Impurity-4	Impurity-5
--	% Level					
Added (%w/w)	LOQ	0.0201	0.0160	0.0155	0.0172	0.0148
	50	0.073	0.076	0.077	0.075	0.078
	100	0.147	0.153	0.155	0.149	0.156
	150	0.219	0.229	0.231	0.224	0.233
Recovered (%w/w)	LOQ	0.0200	0.0167	0.0143	0.0175	0.0139
	50	0.080	0.075	0.075	0.069	0.076
	100	0.160	0.149	0.149	0.134	0.151
	150	0.230	0.216	0.216	0.200	0.220
Recovery (%)	LOQ	99.5	104.4	92.3	101.7	93.9
	50	109.6	98.7	97.4	92.0	97.4
	100	108.8	97.4	96.1	89.9	96.8
	150	105.0	94.3	93.5	89.3	94.4

Robustness

To determine the robustness of the method, experimental conditions were deliberately changed and to evaluate system suitability requirement as per methodology. For this evaluation, system suitability solution and sample solution spiked with impurities at specification level were prepared as per test method and injected into HPLC. To study the effect of flow rate, ±10% variation of flow rate was studied. The effect of column temperature was studied by keeping 20°C and 30°C instead of 25°C. In the same manner, detection wavelength (±3 nm), variation in organic in mobile

phase (±2% absolute in Gradient Composition) has been verified and the results obtained from these experiments are summarized in Table 6.

Table 6 Robustness experiment results

Condition	Variation	System Suitability				Spiked Sample (RRT)				
		USP resoluton	USP Ptailng	USP plaecount	%RSD	Impurity-1	Impurity-2	Impurity-3	Impurity-4	Impurity-5
Original Method	-	4.8	1.0	77094	0.1	1.07	1.13	1.21	1.66	2.50
Flow	-10%	4.7	1.0	77479	0.2	1.07	1.13	1.21	1.62	2.39
	+10%	4.8	1.0	76518	0.2	1.07	1.13	1.20	1.70	2.60
% Organic in gradient variation	-2% absolute	5.2	1.0	65526	0.1	1.08	1.15	1.24	1.55	2.23
	+2% absolute	4.3	1.0	89246	0.1	1.06	1.11	1.18	1.69	2.75
Wavelength	-3 nm	4.8	1.0	76470	0.3	1.07	1.13	1.21	1.66	2.50
	+3 nm	4.7	1.0	76379	0.1	1.07	1.13	1.21	1.66	2.50
Column Oven Temperature	-5°C	4.6	1.0	75601	0.1	1.07	1.12	1.21	1.67	2.55
	+5°C	4.8	1.0	77821	0.3	1.07	1.14	1.20	1.65	2.47

From the tabulated data, it can be concluded that the system suitability results are meet the acceptance criteria at each of the varied conditions. Also the chromatograms of Dapagliflozin drug substance spiked with known related substances at specification level obtained from each of the above varied conditions indicated that RRT's of related substances are comparable to that obtained from STP condition. Hence, it can be concluded that the test method is robust for the determination of related substances in Dapagliflozin drug substance across the extent of change studied for each of the above parameters

Stability of solutions

Standard solution and sample solution spiked with related substances at specification level were prepared and analysed initially and different time intervals by keeping the solutions at 25°C±2°C. Based on experimental data, the standard solution is stable up to 1440 mins (24 hours) at 25°C±2°C and sample solution is stable up to 1200 mins (20 hours) at 5°C±3°C.

CONCLUSION

A reverse phase stability indicating HPLC method was developed and validated for the quantitative determination of related substances of Dapagliflozin drug substance. The present research work will help the manufacturers and suppliers of Dapagliflozin to quantify the quality in

terms of purity based on experimental results. Thus, it can be used for routine analysis, quality control and for determining quality during the stability studies of pharmaceutical analysis.

ACKNOWLEDGEMENTS

The authors express their sincere gratitude to Aurobindo Pharma Limited Research Centre–II, located in Hyderabad for providing the analytical and chemical research support to pursue this work and also grateful to colleagues who helped us in this work.

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