



CODEN [USA]: IAJPBB

ISSN : 2349-7750

**INDO AMERICAN JOURNAL OF
PHARMACEUTICAL SCIENCES**

SJIF Impact Factor: 7.187

Available online at: <http://www.iajps.com>

Review Article

**A REVIEW ON METHOD DEVELOPMENT AND VALIDATION
OF LC-MS/MS FOR ANALYZING POTENTIAL GENOTOXIC
IMPURITIES IN GLIFLOZINS****B. Akhila^{1*}, B.V. Ramana²**¹Research Scholar, Jawaharlal Technological University Anantapur (JNTUA), Anantapuramu
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518218, Andhra Pradesh, India**Abstract:**

The objective of this paper is to review the method development, optimize and validation of the method for the potential genotoxic impurities in gliflozins. The purpose of this validation is to show that processes involved in the development and manufacture of drug, production and analytical testing can be performed in an effective and reproducible manner. This review article provides guidance on how to perform validation characteristics for the analytical method which are utilized in pharmaceutical analysis.

Key words: Analytical method development, Genotoxic impurities, Pharmaceutical analysis, Validation

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Please cite this article in press B. Akhila et al, A Review On Method Development And Validation Of Lc-MS/MS For Analyzing Potential Genotoxic Impurities In Gliflozins, Indo Am. J. P. Sci, 2023; 10 (08).

INTRODUCTION:

SGLT2 Inhibitors are a new class of oral antihyperglycemic agents indicated as an adjunct to diet and exercise to improve glycemic control in adult patients with type 2 diabetes mellitus. The Diabetes Association recently updated the guidelines for pharmacotherapy in type 2 diabetes and added SGLT2 inhibitors as an add-on to metformin or when metformin is not tolerated. SGLT2 Inhibitors can be prescribed in monotherapy or combination with other oral antihyperglycemic agents and Insulin. Pharmacopeia indicated restrictions for active pharmaceutical ingredients (APIs) and formulations for allowable levels of impurities. Moreover, the Food and Drug Administration (FDA) and International Council for Harmonisation (ICH) mentioned strategies for the identification and quantification of impurities along with residual solvent in any in novel dosage forms [1]. Moreover, some impurities in trace levels could affect the efficacy and safety of API, as well as be carcinogenic [2]. Hence, monitoring and control of trace impurities in any API turn into a very tough assignment. Therefore, the process of minimizing such carcinogenic substances became important in pharmaceutical toxicology [3].

Based on the above documented facts, researchers mainly focused on minimizing the production of impurities in any API manufacturing process [4]. However total elimination of impurities in any process is difficult. So, the method development for accurate identification of impurities is the only option for pharmaceutical industries. Furthermore, USFDA and agency of European medicines proposed toxicological threshold limits to be 1.0-1.5 µg/dl [5,6] for genotoxic impurities. In the present work, we aimed to determine genotoxic impurities in Gliflozins using LC-MS method for quantification of these impurities in Gliflozins formulations.

Literature review

Jeyabaskaran M *et al.*, (2013) [7] has developed a simple, precise, accurate, reproducible and specific RP-HPLC method for estimation of dapagliflozin (DGF) in bulk and pharmaceutical dosage forms using an Hypersil BDS 250mm x 4.6 mm, 5µ column in isocratic mode with 0.1% Ortho phosphoric acid buffer and acetonitrile 50:50 % v/v as mobile phase at a flow rate of 1ml/min. The injection volume was 10 µl and the total runtime was set as 5min. The determination of analytes was carried out at 245nm using PDA detector. The retention time for DGF was found to be 2.226min. The proposed method has permitted the quantification of DGZ over linearity in

the range of 25 – 150 µg/ml and its percentage recovery was found to be 100.12 %. The % RSD of intraday and inter day precision were found 0.6% and 0.29%.

Manasa. Sanagapati *et al.*, (2014) [8] has developed and validated an accurate, precise, specific and rapid RP-HPLC method for the determination of Dapagliflozin in API. Better separation of the drug was achieved on BDS column (250×4.5mm, 5µ) with a mobile phase consisting of a mixture of ortho phosphoric acid and acetonitrile (45:55 v/v) at a flow rate of 1ml/min, with detection at 245nm using Photo Diode Array (PDA) detector. The retention time was found to be 2.963 min. The method was found to be linear in the range of 25-150µg/ml with a correlation coefficient (r²) of 0.999. The LOD and LOQ of the method were calculated to be 0.6 and 1.8µg/ml respectively. The Precision was estimated by employing repeatability; intra-day and inter-day studies and the results were calculated as %RSD values and were found to be within the limits. The average recovery of the analyte was found to be 99.8% which confirms the accuracy of the method.

Manasa Sanagapati *et al.*, (2014) [9] have suggested a simple, novel, safe, sensitive and economic UV-Spectrophotometric method for the estimation of a Type II anti diabetic drug, Dapagliflozin was developed and assessed. The developed method was validated as per ICH guidelines. The drug showed two different wavelengths of maximum absorption, at 203nm and 237nm. This method can be successfully applied for the estimation of Dapagliflozin in bulk for routine analysis with UV detection at 237nm. A Labindia UV-Visible spectrophotometer with 1cm matched quartz cells and ethanol solvent were employed in this method. The Developed method obeyed Beer's-Lambert's law in the concentration range of 0.5-0.9µg/ml, having correlation coefficient of 0.994.

Mohammad Yunoos *et al.*, (2015) [10] has developed a simple and precise stability indicating RP-HPLC method for the simultaneous determination of metformin (MET) hydrochloride and dapagliflozin (DAP) in bulk and pharmaceutical dosage form. Chromatography was carried out on hypersil BDS C18 (250 mm × 4.6 mm, 5 µ particle size) column containing mobile phase of buffer (0.1% orthophosphoric acid) adjusted to pH 6.8 with triethylamine:acetonitrile in the ratio of 50:50%/v/v at a flow rate of 1 ml/minutes. The analyte was monitored using photodiode array detector at 240 nm. The retention time was found to be 2.791 minutes

and 3.789 minutes for MET hydrochloride and DAP respectively.

Shyamala NB et al., (2015) ^[11] have reported a new, precise, rapid, accurate RPHPLC method was developed for the Simultaneous Estimation of dapagliflozin and metformin HCL in tablet dosage form. After optimization the good chromatographic separation was achieved by Isocratic mode with a mixture of Phosphate Buffer (pH 6.5):methanol:acetonitrile in the ratio of 50:30:20 v/v/v as the mobile phase with column as stationary phase at flow rate of 1 mL/min and detection wavelength of 240 nm. The retention times metformin HCL and dapagliflozin found to be 2.475min and 3.647min respectively.

Aubry AF et al., (2010) ^[12] have developed a LC-MS/MS method and validated for the quantitation of dapagliflozin in rat plasma. The assay uses solid phase extraction and LC-MS/MS analysis in negative ion electro-spray ionization mode. Because

dapagliflozin readily forms adducts in the presence of formic acid, the mobile phases were simple mixtures of water and acetonitrile. The assay was validated in the concentration range of 5-2000 ng/ml with good intra- and inter-day precisions and acceptable sample stability.

Karuna PC et al., (2015) ^[13] has estimated present work, a selective, specific, sensitive and economical UV spectroscopic method for the estimation of Dapagliflozin in Bulk and its pharmaceutical dosage forms. An absorption maximum was found to be at 233.65 nm. Dapagliflozin obeyed Beer's law in the concentration range from 10-35 µg / ml. precision and other statistical analysis were found to be in good accordance with the prescribed values with correlation coefficient of 0.9998. The percentage recovery of Dapagliflozin ranged from 99.7 in pharmaceutical dosage form. Results of the analysis for accuracy, precision, LOD, LOQ and were found to be satisfactory.

Table 1: Analytical techniques used for estimation of dapagliflozin in pharmaceutical formulations

S. No.	Matrix	Techniques	Mobile phase/ solvent used	Column/ spectrophotometer	Maximum absorbance wavelength (nm)	Flowrate (mL/min)
1.	Dapagliflozin ^[14] (tablet and bulk)	RP-HPLC	Methanol:acetonitrile:orthophosphoric acid (75:20:5)	C-18	246	1
2.	Dapagliflozin (tablet and bulk) ^[15]	RP-HPLC	Orthophosphoric acid:acetonitrile(45:55)	BDS column	254	1
3	Dapagliflozin (tablet and bulk) ^[16]	RP-HPLC	Acetonitrile:water acidified with 0.1% formic acid (42:58)	C-18	245	1
4	Dapagliflozin ^[17] (tablet and bulk)	RP-HPLC	Acetonitrile:di-potassiumhydrogenphosphate (pH-6.5) maintain with ortho phosphoric acid (40:60)	C-18	222	1
5	Dapagliflozin ^[18] (tablet and bulk)	RP-HPLC	Phosphate buffer:acetonitrile (60:40)	C-18	237	1
6	Dapagliflozin ^[19] (tablet and bulk)	RP-HPLC	0.1% orthophosphoric acid buffer andacetonitrile 50:50	BDS	245	1
7	Dapagliflozin ^[20] (tablet and bulk)		Methanol:sodium 1-octanesulphonate(70:3)	C-18	203	1
8	Dapagliflozin (tablet and bulk) ^[21]	RP-HPLC	Acetonitrile:di-potassium hydrogenphosphate with pH-6.5 with OPA (40:60)	C-18	222	1

9	Dapagliflozin and metformin (tablet and bulk) ^[22]	RP-HPLC	0.1% ortho phosphoric acid (pH 6.5) with triethylamine:acetonitrile (50:50)	C-18	240	1
10	Dapagliflozin and metformin (tablet and bulk) ^[23]	RP-HPLC	Phosphate buffer (pH 6.5):methanol:acetonitrile 50:30:20	C-18	240	1
11	Dapagliflozin and metformin (tablet and bulk) ^[24]	RP-HPLC	Acetonitrile: water (75:25)	C-18	285	1
12	Dapagliflozin and metformin (tablet and bulk) ^[25]	RP-HPLC	0.05M potassium dihydrogen ortho phosphate buffer (pH-3.5, adjusted with 0.1% orthophosphoric acid): acetonitrile 50:50	C-18	227	1
13	Dapagliflozin and saxagliptin (tablet and bulk) ^[26]	RP-HPLC	Phosphate buffer (pH 4) and acetonitrile (50:50)	C-18	225	1
14	Dapagliflozin and saxagliptin (tablet and bulk) ^[27]	RP-HPLC	Potassium dihydrogen phosphate buffer (pH 6.0):acetonitrile (45:55)	C-18	220	1.5
15	Dapagliflozin and saxagliptin (tablet and bulk) ^[28]	RP-HPLC	20 mM sodium dihydrogen phosphate (pH 5.5 ± 0.02 with orthophosphoric acid acetonitrile (53:47)	C-18	230	1.2
16	Dapagliflozin and glimepiride (tablet and bulk) ^[29]	RP-HPLC	Acetonitrile:10% orthophosphoric acid in water pH 6 (70:30)	C-18	228	1
17	Dapagliflozin (tablet and bulk) ^[30]	RP-HPLC	Orthophosphoric acid:acetonitrile (60:40)	BDS	245	1
18	Dapagliflozin (tablet and bulk) ^[31]	RP-HPLC	Acetonitrile: 0.1% triethylamine (pH-5) in the ratio of 50:50	C-18	224	1
19	Dapagliflozin (tablet and bulk) ^[32]	RP-HPLC	Phosphate buffer:methanol (35:65)	C-18	215	1
20	Dapagliflozin (tablet and bulk) ^[33]	RP-HPLC	Potassium hydrogen orthophosphate (pH-4.2):methanol (65:35)	C-18	225	1
21	Dapagliflozin canagliflozin empagliflozin metformin	RP-HPLC	Acetonitrile: 0.05M di-potassium hydrogen phosphate with pH-4 (65:35)	C-18	212	1

	(combination) ^[34]					
22	Dapagliflozin and metformin (tablet and bulk) ^[35]	RP-HPLC	Acetonitrile: O.1M orthophosphoric acid (70:30)	C-18	260	1
23	Dapagliflozin and metformin (tablet and bulk) ^[36]	RP-HPLC	0.1M dipotassium hydrogen phosphate:acetonitrile:methanol (60:30:10)	C-8	285	1.2
24	Dapagliflozin (tablet and bulk) ^[37]	UV-Visible	Methanol	Double beam	224	-
25	Dapagliflozin and metformin (Tablet and bulk) ^[38]	UV-Visible	Methanol	Double beam	235 270	-
26	Dapagliflozin (tablet and bulk) ^[39]	UV-Visible	Methanol	Double beam	203	-
27	Dapagliflozin and saxagliptin (tablet and bulk) ^[40]	UV-Visible	Water: Methanol (80:20)	Double beam	223 212	-
28	Dapagliflozin (tablet and bulk) ^[41]	UV-Visible	Methanol	Double beam	233.65	-
29	Dapagliflozin (tablet and bulk) ^[42]	RP-HPLC	Methanol: water (80:20% v/v)	C-18	225	0.8
30	Dapagliflozin saxagliptin ^[43]	UPLC	0.1% orthophosphoric acid (40) :acetonitrile (60)	C-18	254	0.3

Aim and objectives

The literature survey revealed that few analytical methods have been reported for estimation of dapagliflozin alone or in combination with other drugs by UV spectrometry and HPLC. However there is no method was reported about the separation and determination of dapagliflozin impurities. Hence an attempt was made to develop a simple, accurate, precise and sensitive LC-MS/MS method for the estimation of dapagliflozin in presence of its impurities.

Objectives

1. Simultaneous estimation of dapagliflozin and its impurities in tablet dosage form

2. System suitability parameters of dapagliflozin and impurities
3. Linearity evaluation data for dapagliflozin and impurities
4. LOD and LOQ data of dapagliflozin and impurities

MATERIALS AND METHODS:

HPLC grades of ammonium acetate and methanol were purchased from Merck (Mumbai, India). Analytes were obtained from synthink research chemicals (Hyderabad, India). A Shimadzu LC-MS/MS- 8050 system associated with the Nexera X2 HPLC and Lab Solutions software v.5.6 was used. Separations were accomplished on a 5 µm particle

size of Hypersil C18 column (4.6×250 mm) purchased from Thermo Fisher Scientific.

Method development:

Generally, for any analysis, sample preparation plays an important role; it affects the sensitivity, as well as better recovery of impurities. So, preferable combinations of acetonitrile, water, ammonium acetate, and methanol were used as diluents for chromatographic efficiency. In the present work, 0.01 M ammonium acetate in methanol was chosen as a diluent with column oven at 40° due to good response and recovery for impurities. Also, both isocratic and gradient modes of elution were performed. Nevertheless, from the observations, it was noticed that all the impurities were effectively separated by the gradient method. Similarly, a column of dimensions, namely Zorbax C8, Hypersil C18 column Phenomenox, Kromasil C8 and C18, were

also investigated for resolutions. Finally, Hypersil C18 column was selected due to its better response, peak shape, linearity, and reproducibility even at a lower concentration.

Method optimization:

Mobile phase A used was prepared by dissolving 0.77 g of ammonium acetate in 1000 ml Milli-Q water by sonication followed by filtration (0.22 µm). Pure HPLC grade methanol was used as a mobile phase B. An LC-MS/MS system, coupled with an 8050 triple quadrupole detector, was used. Separation was achieved on a 5 µm Hypersil C18 column (250×4.6 mm) with injection volume 10 µl, 1 ml/min flow rate, sample cooler temperature at 15° and column oven temperature 40°. **Table-1** summarized conditions of MRM, Valco valve, and source gas parameters for mobile phases A and B under gradient mode against the blank solution (diluent).

Table 1: Gradient Programme

Gradient program					
Time (minute)	Mobile Phase A			Mobile Phase B	
0.01	45			55	
8.00	45			55	
10.00	20			80	
13.50	Total Flow			0.8 ml	
15.00	45			55	
17.00	45			55	
17.00	Total Flow			0.8 ml	
20.00	Total Flow			1 ml	
21.50	20			80	
32.00	45			55	
37.00	Controller			Stop	
Multiple reactions monitoring conditions					
Parameters					
Impurity	MRM	Q1 Prebias	CE	Q3 Prebias	Dwell Time (milliseconds)
Dapagliflozin Impurity 1	305.70>69.05	20.0	22.0	24.0	100
Dapagliflozin Impurity 2	364.15>291.25	22.0	22.0	30.0	100
Dapagliflozin	314.15>244.10	20.0	19.0	26.0	100
Valco Valve Condition for sample method					
Time (min)	Command		Value		
17.00	FCV2=		1		
24.00	FCV2=		0		

Standard preparation:

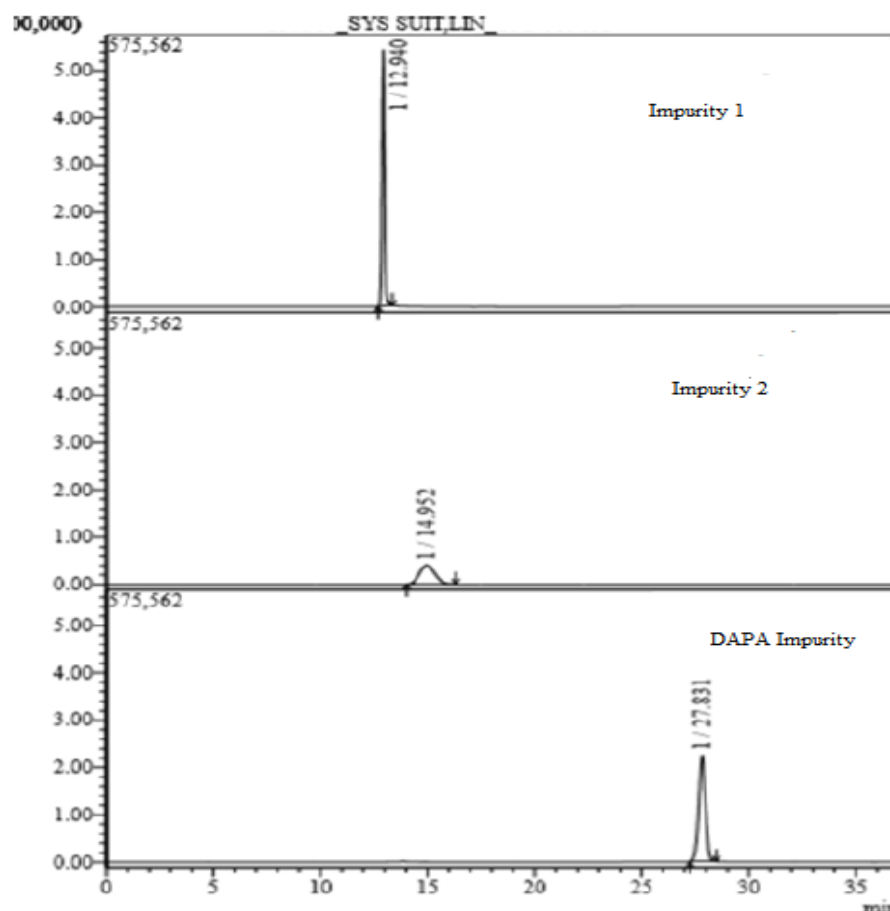
Separately, 2.6 mg of impurity 1, 2 and DAPA were weighed accurately and dissolved completely in 100 ml diluent via sonication. One milliliter of the above impurity/ intermediate standard stock solution was further diluted to 100 ml with diluent. One hundred milligrams of accurately weighed DAPA was diluted to 5 ml. To evaluate the system suitability parameters, 10 μ l of the above-prepared solution was separately injected namely blank, standard, and sample preparations and their peak area responses were monitored. As per the pharmacopeias, the average peak area response of % relative standard deviation (RSD) of impurity 1, 2, and DAPA impurities should not be more than 15.0.

Method validation:

Method was validated according to the USFDA and ICH guidelines. The appropriateness and efficacy of the chromatographic scheme were obtained from the system suitability test and it is proficient in the investigation without any bias. To guarantee the capacity of the chromatographic systems, these must placate pre-defined acceptance conditions to implement the examination of various samples. In the contemporary experiment, impurity 1, 2, and DAPA impurity solutions were injected into the LC-MS/MS system for determining system suitability parameters such as peak area and its RSD and retention time, which were detailed after data incorporation using software (Table-2, Fig-2).

Table 2: Results for System Suitability

System suitability parameters	CPP	CPE	EFI
%RSD of Peak areas obtained from six replicate injections of the standard solution	0.8	1.2	1.6

**Figure 2: System suitability**

The impurity 1, 2, and DAPA impurities were also checked for specificity by injecting them against the blank solution. The outcomes showed that the chosen method is unbiased concerning the presence of further components and interestingly, no nosiness was recorded at the RTs of impurity 1, 2, and DAPA impurities. (Table-3, Fig-3).

Table 3: Blank Interference Results for impurity 1, 2, and DAPA impurities

ame	Retention time (min)	Interference found at the retention time of CPP, CPE, and EFI
CPP	12.940	No
CPE	14.952	No
EFI	27.831	No
Blank	NA	No

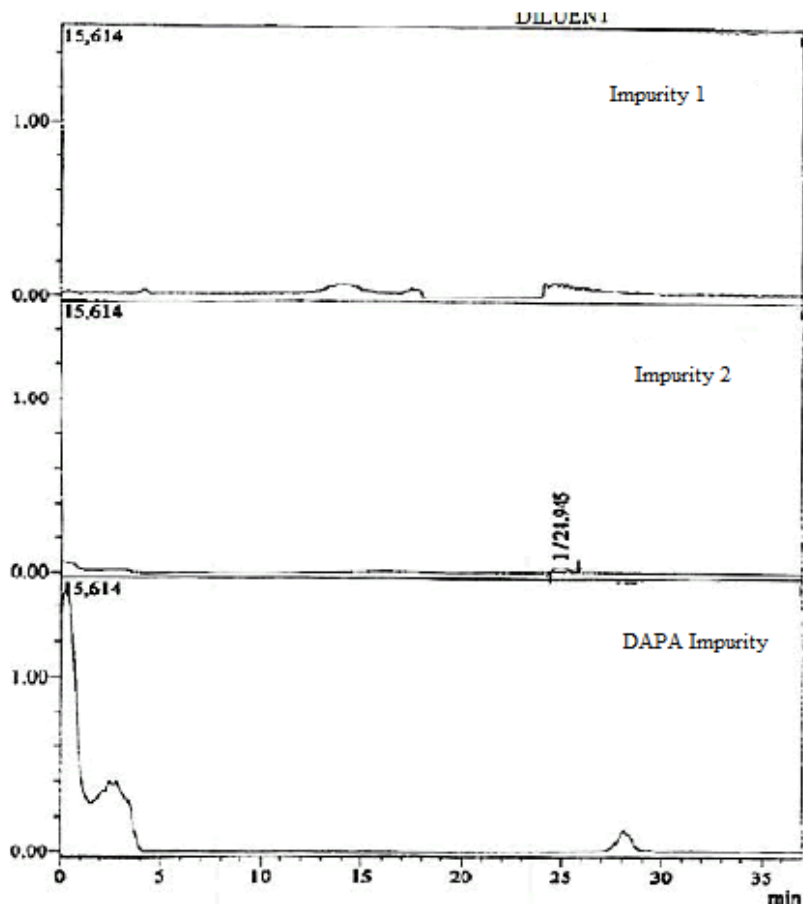


Figure 3: Specificity

Table 4: standard drug and its procurement

Drug standard	Procured from
Dapagliflozin (99.67%) and Impurities	Veeprho laboratories pvt ltd, Pune, India

Table 5: Commercial drug information

Commercial formulations	Components	Manufacturer
FORZIGA Tablet	Dapagliflozin 10 mg	AstraZeneca Pharma India Limited, Bangalore, India.

RESULTS AND DISCUSSION:

At limit of quantification (LOQ), 4 levels of the precision method, namely system precision, intermediate precision (ruggedness), method precision (repeatability) and precision were evaluated. System precision suggested inconsistency in the dimensions of the analytical system, while repeatability (method precision) indicated the reproducibility of the method. Standard solution was prepared with impurity 1, 2, and DAPA impurities and injected (n=6) into the LC-MS/MS system from which peak area and RSD were derived, whereas from method precision, the % RSD data was obtained (**Table 6**).

Table 6: Method Precision Results for impurity 1, 2, and DAPA impurities

Preparation	Impurity 1 (ppm)	Impurity 2 (ppm)	DAPAI (ppm)
1	2.5	2.8	3.0
2	2.6	2.9	3.1
3	2.7	3.0	3.2
4	2.7	2.9	3.2
5	2.8	3.1	3.4
6	2.5	2.7	3.1
Average	2.6	2.9	3.2
% RSD	4.6	4.9	4.3

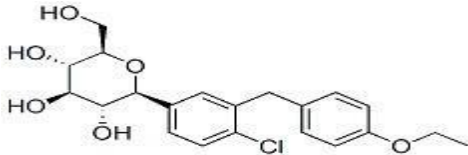
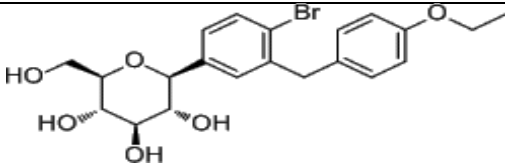
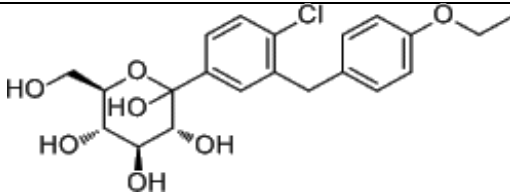
The peak area of each sample was noted and plotted against respective concentrations. The Eqn. $y=mx+b$, defined the linear relation between impurity concentration (x) and respective peak area (y). From this analysis, a correlation coefficient (must be above 0.99) and slope-intercept values were derived (**Table 7**).

Table 7: Linearity of impurity 1, 2, and DAPA impurities

Linearity results						
Levels	IMP 1		IMP 2		DAPA IMP	
	Conc. in ppb	Area	Conc. in ppb	Area	Conc. in ppb	Area
5 %	2.508	259187	2.531	117538	2.569	305901
10 %	5.224	298024	5.272	137684	5.352	388002
25 %	12.538	1214979	12.653	619137	12.845	1482624
50 %	25.075	2661829	25.306	1341802	25.690	3110678
75 %	37.613	3873591	37.958	1991286	38.534	4712314
100 %	50.046	4817911	50.506	2451722	51.272	5857968
125 %	62.688	6522284	63.264	3349419	64.224	8271495
150 %	75.748	8502919	76.444	4296518	77.604	11062322
Slope	107848		55129		129684	
Intercept	-165458.4		-87086.3		-234051.5	
Correlation	0.9974		0.9994		0.9976	

Dapagliflozin and its impurities

Table 8: Dapagliflozin and its impurities chemical name and structure

Name of compound	Chemical Name	Structure
Dapagliflozin	(2S,3R,4R,5S,6R)-2-[4-chloro-3-(4-ethoxybenzyl) phenyl]-6- (hydroxymethyl)tetrahydro-2H-pyran-3,4,5- triol	
Impurity-A	(2S,3R,4R,5S,6R)-2-(4-bromo-3-(4-ethoxybenzyl)phenyl)-6- (hydroxymethyl)tetrahydro-2H-pyran-3,4,5-triol	
Impurity-B	(3R,4S,5S,6R)-2-(4-chloro-3-(4-ethoxybenzyl)phenyl)-6- (hydroxymethyl)tetrahydro-2H-pyran-2,3,4,5-tetraol	

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