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Qualitative and Quantitative Determination of Dapagliflozin Propanediol Monohydrate and Its Related Substances and Degradation Products Using LC-MS and Preparative Chromatography Methods

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Abstract:

Dapagliflozin is a novel sodium-glucose cotransporter type 2 inhibitor. This work aims to develop a new validated sensitive RP-HPLC coupled with a mass detector method for the determination of dapagliflozin, its alpha isomer, and starting material in the presence of dapagliflozin major degradation products and an internal standard (empagliflozin). The separation was achieved on BDS Hypersil column (length of 250mm, internal diameter of 4.6 mm and 5-µm particle size) at a temperature of 35°C. Water and acetonitrile were used as mobile phase A and B by gradient mode at a flow rate of 1 mL/min. A wavelength of 224nm was selected to perform detection using a photo diode array detector. The method met the requirement of the International Conference on Harmonisation for Registration of Pharmaceuticals for Human Use (ICH) for validation. The molecular weight of impurities and degradation products was estimated using positive ESI-MS. Fifteen impurities were detected during the analysis of dapagliflozin APIs and the brand Farxiga ® and some generic products. Three of fifteen detected impurities (H, J and K) exceeded the impurities acceptable limits 0.1%. Those impurities were isolated using new preparative chromatography then characterized using elemental analysis, FTIR and NMR.

Keywords: Dapagliflozin, Degradation products, Impurity profile, LC/MS, Preparative chromatography.

Introduction:

Dapagliflozin propanediol monohydrate (DPG), with the chemical structure represented in the (Fig.1), is a novel sodium cotransporter type 2 (SGLT-2) inhibitor. SGLTs play a significant role in glucose absorption in the kidney, wherein SGLT-2 usually takes up nearly 90% glucose [1]. DPG was first manufactured by AstraZeneca and Bristol Myers Squibb under the brand name of Farxiga® in 2014 [2]. It is prescribed as an oral drug for the management of type 2 diabetes mellitus (T2DM) [2]. DPG has also been approved to manage T2DM in patients with heart failure[3]. DPG is the first SGLT-2 inhibitor authorized as an adjunct to insulin in adults with type 1 diabetes (T1DM)[4]. DPG is available commercially as a solvate, which is a mixture of dapagliflozin with (S)-propylene glycol and water in a ratio of 1:1:1[5]. According to ICH guideline Q3A (R2), impurities were classified into three main categories (organic, inorganic and residual solvents) [6]. The organic impurities include process-related impurities (drug-related substances) that might arise during the manufacturing of the raw materials, intermediates and/or by-products. Drug related substances may also produce from the degradation of active pharmaceutical ingredient API or formulated drug product[6-9]. The determination of dapagliflozin alone or in combination with other drugs was performed using (UV) spectrometry[10-11], thin layer chromatography TLC[11- 13], reverse phase high-performance liquid chromatography (RP-HPLC)[11, 14- 17] and LC-mass spectrometry[11, 18- 20]. However, one HPLC method was reported for the determination of DPG with six impurities in tablets dosage form. 'The chromatographic separation of dapagliflozin and its six impurities was performed using Hypersil BDS C_{18} column (length of 250 mm, 4.6 mm i.d and 5 µm

particle size) with the eluent consisted of buffer pH 6.5 (A) and acetonitrile: water 90:10 (B) by gradient elution mode at a flow rate of 1 mL/min with UV detection at 245 nm[14].

The present study is interested in the determination of some dapagliflozin-related substances (DRS) in the presence of empagliflozin (EMG) as an internal standard (IS) (Fig.1). DRS1 is the dapagliflozin alpha isomer while DRS2 is the starting material used for DPG synthesis [21]. EMG has also a chemical structure related to DPG, where an ethyl group was replaced with an oxolane moiety [22]. To the best of our knowledge, DPG is not embodied in any of the pharmacopeias until now and there are no analytical RP-HPLC/MS and preparative chromatographic methods available for estimation of the impurity profile and stability profile of DPG. The aim of this work is to develop both validated analytical RP-HPLC/MS and preparative chromatographic methods for the qualitative and quantitative determination of DPG-related substances in the active pharmaceutical ingredient API and pharmaceutical dosage forms. The developed method was successfully validated as per ICH Q2 (R1)[23-26].

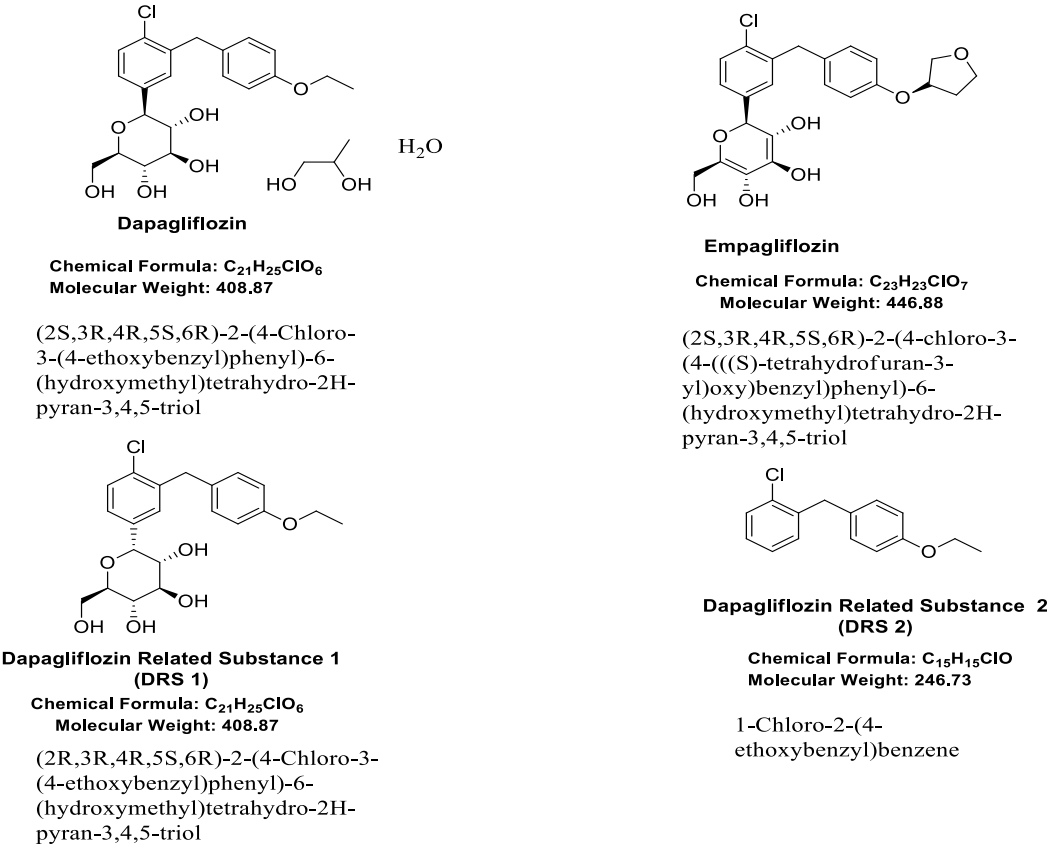


Figure 1. Chemical structure of dapagliflozin propanediol monohydrate (DPG) [5], Empagliflozin (EMG) [27] and dapagliflozin-related substances (DRS1 and DRS2).

Material and Methods:

Chemical and Reagents

Dapagliflozin propanediol monohydrate (DPG) reference standard was purchased from Shunlong Pharmaceutical Co. Ltd., China. Two dapagliflozin-related substances (DRS1 and DRS2) standards were purchased from (SimSon Pharma Limited, India). Empagliflozin (EMG) standard was purchased from Hubei Derun Pharmaceutical Co., Ltd. and was used as an internal standard (IS). Two Active pharmaceutical ingredients (APIs) were obtained from Hubei Derun Pharmaceutical Co., Ltd., China, and Anhui Lianchuang biological medicine Co., Ltd., China. DPG Crude API, with purity, claimed to be 82.5% based on the manufacturer's analysis certificate, was purchased from Hubei Derun Pharmaceutical Co. Ltd, China. HPLC grade solvents (methanol and acetonitrile), sodium hydroxide (NaOH), hydrochloric acid (HCl) and hydrogen peroxide (H_2O_2) were purchased from Merck®. Water for HPLC was obtained from Siemens Evoqua LaboStar® D2. Farxiga® film-coated tablets were purchased from AstraZeneca (USA) and kindly gifted to us by Zein for pharmaceutical industries in Syria. Two generic products, Dapagold® 5 mg film-coated tablets from Golden Med Pharma (Syria) and Diadab® 5 mg film-coated tablets from Alfares Pharmaceutical (Syria)

were purchased from the local market in Syria. FT-IR purity Potassium bromide (KBr) was purchased from Sigma-Aldrich® (India). NMR solvents (Dimethyl sulfoxide-d_6 (DMSO-d_6) and Chloroform-d_1 ($CDCl_3$-d_1)) were purchased from Sigma-Aldrich® (Germany).

Preparation of Solution

Solvent (Diluent)

A mixture of water: acetonitrile 70:30 % v/v was prepared, degassed by sonication and also used as the blank solution.

Standard Solutions (Stock and Working)

Dapagliflozin propanediol monohydrate stock solution 1250 ppm equivalent to dapagliflozin 1000 ppm, was obtained by transferring an accurate amount of 62.5 mg of DPG reference standard into a 50 mL volumetric flask containing 15 mL of solvent, shaking well, and sonicated, then made up to volume 50 mL with solvent. To obtain DPG working solution 100 ppm, 1 mL of DPG stock solution was diluted into a 10 mL volumetric flask using the solvent. EMG stock solution (100 ppm) was obtained by transferring an accurate amount of 10 mg of EMG reference standard into a 100 mL volumetric flask containing 75 mL of solvent, shaking well and sonicated, then made up to volume 100 mL with solvent. Each of the DRS1 and DRS2 stock solutions was prepared at a concentration of 100 ppm.

Preparation of the Standard Mixture Solution

To obtain the mixture solution 100% dapagliflozin, 1.0% impurities, 12.5 mg of DPG standard was accurately weighted into a 100 mL volumetric flask, and precisely 1 mL of reference stock solution of DRS1, DRS2, and EMG was added, the volume was made up and the solution was shacked well. The final solution contains 100 ppm of DPG and 1.0 ppm of each impurity and EMG.

Preparation of Samples Solution

The average weight of twenty film-coated tablets of each dosage form (Farxiga ® and generic products was calculated. Then, a quantity of 25 mg of dapagliflozin propanediol monohydrate equivalent to 20 mg of dapagliflozin was weighed and transferred quantitatively into a 25 mL volumetric flask containing 15 mL of the diluent, the solution was sonicated for 10 minutes, and made up to 25 mL final volume with diluent to make 1000 ppm solution. Then, 1 mL of the solution was accurately transferred into a 10 mL volumetric flask and made up to the mark using the diluent to get the final concentration of 100 ppm of dapagliflozin. The resulted solution was filtered using a 0.45 µm disposable filter and used to analyze.

Calibration of Standard

The standard calibration curve was constructed for DPG, EMG, DRS1, and DRS2. Nine different volumes of stock solutions each were transferred into 10 mL volumetric flasks the volume was made up and the solution was shaken. The final concentration ranged from 2.0 to 200 ppm for DPG and 0.02 to 2.0 ppm for each EMG, DRS1, and DRS2.

RP- HPLC/LC- MS Conditions

The analysis was conducted on a Shimadzu LC prominence system (Shimadzu®, Japan) equipped with photo-diode array (PDA) detector SPD-M20A, solvent delivery units LC-20A, auto-sampler SIL-20A, and column oven CTO-20A. The mixture of water: acetonitrile 70:30 v/v was used as the solvent (Diluent). The separation of DPG with other impurities and EMG was achieved on BDS Hypersil column length of 250mm, 4.6 mm i.d, 5-µm particle size (Agilent®, USA) at column temperature 35 ℃. For gradient elution mode, water and acetonitrile were used as mobile phases A and B, respectively, and the flow rate was 1 mL per minute. The gradient program was set as follows; [Time (min) /Acetonitrile (%)] [0.01/28, 5.0/20, 10.0/28, 40.0/90, 55.0/90, 56.0/28, 70.0/28]. A volume of 20.0 µL of each sample was injected. The wavelength of 224nm was selected for detection. LabSolutions software (Schimadzu®, Japan), was used for data analysis and system control. Analytical balance (Sartorius®, Germany) with sensitivity of ± 0.0001g was used in preparation of all samples. The LC-MS studies were performed by applying the same chromatographic conditions used in analytical HPLC and using MS detector 2020 (Schimadzu, Japan). After LC separation, the analytes were determined by positive electrospray ionization mass spectrometry (ESI^+–MS). The protonated molecular ions $[M + H]^+$ were found to be abundant and therefore selected for monitoring and quantification. The value of ion source voltage was 3.5 kV and its temperature was maintained at 350℃. The flow rate of Nitrogen (curtain gas) was 10 liters per minute. All samples were prepared using diluent of water: acetonitrile 70:30 % v/v at a concentration of 100 ppm. Mass to charge m/z ratio was scanned across the range of m/z 50–1000. This RP-HPLC method coupled with mass spectrometry was successfully validated as per ICH guidelines.

Preparative HPLC Conditions

The impurities were isolated using Jasco PU-2087 prep-HPLC system (Japan) equipped PDA-detector, and preparative fraction collector system, Waters C_{18} column 250 mm, 20.1 mm i.d, 5 -µm particle size (Waters®, USA) at temperature 35℃. The injected volume was 500.0 µL. The flow rate of mobile phase was adjusted at 16.0 mL per minutes. The eluents were monitored at 224 nm during the chromatographic run time of 70 minutes.

NMR Spectroscopy

Using a Bruker AVANCE 400 MHz spectrometer (Bruker® Biospin, Karlsruhe, Germany), H_1-NMR spectra was recorded at 400 MHz and C_{13}, depth 90 and depth 135 NMR spectra were recorded at 100 MHz. All samples were dissolved in DMSO-d_6 at 25 °C. Only (impurity J) was dissolved in $CDCl_3$-d_1 at 25 °C. The H_1 and C_{13} chemical shift values were reported on δ scale in ppm, relative to tetra methyl silan (TMS) of chemical shift δ = 0.00 as an internal standard.

Infrared Spectroscopy

The IR spectrum for DPG and the isolated impurities were recorded using FT-IR Nicolet 6700 (USA) equipped with DTGS detector. The samples in solid state were prepared as KBr dispersion. The data collected within range 400-4000 cm^{-1} ± 4.0 cm^{-1}. Thirty-two scans were obtained and processed using the OMNIC software version 7.3 (Thermo Nicolet, USA).

Degradation Protocols

As per ICH Q1A guideline recommendations[18], stress testing is used to evaluate the validity and applicability (stability indication) of the method by determining the resolution factors between peaks of degradation product and dapagliflozin and between peaks of degradation products and known impurities, comparing the result of related substances determination test and assay. For acidic degradation, accurately 12.5 mg of DPG was added to a 50 mL volumetric flask containing 5mL (1N) HCl, and the solution was heated for 8 hours at a 60℃ water bath under reflux. The solution was cooled to room temperature then 5.0 mL of NaOH (1N) was added to neutralize the solution. Finally, the volume was made up to the final volume 50.0 mL using the diluent. Basic degradation was performed using NaOH (1N) for 8 hours at a 60 ℃ water bath under reflux and HCl (1N) to neutralize the solution. The stressed oxidation study was carried out using a 30% hydrogen peroxide solution for 8 hours in a 60℃ water bath under reflux. The photolytic degradation was studied by placing DPG powder for 10 days in a 4500 ± 500 lux light cabinet (Memert®, Germany). The thermolytic degradation was carried out in a dry state for 72 hours in an 80 ℃ oven. Each stressed solution was spiked with EMG solution 1.0 ppm as an internal standard. All of the stressed solutions were obtained at a concentration of 100 ppm using diluent. In addition, the degradation of the solvent in each condition was performed at the same time. The degraded samples were quantified against the undegraded DPG reference standard 100 ppm.

Validation Study

The developed RP-HPLC/MS was validated according to the ICH validation of analytical method guidelines. Method specificity was studied by injection of each blank solution, DPG standard solution, EMG standard solution, and mixed standard solution. The obtained chromatograms were checked for the interference of blank with the determination of DPG, EMG, DRS1, and DRS2. Resolution (R), tailing factor (T), and theoretical plates number (N) were calculated. To study method linearity, a series of concentrations of 2.0-200 ppm for DPG and 0.02-2.0 ppm for each impurity and EMG were prepared from a standard mixture solution. The method accuracy was demonstrated by spiking of DPG sample solution with DRS1, DRS2, and EMG at different levels of 80-120%. Three replicates were measured in order to calculate the recovery percentage. To verify the precision of the method, a standard mixed solution was analyzed, and the percentage relative standard deviation (%RSD) of peak area and retention time for DPG, EMG, DRS1, and DRS2 were calculated after injection of six replicates. To investigate the impact of random variation factors on precision, six replicates of standard mixed solution were prepared and tested using different instruments on different days. The percentage recovery and %RSD for peak area were calculated in order to evaluate the intermediate precision. Signal-to-noise (S/N) ratio was used to determine the detection limit (LOD) and quantification limit (LOQ). The robustness of the method was verified using slightly modified chromatographic conditions flow rate 0.9 - 1.1 mL/min, column temperature 30℃ - 40℃, and wavelength 222nm - 224nm. The relative retention time RRT and RSD % of peak area were calculated.

Results and Discussion:

Method Development

DPG and its related substances and major degradation products have maximum absorption at 224nm and 277nm based on UV spectrum obtained by HPLC-PDA (Fig. 2). The unknown impurities ≥

0.1% exhibited strong maximum absorption at about 224nm. The results are mentioned in Table 1. Various columns were tested for the separation of dapagliflozin from its related substance. Various mobile phases with different pH and composition were prepared and tested. Finally, the separation of DPG from EMG and all impurities peaks was achieved on BDS Hypersil column C_{18} (length 250 mm × 4.5 mm i.d, 5-µm particle size) using a gradient mixture of water and acetonitrile at 35℃. The retention time (RT) of DPG, EMG DRS1, and DRS2 was identified by analyzing the chromatograms of individual standard solutions (Figs. 3, 4, 5 and 6). To avoid overlap between peaks, 1 mL per min was chosen as flow rate.

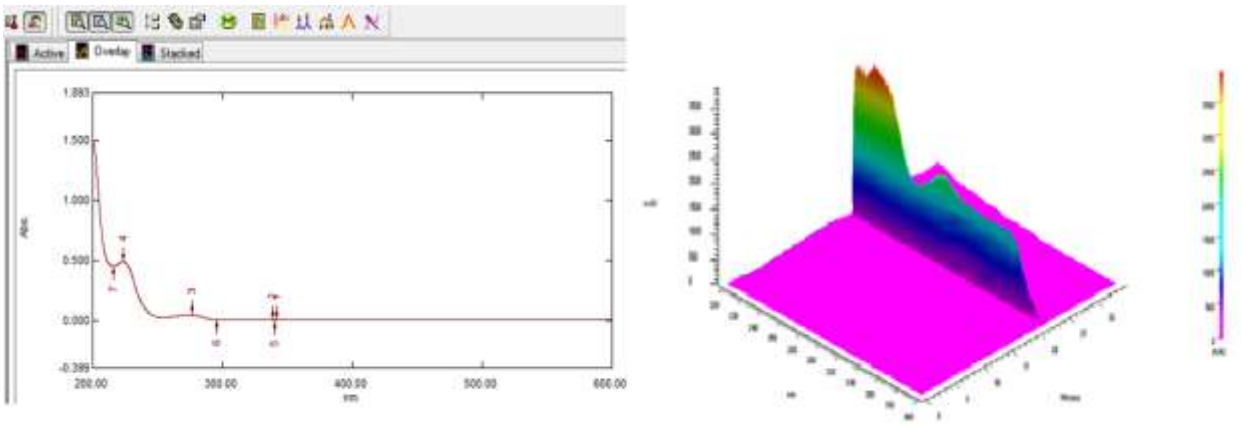


Figure 2. Ultra-violate spectrum of standard solution of dapagliflozin by using HPLC-PDA.

Table 1. Maximum UV absorbance for different solution by HPLC-PDA.

Sample	Material Name	Retention Time (min)	The wave Length of Maximum Absorption (nm)
Standard #1	**DPG**	22.970	221.34, 276.83
Standard #2		22.965	224.59
Standard #3		22.967	223.75
Standard #4		22.966	224.44
Standard #5		22.968	224.13
Standard #6		22.969	223.67, 276.69
Standard	**DRS1**	25.547	
Standard	**DRS2**	41.265	
Standard	**EMG**	20.070	
Acid Hydrolysis	**DP1***	4.489	213.38, 252.38
	DP2	3.780	221.56
Oxidation	**DP1**	4.460	217.35, 251.56
	DP3	10.469	222.58 , 277.01
High Temperature	**DP4**	21.617	222.01 , 296.58

*DPs= Degradation products.

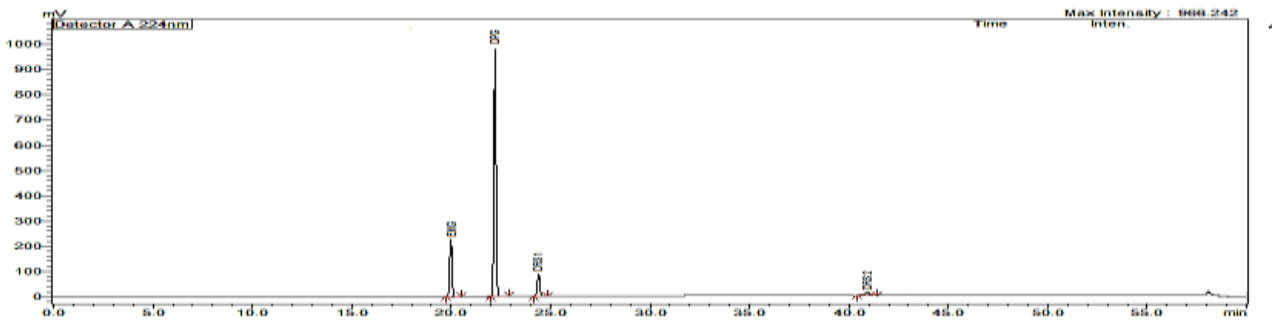


Figure 3. The typical HPLC-PDA chromatogram of standard mixed solution.

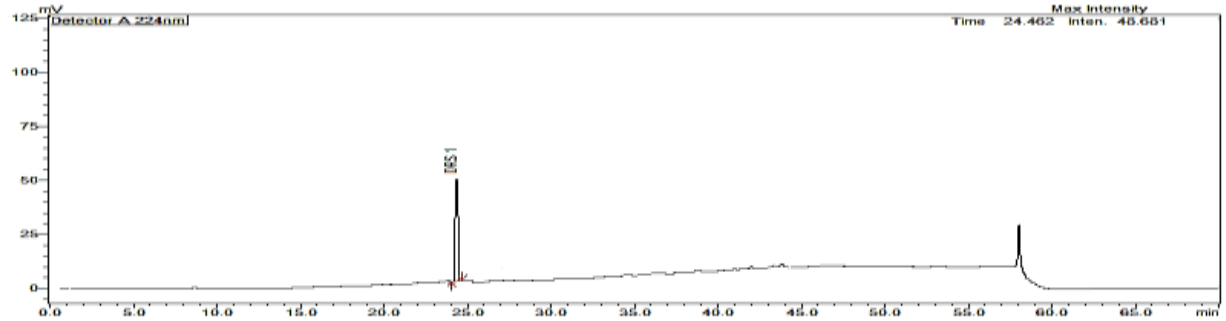


Figure 4. The typical HPLC-PDA chromatogram of dapagliflozin related substance (DRS1).

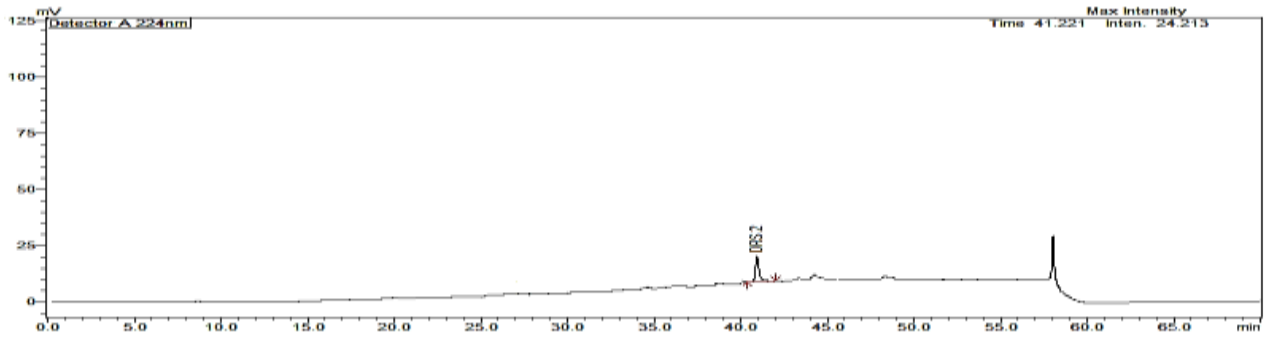


Figure 5. The typical HPLC-PDA chromatogram of dapagliflozin-related substance (DRS2).

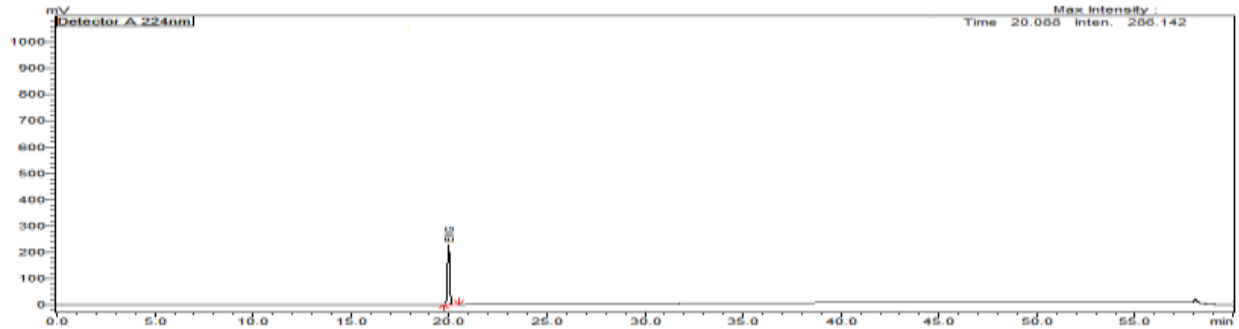


Figure 6. The typical HPLC-PDA chromatogram of the Internal Standard (EMG).

Method Validation Results

The method was found to be compatible with the ICH requirements for system suitability. The developed method was found to have a good specificity as no interference of blank with the determination of DPG, EMG, DRS1 and DRS2 was detected. The retention time was 22.968 min for DPG and 20.067 minutes, 24.135 minutes and 41.261 minutes for EMG, DRS1 and DRS2, respectively. The resolution between dapagliflozin and other impurities and degradation products were found to be greater than 1.5. The tailing factor was less than 2.0 and the number of theoretical plates were more than 5000. The results are presented in the Table. 2.

Table 2. The results of system suitability.

Substances	Retention time (min)	Resolution (R)	Tailing Factor (T)	Theoretical Plates Number
DPG	22.968	8.71	1.01	236073
EMG	20.067	6.42	0.98	185632
DRS1	24.135	3.26	1.01	244446
DRS2	41.261	2.68	1.04	442037

The calibration curves were plotted between the concentration and the response (peak area). Correlation coefficient R^2 was 0.9998, 0.9996, 0.9998 and 0.9997 for DPG, EMG, DRS1 and DRS2, respectively. As R^2 values were more than 0.999, the linear relationship between the peak area and the concentration was demonstrated. The estimated regression equation is presented in the Table 3.

Table 3. Linearity study results.

Sr. No.	Substances	Range	Regression equation	Correlation coefficient
1	DPG	2.0 -200 ppm	y = 53617x + 32060	R^2 = 0.9998
2	EMG	0.02-200 ppm	y = 62677x - 16610	R^2 = 0.9996
3	DRS1	0.02-200 ppm	y = 58496x - 331.52	R^2 = 0.9998
4	DRS2	0.02-200 ppm	y = 62114x + 66.062	R^2 = 0.9997

In precision study, the RSD values were less than 2% for peak area and 1% for retention time for all injected samples indicating that developed method have good precision. The percentage recoveries were in the range 98.24 -104.12% and met the acceptance criteria 115-85%. Slightly modification of the chromatographic conditions (flow rate, column temperature and detection wavelength) did not influence the system suitability parameters of the method and the tailing factors of DPG, EMG DRS1 and DRS2 peaks were less than 2.0. The resolution factors between peaks of DPG and EMG, DRS1 and DRS2 were greater than 1.5. LOD and LOQ is the concentration of S/N ratio about 3/1 and 10/1, respectively. The estimated LOD and LOQ values showed that the method has a good sensitivity. The result of validation is summarized in the Table. 4.

Table 4. Summary of validation study.

Parameter			DPG	DRS1	DRS2	EMG
Retention Time (RT) min			22.968	24.135	41.261	20.067
Peak Area			11460667	115881	125014	130169
Relative Retention Time RRT*			1.00	1.03	1.66	1.18
Accuracy	**Recovery (80%)**		100.20	99.56	104.12	98.24
	Recovery (100%)		103.35	100.40	99.67	99.85
	Recovery (120%)		102.63	99.41	103.5	99.75
	Average recovery ± SD		102.39 ± 2.08	99.79 ± 0.55	102.43 ± 2.41	100.61 ± 1.41
Repeatability	**RSD (Peak area)**		0.76	0.63	0.53	0.82
	RSD (RT)		0.01	0.02	0.01	0.03
Intermediate Precision	**Recovery %**		100.30	100.40	99.65	99.84
	RSD (Peak area)		0.89	0.91	0.68	0.75
Robustness (Flow Rate)	**0.9 mL/min**	**RRT**		1.75	1.64	1.06
		RSD**	0.85	1.21	0.77	0.84
	1.2 mL/min	**RRT**		1.80	1.69	1.17
		RSD	1.10	1.05	0.88	0.92
Robustness (Column Temp.)	**30℃**	**RRT**		1.03	1.67	1.17
		RSD	0.94	1.23	0.91	1.01
	40℃	**RRT**		1.03	1.66	1.16
		RSD	0.81	1.21	1.01	1.06
Robustness (Wavelength)	**222nm**	**RRT**		1.02	1.66	1.18
		RSD	1.12	1.20	0.95	0.99
	226nm	**RRT**		1.02	1.66	1.17
		RSD	1.05	1.08	0.89	1.26
LOD (ng/mL) =			8.59	10.05	5.25	9.75
LOQ (ng/mL)			28.34	33.16	17.32	32.17

*RRT= relative retention time = Retention time of impurity / Retention time of dapagliflozin.
** RSD of peak area = 100 × (Standard deviation / Average).

Results of Dapagliflozin Degradation Study

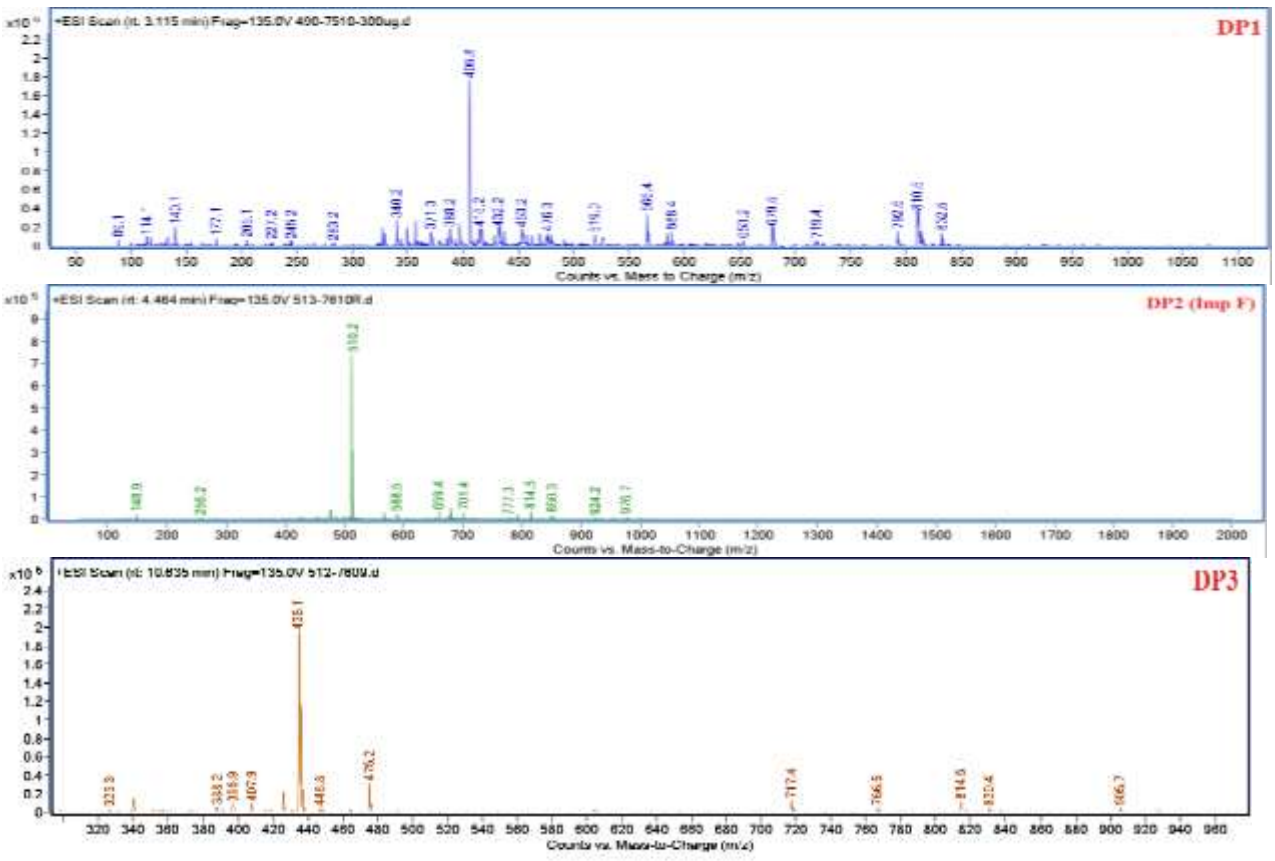
There were significant reductions in the peak areas of samples of DPG exposed to different stress conditions as compared to the standard DPG. Mass to charge (m/z) ratio was scanned across the range of 50–1000 m/z to produce spectra of molecular weight of DPG (Fig.7.) and its degradation product (DPs) by positive ESI-MS. The degradation products are named "DPn", where n accounts for the elution order. Recorded loss of DPG was 31.88% after 8 hours in acidic conditions and the major degradation product detected was DP2 with $[M + H]^+$ 510.2 m/z at retention time of 4.464 minutes. After 8 hours in basic conditions, the recorded loss of DPG was 29.15% and degradation product DP5 that has $[M + H]^+$ 453.6 m/z was detected at 36.135 minutes. About 22.15% loss was observed in oxidative conditions and two major degradation products (DP1 and DP3) were detected. The recorded $[M + H]^+$ for DP1 and DP3 was 405.8 m/z and 435.5 m/z, respectively. About 34.16 % of DPG was lost after exposed to thermal condition at 80 ℃ for 72 hours. In addition,

the degradation product DP4 appeared at retention time of 21.617 minutes and has $[M + H]^+$ 348.1 m/z . However, the lower reduction was observed in photolytic degradation condition (about 9.5%). Mass balance of all the stressed samples of DPG was obtained in the range of 99.14–99.77%. The findings of the degradation study are reported in the Table. 5. Chromatograms of the samples of DPG exposed to stress conditions are shown in the Figs. (8 and 9). A good resolution was observed between the peaks of DPG and its degradation products and the peak of EMG.

Table 4. Summery of degradation study.

Stress Testing Condition	Peak Name	RT (min)	[1] IS peak area	[2] DPG Average Area (%) ± RSD%	[3] Total Degradation (%)	[4] Mass Balance
Undegraded	DPG	22.969		99.98 ± 1.92	-	-
	EMG	20.060	99.64		-	-
Acid Hydrolysis	DPG.	22.972				
	EMG	20.065	99.85	67.42 ± 1.16	31.88	99.30
	DP2	4.464				
Base Hydrolysis	DPG	22.963				
	EMG	20.065	99.77	69.99 ± 1.06	29.15	99.14
	DP5	36.135				
Oxidation	DPG	22.987				
	EMG	20.062	99.53	77.62 ±1.91	22.15	99.77
	DP1	3.115				
	DP3	10.635				
Thermolytic Degradation	DPG	22.962				
	EMG	20.068	99.86	65.25 ± 0.86	34.16	99.41
	DP4	21.617				
Photolysis (UV Light)	DPG	22.969		89.65 ± 1.23	9.87	99.52
	EMG	20.061	99.76			

[1]Internal standard (Empagliflozin).
[2]Area of undegraded dapagliflozin calculated based on dapagliflozin reference standard.
[3] Total area of degradation product peaks.
[4]Mass balance = [Assay % + total degradation %]. Accepted values (95.0% -105%).



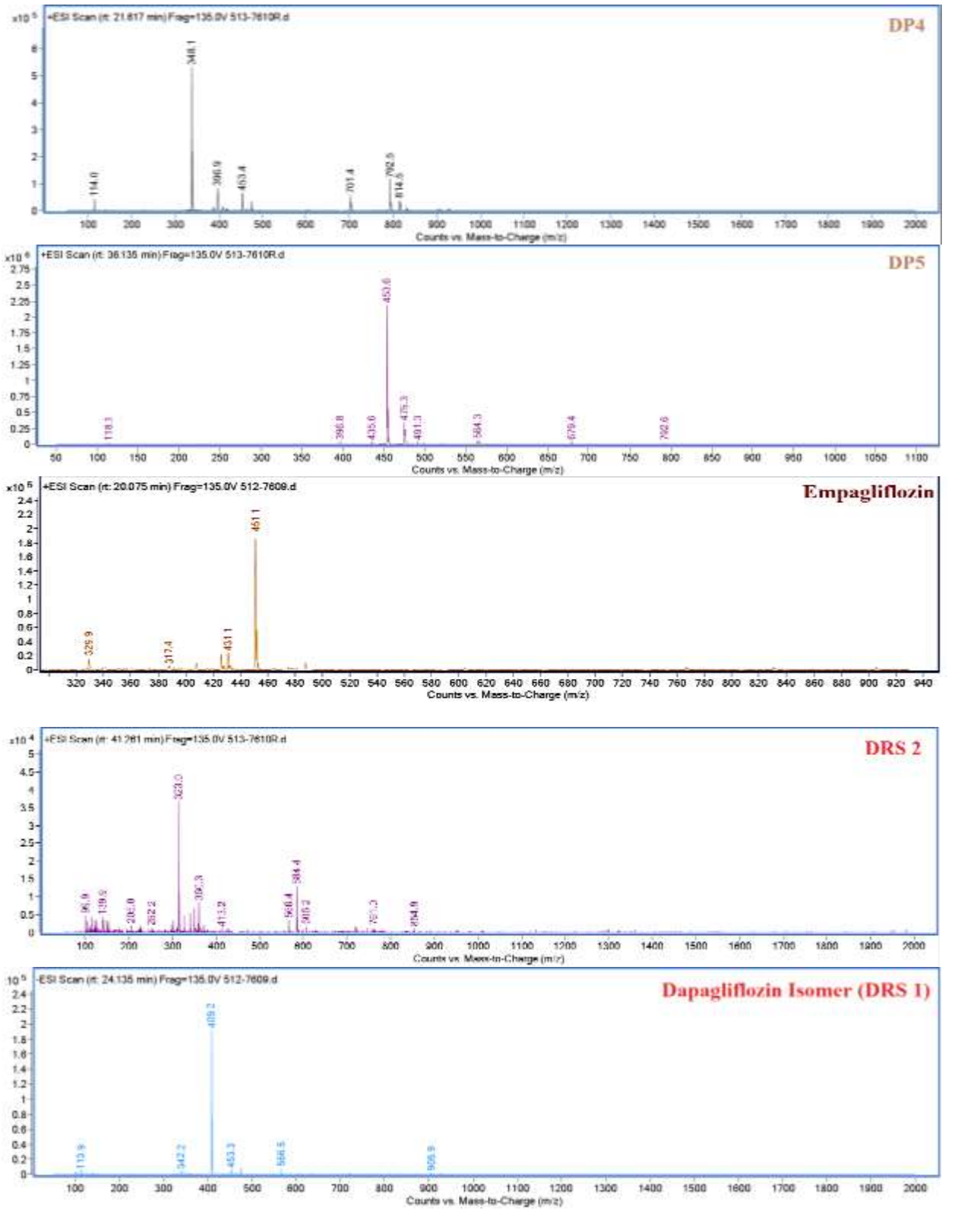


Figure 7. LC-MS spectrum of dapagliflozin (DPG), empagliflozin (EMG), DRS1, DRS2 and major degradation products of dapagliflozin DP 1, 2, 3, 4 and 5.

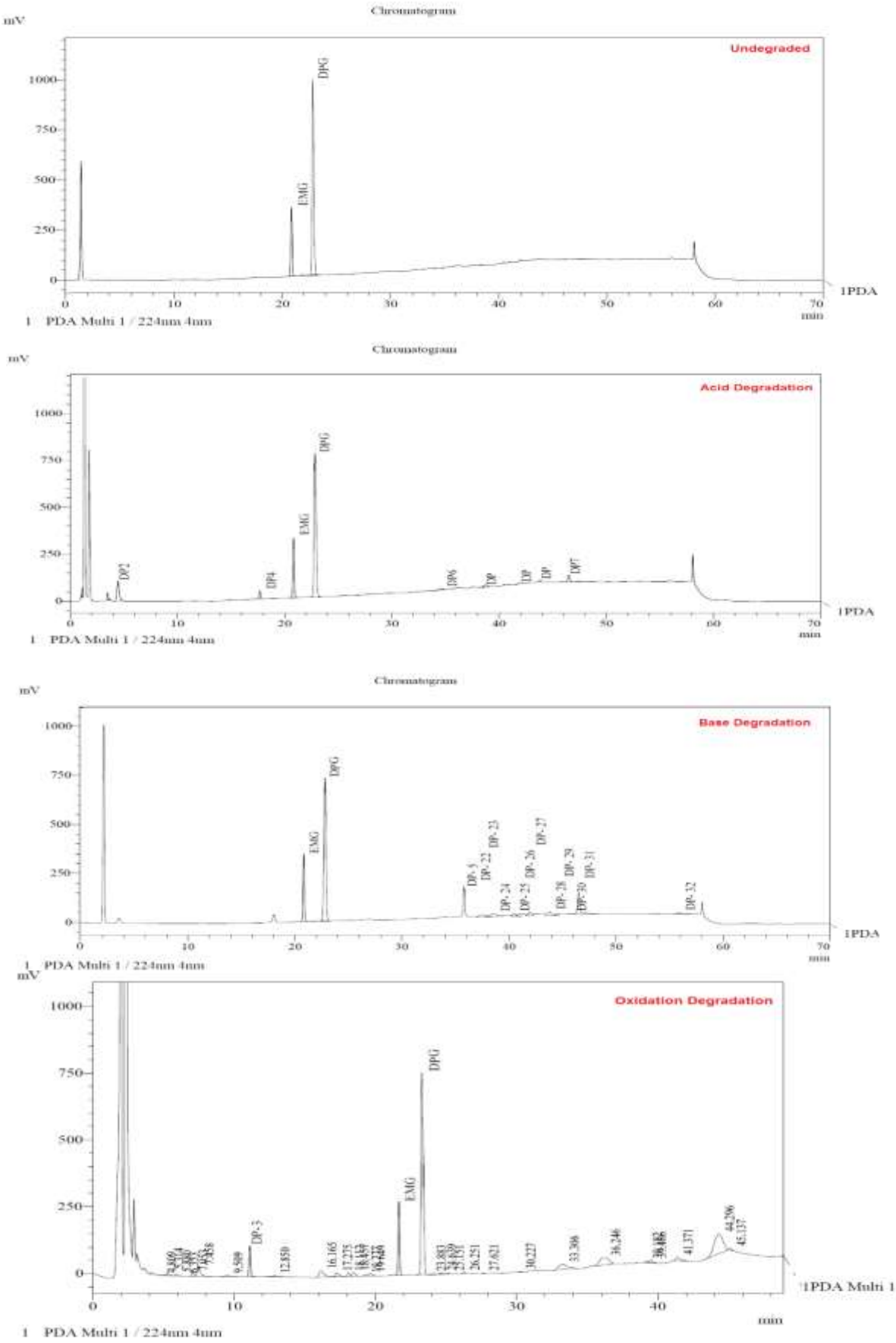


Figure 8. Chromatogram of degradation solution (undegraded, acid degradation, base degradation, and oxidation)

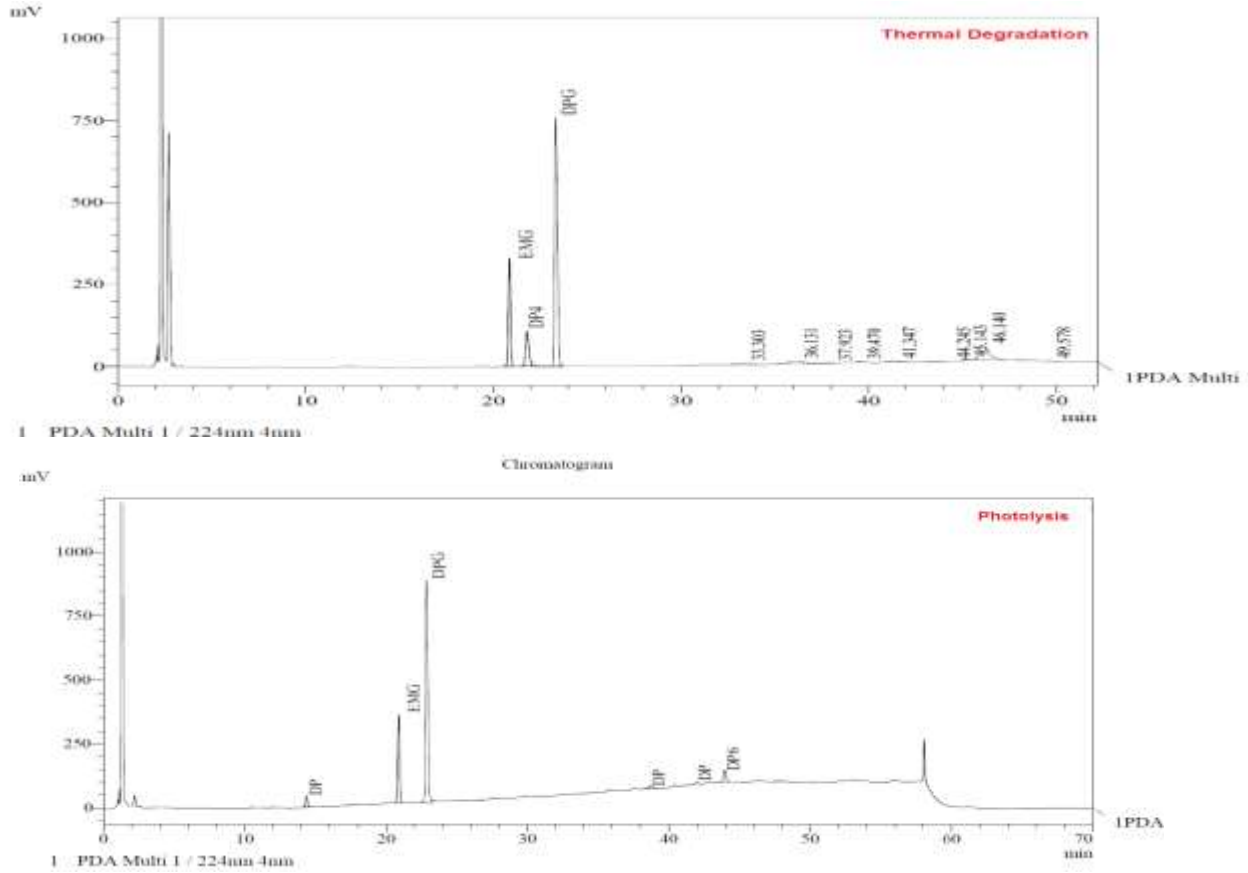


Figure 9. Chromatogram of degradation solution (thermal degradation and photolysis).

Comparison with Reported Method

The published HPLC-VIS method for the determination of DPG and its six impurities used different chromatographic conditions (mobile phase and detection wavelength) [14]. The retention time of DPG was 16.95 min, with a LOD of 0.065 µg/mL, a linearity range was 0.20-13.00 µg/mL, and a total runtime of 75 minutes. Another HPLC-PDA method was published for the estimation of DPG in API and pharmaceutical dosage forms. It used Agilent® C_{18} column 150 mm, 4.6 mm, 5-µm and a mixture of K_2HPO_4 with a pH of 6.5 adjusted with OPA 40:60 %v/v as a mobile phase with the flow rate of 1 ml/min in isocratic mode. The detection wavelength was 220 nm [15]. The retention time of DPG was 3.160. The published methods have not been tested in the estimation of DPG in the presence of degradation products or related substances. One stability-indicating LC-MS/MS method was reported for the determination dapagliflozin propanediol hydrolytic degradation products [18]. Three major degradation products have been detected and their structures were proposed by using positive mode electrospray ionization tandem mass spectrometry and high-resolution mass spectrometry. This method used an Agilent Zobrax Eclipse C_8 column length of 150 mm, internal diameter of 4.6 mm, 5-µm particle size. The mobile phase consisted of acetonitrile and ammonium formate 10mM, 4.0 pH. The runtime was 15 minutes. This LC-MS/MS method has not been tested in the determination of DPG in the presence of process-related impurities. Another HPLC/MS was reported for quantification of three SGLT-2 inhibitors (dapagliflozin, empagliflozin, and canagliflozin) in human plasma [19]. The run-time of the method was 1 minute and the linearity range of DPG was 1-500 µg/L. The proposed method in our study was found more sensitive LOD of 8.59 ng/mL with shorter runtime of 70 minutes than the reported method. The retention time of DPG was longer 22.965 minutes in the proposed method compared to the reported method. However, the proposed method in our study was found more sensitive (LOD of 8.59 ng/mL) with shorter runtime of 70 minutes than the reported one[14]. The proposed method also offers an advantage in determining the relationship between the impurity profile and the stability profile.

Result of Analyzing Samples (Dapagliflozin API, Crude Dapagliflozin and Drug Products)

Two samples of DPG API from different sources, one DPG working standard, one EMG working standard, Farxiga®, and two generic products were analyzed using the developed

chromatographic method. The concentrations in ppm of these samples were calculated from the peak areas and the impurities were successfully separated from DPG and EMG. The results are presented in Table. 6. Fifteen impurities were characterized, three of them were known impurities (DRS1, DRS2, and EMG) and the other twelve were unknown, their $[M + H]^+$ m/z are shown in Table 6. The impurity (Imp C) has $[M + H]^+$ 405.6 m/z with retention time of 3.115 minutes and the Imp F has $[M + H]^+$ 510.2 m/z with retention time of 4.464 minutes, were detected in oxidation and acid degradation solution, respectively. Impurity C and Impurity F were found in all dapagliflozin samples. However, twelve of fifteen impurities were detected in concentration below identification threshold 0.10% in accordance with ICH guidelines[6, 9, 28], so no necessary to identification them. All analytical results are acceptable according to the pharmaceutical requirements.

The unknown impurities, Impurity J has $[M + H]^+$ 439.78 m/z and Impurity K has $[M + H]^+$ 579.12 m/z were detected in all DPG samples, at retention time 26.521 minutes and 39.658 minutes, respectively. In DPG API 2 sample, Impurity H, which has $[M + H]^+$ 427.12 m/z, was appeared at retention time of 19.075 minutes. The impurities H, J and K were found at concentration greater than 0.10%, so it was necessary to characterize their structures[6, 9, 28].

The method was tested in the analysis of the dapagliflozin crude sample, five impurities were found. However, two of them were known (DRS1 and DRS2), and three were unknown which are Impurities H, J and k and had to be isolated by preparative chromatography to elucidate their structures according to elemental analysis CHN, FT-IR, MS, NMR H1, C13, DEPT 90-135.

Impurity H, appeared at retention time of 19.075 minutes, and has a molecular weight 424.87 $g.mol^{-1}$. According to elemental analysis, Impurity H has the molecular formula $C_{21}H_{25}ClO_7$. By analyzing of FTIR and NMR spectrums, Impurity H has characterized as benzylic hydroxy dapagliflozin, which was first reported as a metabolite for dapagliflozin [29]. The recorded spectra are shown in Fig.10 and Fig. 11.

Impurity J, appeared at retention time of 26.521 minutes, and has a molecular weight 438 $g.mol^{-1}$. According to elemental analysis, Impurity J has the molecular formula $C_{22}H_{27}ClO_7$. By analyzing of NMR spectrum Impurity J was characterized as dapagliflozin methoxy pyranose, which is an intermediate of starting material rising during synthesis process. The recorded spectra are presented in Figs.10 and 12.

According to spectral date of Imp K, the molecular weight is 577.02 g.mol-1. In addition, the CHN analysis confirms the molecular formula is $C_{29}H_{33}ClO_{10}$. Moreover, FT-IR spectrum shows C=O stretching absorptions at $1741cm^{-1}$ (Fig. 10) and with aid of NMR (Fig. 13), the Imp K was identified as dapagliflozin tetraacetate, which is an intermediate product. The isolated impurities (H, J and K) are listed as impurities of dapagliflozin.

Table 5. Impurities of dapagliflozin API samples and different pharmaceutical dosage form (Farxiga ® and generic products).

Peak Name	RT (min)	DPG. API 1	DPG. API 2	DPG. WS.	EMG. WS	Crude	Farxiga ®	Product 1	Product 2	$[M + H]^+$ m/z
Imp A	2.323	0.009	ND	0.013	ND	0.009	ND	0.029	0.035	509.9
Imp B	2.664	0.023	0.032	0.011	ND	0.077	0.001	0.024	0.018	396.9
Imp C	3.115	0.006	0.013	0.001	ND	0.044	0.002	0.005	0.01	405.8
Imp D	3.440	0.031	0.021	0.01	0.001	0.066	0.016	0.022	0.052	475.3
Imp E	3.883	0.005	0.008	0.001	ND	0.035	0.001	0.012	0.006	366.9
Imp F	4.464	0.019	0.026	0.014	0.001	0.086	0.016	0.02	0.045	510.2
Imp G	10.635	0.022	0.04	0.021	0.004	0.036	0.024	0.035	0.044	435.5
Imp H	19.075	0.031	0.125	0.011	0.002	1.268	0.025	0.072	0.035	427.2
EMG	20.075	0.016	0.013	ND	99.95	ND	ND	0.02	0.011	451.8
DPG	22.961	98.420	99.411	99.521	0.005	82.320	99.621	96.521	97.91	409.0
DRS1	24.135	0.086	0.061	0.030	0.016	1.115	0.025	0.063	0.054	409.2
Imp I	25.015	ND	ND	ND	ND	1.14	ND	ND	ND	425.3
Imp J	26.521	0.124	0.160	0.005	ND	4.625	0.002	0.005	0.006	439.7
Imp K	39.658	0.132	0.121	0.072	0.002	6.420	0.055	0.065	0.047	579.1
DRS2	41.261	0.088	0.088	0.088	ND	0.262	ND	0.018	0.041	323.0
Imp L	44.165	0.064	0.062	0.052	0.098	0.062	0.075	0.089	0.092	326.4
Imp M	46.231	0.039	0.027	ND	ND	0.027	ND	0.027	0.051	371.0
Total Area %		99.52	100.16	99.87	100.08	98.74	99.89	97.07	98.56	

ND = Not detected in sample or peak is too small to be extracted

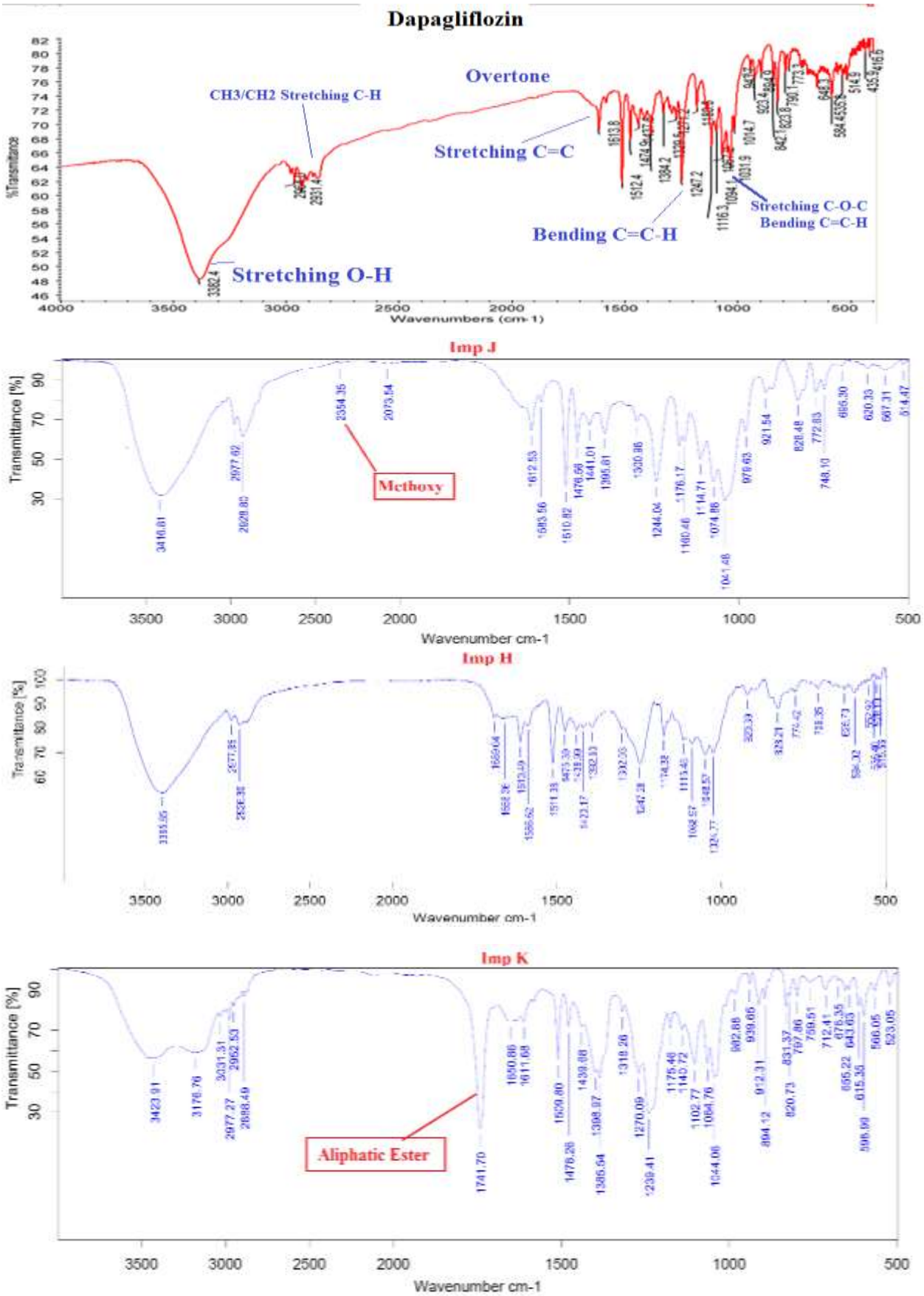
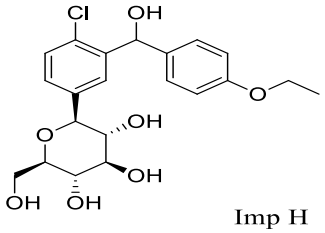


Figure 10. FTIR spectrum of dapagliflozin, impurities H, J and K.



Imp H

Chemical Formula: $C_{21}H_{25}ClO_7$
Molecular Weight: 424.87
Elemental Analysis: C, 59.36; H, 5.93; Cl, 8.34; O, 26.36

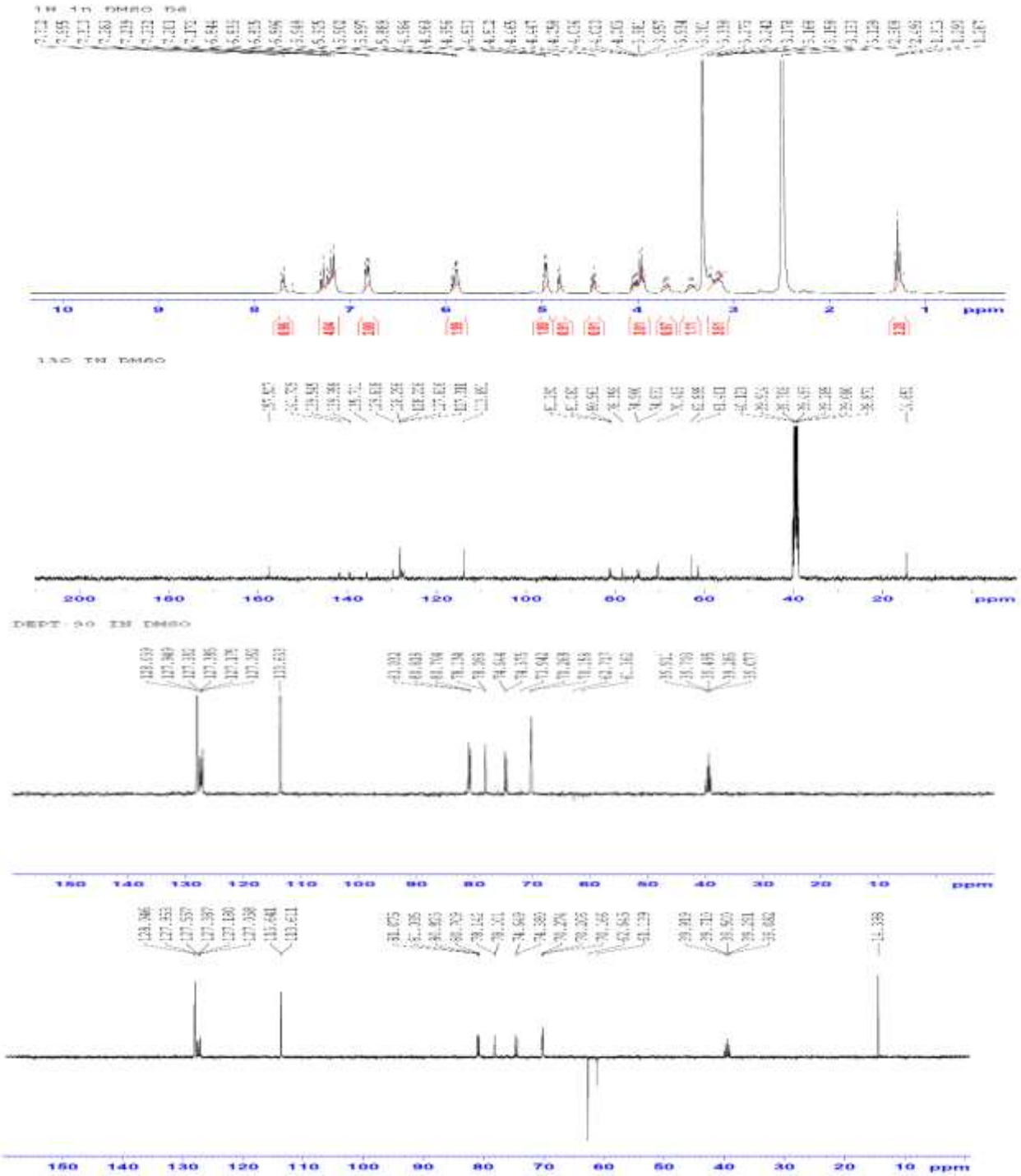
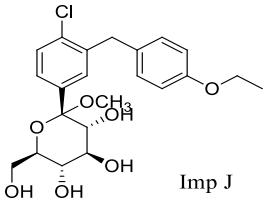


Figure 11. NMR spectrum of Impurity H using DMSO-d_6; H1-nmr (400MHz) and C13-nmr (100MHz), DEPT 90 and DEPT 135.



Imp J

Chemical Formula: $C_{22}H_{27}ClO_7$
Molecular Weight: 438.90
Elemental Analysis: C, 60.20; H, 6.20; Cl, 8.08; O, 25.52

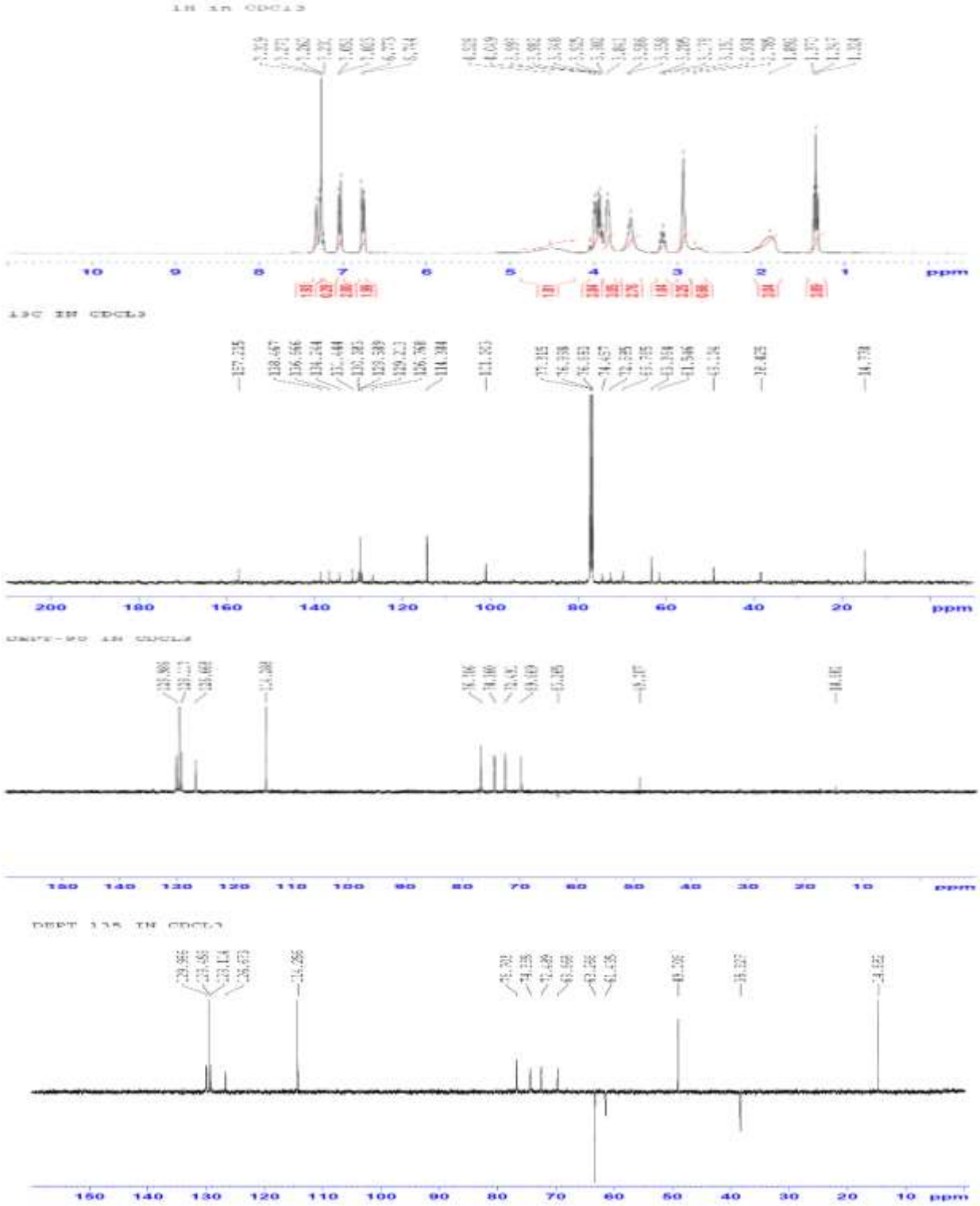


Figure 12. NMR spectrum of Impurity J using $CDCl_3$; H1-nmr (400MHz) and C13-nmr (100MHz), DEPT 90 and DEPT 135.

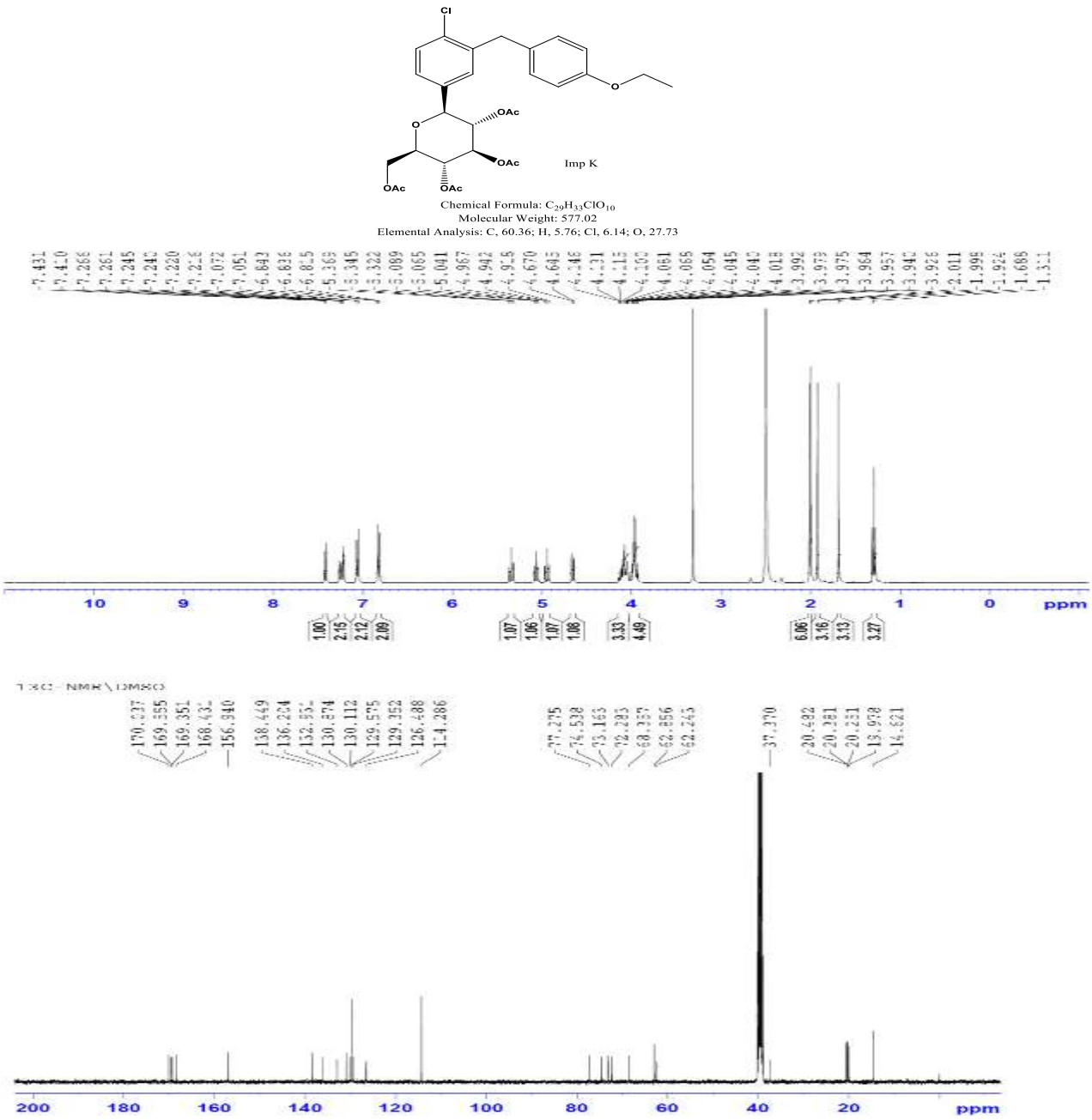


Figure 13. NMR spectrum of Imp K using DMSO-d_6; H1-nmr (400MHz) and C13-nmr (100MHz).

Conclusion:

A linear, very sensitive, and precise RP-HPLC/MS method was successfully developed and validated as per ICH guidelines. The method could be successfully applied for the simultaneous determination of dapagliflozin propanediol monohydrate and its related substances in purchased APIs and formulated dosage forms. The developed method showed stability-indicating properties and can be used for the estimation of the stability profile of dapagliflozin. The detected impurities in different samples that exceeded the acceptance limits of the ICH could be successfully isolated by the new preparative HPLC method.

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Authors' declaration:

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are mine ours. Besides, the Figures and images, which are not mine ours, have been given the permission for re-publication attached with the manuscript.
- Ethical Clearance: The project was approved by the local ethical committee in Tishreen University, Syria.

Authors' contributions Statement:

The authors confirm contribution to the paper as follows: Conception, design, acquisition of data, analysis, drafting the MS, interpretation were done by A. I. Revision and proofreading were done by M. H. and Y. A.

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التحديد الكيفي والكمي لداباغليفلوزين بروبانديول مونوهيدرات وشوائبه المتعلقة بالبنية ومنتجات تحلله باستخدام طرائق الكروماتوغرافيا السائلة المقترنة بمكشاف الكتلة والكروماتوغرافيا التحضيرية

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الخلاصة:
داباغليفلوزين هو مثبط جديد للناقل المشترك صوديوم-غلوكوز من النمط الثاني. يهدف هذا العمل لتطوير والتحقق من مصداقية طريقة جديدة وحساسة من نمط الكروماتوغرافيا السائلة عالية الأداء ذات الطور العكوس والمقترنة بمكشاف الكتلة وذلك لتحديد داباغليفلوزين ومماكبه ألفا ومادته الأولية بوجود منتجات التخرب الرئيسة لداباغليفلوزين ومعياري داخلي (إيمباغليفلوزين). أجري الفصل على عمود BDS C_{18} Hypersil ذو الطول 250 مم، والقطر داخلي 4.6 مم، وأبعاد الأجزاء 5 ميكروميتر وبدرجة حرارة للعمود 35 درجة مئوية. استخدم الماء والأسيتونيتريل كطور متحرك A وB على الترتيب، في النمط المتدرج وبمعدل تدفق 1 مل / دقيقة. تم الكشف بطول موجة 224 نانومتر وذلك باستخدام كاشف PDA. استوفت الطريقة متطلبات ICH للتحقق من مصدوقيتها، وتم تقدير الوزن الجزيئي للشوائب ونواتج التخرب باستخدام مطيافية الكتلة ذات التأين بالرذّ الالكتروني في المجال الموجب. تم الحصول على القيم المقبولة للخطية والانتقائية والدقة والمتانة للطريقة المطورة، تم الكشف عن خمسة عشر شائبة أثناء تحليل المادة الفعالة الصيدلانية والدواء ذو العلامة التجارية Farxiga وبعض منتجاته الجنيسة. تجاوزت ثلاث شوائب (H، J، K) من بين خمسة عشر شائبة مكتشفة الحدود المقبولة للشوائب 0.1٪ . عُزلت هذه الشوائب الثلاث باستخدام طريقة كروماتوغرافيا تحضيرية جديدة. ثم تم توصيفها باستخدام التحليل العنصري، ومطيافية الأشعة تحت الحمراء، ومطيافية الرنين النووي المغناطيسي.

الكلمات المفتاحية: داباغليفلوزين، منتجات التحلل مرتسم شوائب، الكروماتوغرافيا السائلة / مكشاف الكتلة، الكروماتوغرافيا التحضيرية.