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Development of novel gradient RP-HPLC method for separation of dapagliflozin and its process-related impurities: insight into stability profile and degradation pathway, identification of degradants using LCMS

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Abstract

Background In the dapagliflozin (DPF) synthesis, 5-Bromo-2-chlorobenzoic acid (5-BC impurity) and 4-Bromo-1-chloro-2-(4-ethoxybenzyl) benzene (4-BC impurity) are used as starting and reagent sources, respectively. The presence of 5-BC and 4-BC impurities in DPF could potentially affect the effectiveness of the final DPF product. The purpose of this investigation was to develop a stability indicating HPLC methodology for the separation of DPF, its process-related impurities and degradants. The method of analysis was developed on Xbridge Phenyl C18 column of dimensions, 250 × 4.6 mm, 5 µm with gradient elution using mobile phase made up of 0.05% aqueous trifluoro-acetic acid and acetonitrile (AcN).

Results The method proposed indicates a good linearity ($R^2 = 0.9996$ and 0.9993), good system precision (RSD ≤ 2%), good method precision (RSD ≤ 2%), accuracy (50–150%), LOD (0.000053 ppm and 0.0000165 ppm) and LOQ (0.00016 ppm and 0.00005 ppm) for 4-BC impurity and 5-BC impurity, respectively. LC–MS was used to detect and characterise degradants that were obtained in acid and base condition were identified and characterised. A comparison of the fragmentation pattern of the [M + H] + ions of DPF and its degradation products revealed the most likely processes for the generation of degradation products.

Conclusion DPF sample quality can be evaluated using the suggested method for the presence of 4-BC impurity, 5-BC impurity and 2-(3-(4-ethoxy benzyl)-4-chloro phenyl)-tetrahydro-6-(hydroxy methyl)-2H-pyran-3,4,5-triol.

Keywords Process impurities, Dapagliflozin, HPLC, Xbridge Phenyl, Gradient elution

Background

Estimation of process-related impurities is vital for the improvement of pharmaceuticals. Impurities a part of production process are known as process associated impurities and can include precursor substrates, Intermediates formed during the synthesis, and diluents employed in production or purification [1, 2]. The ICH mentioned that for the drug material to qualify, the allowed threshold for known process associated impurities must be less than 0.15% and the impurity linked to the unidentified process must be less than 0.10% [3, 4]. Identification of impurities and close monitoring of their concentrations are required in order to adhere to comply regulatory requirements.

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Dapagliflozin (DPF) is an antidiabetic drug with Na^{2+}-glucose cotransporter 2 blocking activity [5, 6]. DPF limits the resorption of Na^{2+} and glucose into the renal proximal tubule by blocking the cotransporter system resulting in greater efflux of urinary glucose. 5-BC impurity and 4-BC impurity are utilised as starting and reagent materials, respectively, in the dapagliflozin synthesis process [7, 8]. Moreover, 5-BC impurities and 4-BC impurities that copurify with the DPF frequently appear after so many purification stages. The presence of 5-BC and 4-BC contaminants in DPF has the potential to affect the final DPF drug's efficacy. Consequently, to identify and assess 5-BC and 4-BC impurities in DPF, a sensitive, trustworthy, and efficient approach is needed. According to the new drug substances policy stated in ICH Q3A, the specification levels for 5-BC and 4-BC impurities were taken into consideration as 0.15% concentration. To obtain meaningful information in a short period of time, forced degradation studies are an unavoidable element of the drug development cycle [9]. Determining the potential degradation products, pathways, and inherent stability of the drug molecule through stress testing is helpful [10]. Assessing the toxicity of degradants is critical because the ICH Q3 criteria include strict reporting, identification, characterisation, and qualification limits [11, 12].

Caroline et al. [13] reported quantification of DPF and its associated six impurities among employing BDS Hypersil C18 column, and mobile phase made up of phosphoric acid buffer with pH 6.5 and acetonitrile/water (90:10) through gradient programme. Detection and evaluation of 5-BC impurity in DPF using HPLC approach are not yet documented. Hence, the goal of the study was to develop an RP-HPLC method for studying the separation profile of drug and its impurities and identify whether any new degradants are formed during stress conditions or the starting materials itself using LCMS.

Method

Chemicals

HPLC grade Qualigens (Mumbai, India) trifluoroacetic acid, methanol and acetonitrile, (Pune, India).

Instrumentation

Waters (USA) HPLC2105 system, Waters (USA) photodiode array detector 2487 system, Waters (USA) Empower version 2 software, Scaletec (Vadodara, India) SAB224CL electronic balance, Enertech (Mumbai, India) SE60USultrasonicator, Smis (New Delhi, India) PH-7000 pH meter and Merck (Bangalore, India) Millipore 0.45 microns filter paper.

Conditions of chromatography

Xbridge Phenyl C18 column, 250×4.6 mm, 5 µm was used at room temperature with gradient elution occurring at flow rate of 1 ml per min. 0.05% aqueous trifluoroacetic acid (Phase I) and acetonitrile made up the mobile phase (Phase II). The gradient programme was: 0 min (80% Phase I and 20% Phase II), 3 min (50% Phase I and 50% Phase II), 10 min (30% Phase I and 70% Phase II), 13 min (30% Phase I and 70% Phase II), 19 min (80% Phase I and 20% Phase II) and 25 min (80% Phase I and 20% Phase II). In detection and evaluation of 5-BC and 4-BC impurities in DPF, sample volume of 10 µl and wavelength of 240 nm were utilised. For LC–MS study, the same methodology as for HPLC was used. To achieve high sensitivity and a good signal, the ESI source conditions were also tuned. Various conditions such as drying gas flow, nebulizing gas flow, capillary voltage, and spray voltage were tuned to increase sensitivity at low concentrations in order to identify and characterise degradation products.

LC MS study:

1. Optimised MS Conditions:
   - Collision energy: 15 V.
   - Ion spray voltage: 5500 V.
   - Source temperature: 550 °C.
   - Drying gas temperature: 120–250 °C.
   - Collision gas: nitrogen.
   - Drying gas flow stream: 5 L/min.
   - Declustering potential: 40 V.
   - Entrance potential: 10 V.
   - Exit potential: 7 V.
   - Dwell time: 1 s.
2. LC–MS/MS instrument details: LC–MS: Waters Alliance e2695 HPLC coupled to SCIEX QTRAP 5500 mass spectrometer equipped with electrospray ionisation (ESI).
3. Software: SCIEX.

The mass spectrometer was managed in positive ion electrospray ionisation interface mode.

Solutions of 5-BC and 4-BC impurities

In methanol, a mixed impurity stock solution (0.5%w/v) for 5-BC and 4-BC was made. A series of working solutions were created by adding methanol to the suitable

aliquots of the mixed impurity stock solution (0.5% w/v), which ranged in concentration from 0.01 to 0.225% for 5-BC and 0.03 to 0.225% for 4-BC. By combining a suitable amount of mixed impurity stock solution (0.15%w/v concentration) with methanol, a working solution of 5-BC and 4-BC with 0.15%w/v concentration was also created.

PF sample

This methanol-prepared solution had a concentration of 0.5 mg/ml. The DPF sample was sonicated for 20 min and filtered through Millipore 0.45µ microns.

Procedure to evaluate 5-BC and 4-BC impurities in DFP sample

Diluent solution, working solutions of 5-BC and 4-BC, and DPF solution were infused (10 µl) after column equilibration for 30 min. Chromatograms were then recorded using the recommended HPLC procedure. The peak area of 5-BC and 4-BCimpurities in DFP solution and in working solution were documented.

Trails

Inertsil ODS 3 V (150 mm × 4.6 mm, 5 µm), Inertsil ODS (250 mm × 4.6 mm, 5 µm), and Inertsil C8 column (150 mm × 4.6 mm, 5 µm) with isocratic elution using solvents combinations like 0.1% aqueous formic acid/MeOH (methanol) (50:50 ratio), 0.1% aqueous formic acid/MeOH (methanol) (40:60 ratio), and 0.1% trifluoroacetic acid/acetonitrile (40:60 ratio) were tried during trail experiments. A Xbridge Phenyl C18 (250 mm × 4.6 mm, 5 µm) column with gradient elution with 0.05% trifluoroacetic acid/AcN (acetonitrile) combination as mobile phase was also tried. The sample volume for analysis, temperature, and flow rate were all held constant during trials at 10 µl, room temperature, and 1.0 ml/min, respectively. Xbridge Phenyl C18 (250 mm 4.6 mm, 5 m) column with gradient elution using mobile phase 0.05% trifluoroacetic acid (Phase I)/acetonitrile (Phase II) was chosen as the best conditions to identify and estimate 5-BC and 4-BC impurities concurrently in DPF based on resolution, peak shape, and sensitivity values attained during trials. The gradient programme opted was: 0 min (80% Phase I and 20% Phase II), 3 min (50% Phase I and 50% Phase II), 10 min (30% Phase I and 70% Phase II), 13 min (30% Phase I and 70% Phase II), 19 min (80% Phase I and 20% Phase II), and 25 min (80% Phase I and 20% Phase II). 5-BC and 4-BC impurities were studied at 210 nm because this is the wavelength at which they were most sensitive.

Validation

The method for 5-BC and 4-BC impurities evaluation in DPF was proved in harmony through ICH approaches [14].

System suitability

To check suitability of the HPLC system, DPF samples spiked at 0.15% concentration with 5-BC and 4-BC impurities were analysed six times by way of suggested HPLC method.

Specificity

To confirm that the DPF and diluent did not interfere with the analysis of 5-BC and 4-BC impurities, the specificity of this procedure was examined. The recommended HPLC method was used to prepare and analyse the DPF sample (0.5%), each impurity solution (0.15%), solution of DPF spiked with 5-BC and 4-BC impurities (0.15%), and diluent blank and proves that DPF shows no effect on analysis of 5-BC and 4-BC impurities is unaffected by DPF. The blank peak, in contrast, did not overlap the impurity peaks of 5-BC and 4-BC. It is therefore a very selective procedure.

Quantification and detection limits

The quantification and detection limits for 4-BC and 5-BC impurities at concentrations that result in S/N fractions ≥ 10 and ≥ 3, respectively, were confirmed. The quantification limit for 4-BC and 5-BC impurities was 0.00016 ppm and 0.00005 ppm respectively. The detection limits were 0.000053 ppm and 0.0000165 ppm for 4-BC and 5-BC impurities, respectively.

Linearity

The quantification limit level (0.03% for 4-BC impurity and 0.01% for 5-BC impurity) to 150% of the specification quantity limit (0.225% for both 4-BC and 5-BC impurities) was used to confirm the linear quantity range for 4-BC and 5-BC impurities.

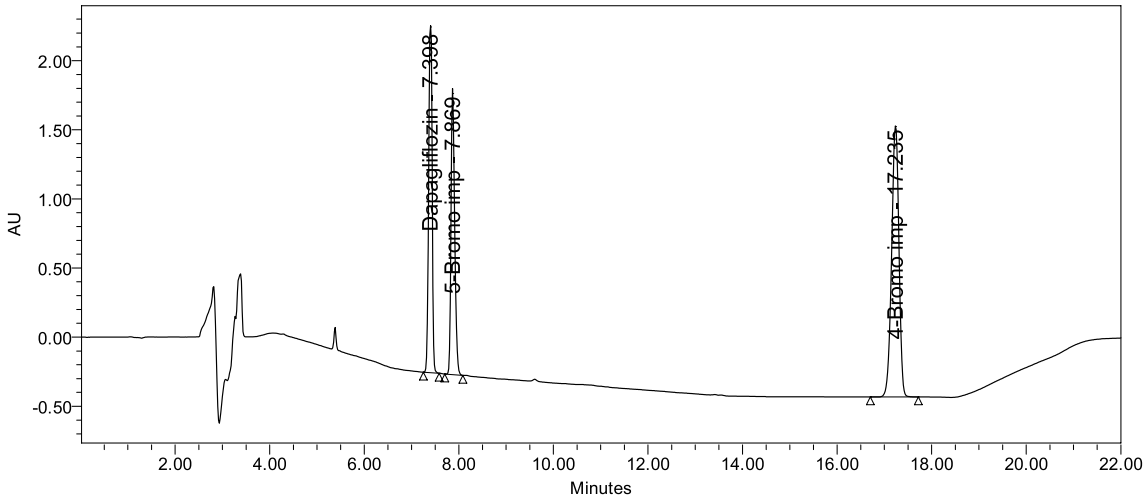

Fig. 1 Typical chromatogram of DFP, 5-BC impurity, and 4-BC impurity

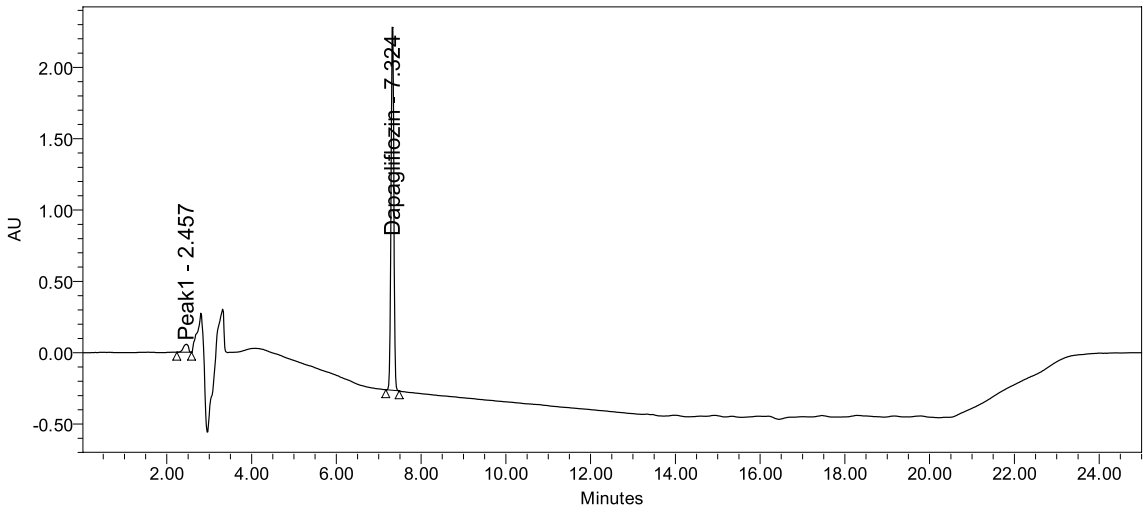

Fig. 2 Chromatogram for acid degradation

Accuracy

Replicates ($n=3$) of the DFP sample were spiked with the appropriate concentrations of 4-BC and 5-BC impurities at LOQ levels, 50%, 100%, and 150% of the specified quantity limit.

Robustness

To validate robustness, DPF sample solution spiked with 5-BC (0.15%) and 4-BC (0.15%) impurities was analysed by way of recommended HPLC method with slight dissimilarities in wavelength (± 2 nm) and flowrate (± 0.1 ml per min) and column temperature (± 2 °C).

Precision

The system and method precision were verified by analysing the working solution (0.15% of 5-BC and 0.15% of 4-BC) and DFP sample spiked with 5-BC (0.15%) and 4-BC (0.15%), respectively. The system precision was expressed as mean area response and RSD of six peak area responses of 4-BC impurity and 5-BC impurity. The mean concentration quantified and RSD of six quantified values of the 4-BC impurity and 5-BC impurity were used to express the method's precision (Fig. 1).

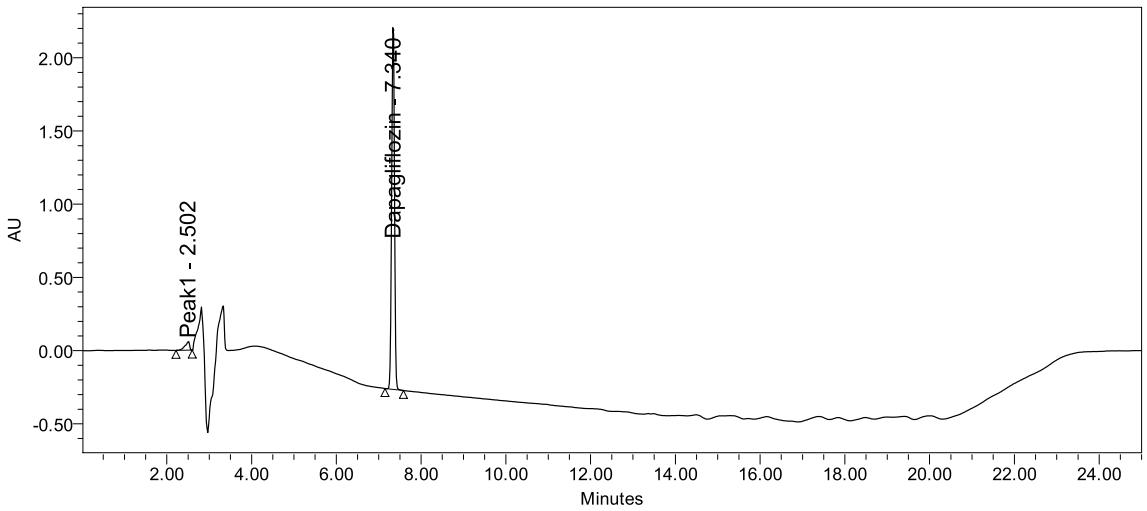

Fig. 3 Chromatogram for base degradation

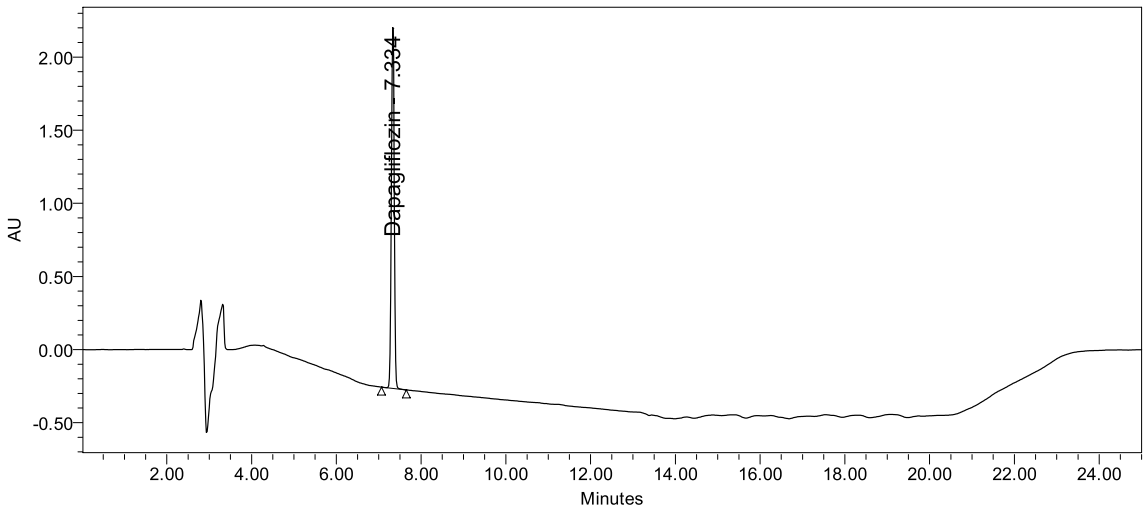

Fig. 4 Chromatogram for H_2O_2 degradation

Stability studies

According to ICH guidelines, stress degradation tests of Dapagliflozin were performed in the presence of heat, oxidation, photolytic, and hydrolysis (acid, base, and neutral). Acidic and basic hydrolysis were executed for 24 h in 2N HCl and 2N NaOH, respectively (Figs. 2, 3). Oxidative degradation was carried out at ambient temperature using 30% H_2O_2 (Fig. 4), and in the photostability chamber, solid drug in the form of thin layer taken in a Petri dish was exposed to a UV lamp (200-Wh/m^2) at 240 nm for 7 days (Fig. 5). Sample is thermally degraded by placing the sample in an oven at 105 °C for 6 h (Fig. 6).

Table 1 Robustness data

Parameter	Resolution b/w DFP and 5-BC impurity
Flow rate 1.0 ml/min (actual)	3.4
Flow rate 0.9 ml/min, (low flow)	3.8
Flow rate 1.1 ml/min, (high flow)	3.3
Wavelength 210 nm (actual)	3.4
Decreased wave length 208 nm	3.8
Increased wave length 212 nm	3.7
Temp minus (28 °C)	3.7
Temp plus (32 °C)	3.8
Temp optimum (30 °C)	3.6

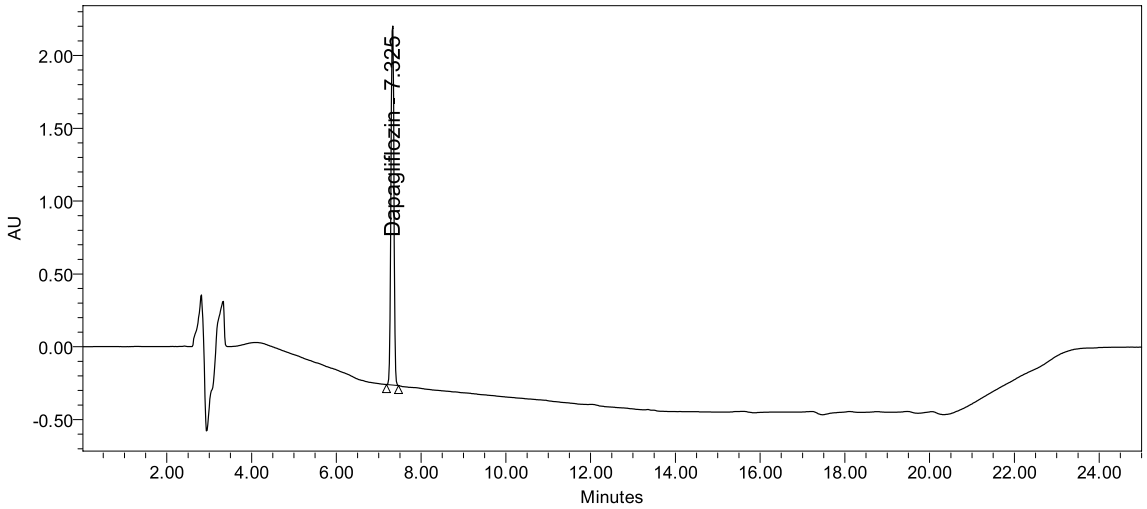

Fig. 5 Chromatogram for photodegradation

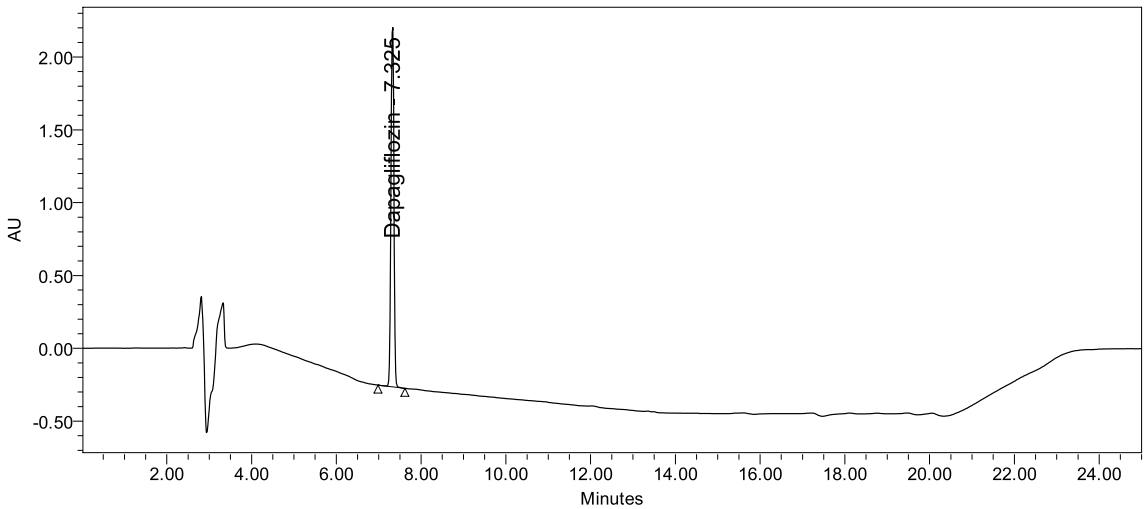

Fig. 6 Chromatogram for thermal degradation

Results

Optimised chromatographic conditions

Buffer 0.05% trifluoroacetic acid (TFA) in Water.
Mobile Phase Buffer: Acetonitrile.
Column Xbridge Phenyl 250*4.6, 5um.
Flow Rate 1.0 ml/min.
Temperature Ambient.
Volume 10ul.
Detector 210 nm.
Diluents Methanol.

Gradient method

Time	Flow	A	B
0.0	1.0	80	20
3.0	1.0	50	50
10.0	1.0	30	70
17.0	1.0	30	70
19.0	1.0	80	20
25.0	1.0	80	20

Validation

System suitability

The retention time, theoretical plates, peak symmetry, and resolution for peaks of DPF, 5-BC, and 4-BC impurities were observed. The suitability of the HPLC method

Table 2 Results of validation parameters

Parameter	Results			ICH limits
	DPF	5-BC	4-BC	
Retention time	7.389 min	7.952 min	17.142 min	–
System suitability				
% RSD	1.0	1.1	1.5	NMT 2.0
Theoretical plates	53,695	53,031	66,564	> 2000
Tailing factor	1.29	1.14	0.98	NMT 2.0
Linearity	50–150%	0.01–0.225	0.03–0.225	–
% recovery	98.6–101.2	100.1–102.8	100.1–102.8	98–103%
System precision (% RSD)	0.4	1.26	1.49	NMT 2.0
Method precision (% RSD)	0.4	0.82	0.71	NMT 2.0
LOD (ppm)	0.0000627	0.0000165	0.000053	–
LOQ (ppm)	0.00019	0.00005	0.00016	–

Table 3 Results of forced degradation studies of DPF

Sr.no	Degradation condition	DPF %	Degraded %
1	2N HCl	96.18	3.82
2	2N NaOH	96.40	3.60
3	30% H_2O_2	100	0
4	Heat	100	0
5	U.V	100	0

Table 4 Multiple reaction monitoring of acid and alkali dapagliflozin

Analogue	Precursor ion (*m/z*)	Daughter ion with highest intensity(*m/z*)
Acid and alkali DPF(RT-7.324)	409.27	320.42

for the evaluation of 5-BC and 4-BC impurities in the DPF sample was verified by the measured values.

Linearity

A linear correlation was observed a mid-peak area responses and concentrations of 4-BC and 5-BC impurities in 0.15–0.225% range and 0.15–0.225%, respectively.

The coefficient of correlation was 0.9993 for 5-BC impurity and 0.9996 for 4-BC impurity which reveals that the process was linear in the concentration range investigated. The regression equations were: $y = 80{,}321{,}692.31x - 88.89$ for 5-BC impurity and $y = 35{,}517{,}268.23x - 1407.49$ for 4-BC impurity.

Precision

The relative standard deviation calculated for 4-BC impurity and 5-BC impurity was noticed as < 2% which demonstrates that system and method were precise for evaluation of 4-BC impurity and 5-BC impurity.

Robustness

In all different and ideal circumstances, the resolution between DPF and 5-BC impurity was reported (Table 1). The findings had indicated that resolution value changes are not significant; hence, method was considered as robust.

Accuracy

Samples were evaluated using the proposed HPLC method, and the recoveries of DPF, 4-BC impurity, and 5-BC impurity at each level were determined. The evaluated values of recoveries of 4-BC impurity and 5-BC impurity for the advised method used were in the range 100.1–102.8% which shows that method was exact enough for estimation of 4-BC impurity and 5-BC impurity in DPF sample.

Quantification and detection limits

Precision examination was used to confirm the quantification limit values for the 4-BC and 5-BC impurities. %RSD of six peak area responses of 4-BC impurity and 5-BC impurity at their quantification limit level were 1.04% and 1.06%, respectively (Tables 2, 3).

Degradation behaviour'

The drug was 96% degraded in both acidic and basic hydrolysis, resulting in the emergence of one more peak. Both degradates total ion chromatograms were similar, revealing a molecular ion at *m/z* 320.42, confirming the existence of the same degradation product in both cases, as determined by LC–MS. The degradant obtained in the LC–MS study requires further analysis for the identification of that particular compound. The *m/z* values of all degradates and associated fragmentation ions are listed in Table 4. Figure 7 depicts the LC–ESI–MS–spectra of all degradation products. Figure 4 depicts the hypothesised fragmentation mechanism for DPF's hydrolysis (acidic and basic) breakdown product. LC–MS was used to evaluate the chromatograms of acid and base hydrolysis degradation products.

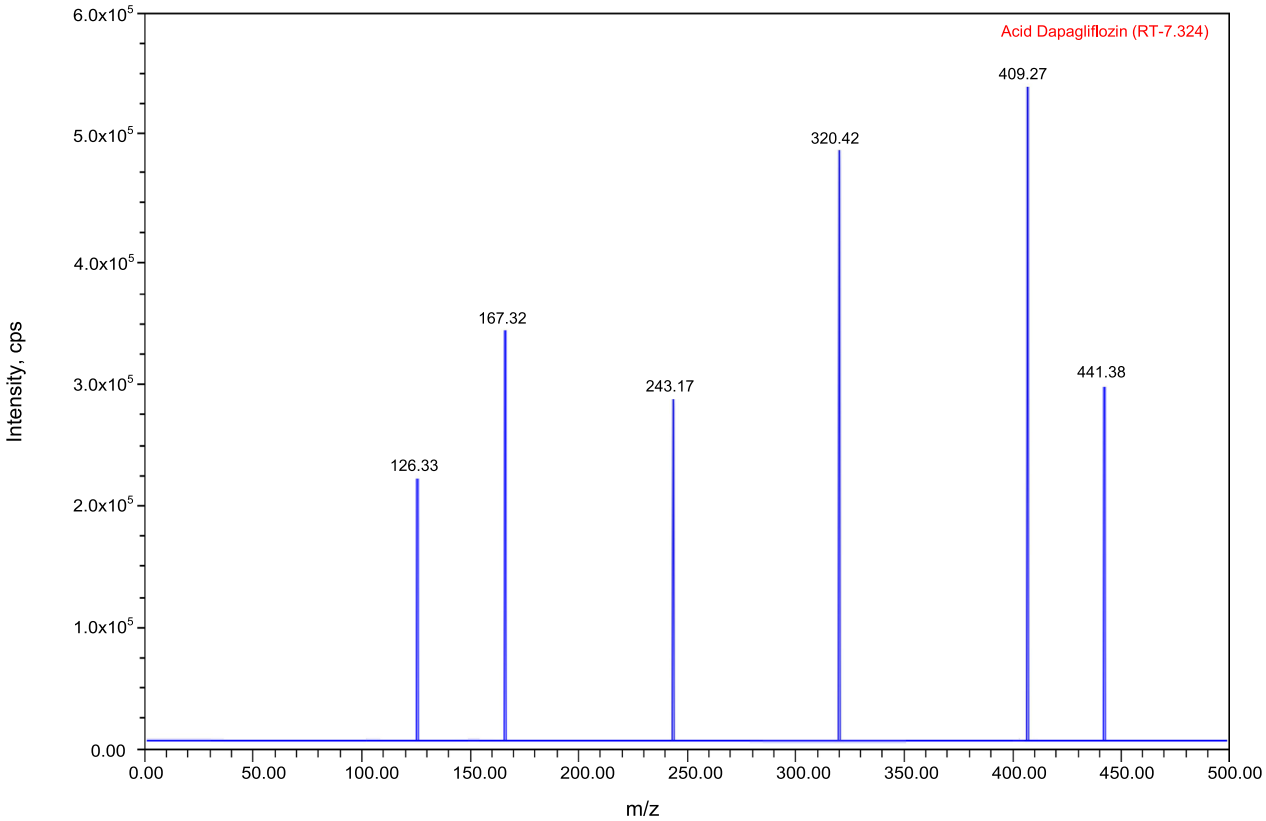

Fig. 7 Mass spectra of acid and alkali dapagliflozin

Discussion

Very few methods were reported for the estimation of impurities of dapagliflozin. Efforts were made to develop an effective HPLC method compared to the other methods. This study develops and validate a reliable and efficient HPLC-dependent approach to concurrently evaluate and detect 5-BC and 4-BC contaminants in DPF sample. All the analytes were eluted below 20 min when compared to the other methods which proves the method is preferable, RT in the current method was 7.3 min for DPF, 7.9 min for 5-BC, and 17.1 min for 4-BC. System suitability values prove the method can be applied for the detection of impurities in the drug. We can say that the peaks were eluted well and in good shape according to the data obtained and hence it is specific. An excellent correlation was found between peak area and concentration of dapagliflozin and impurities, which proves the method is linear. The % recovery values were within the acceptance range proving the method is accurate. The validation parameters for DPF, 5-BC impurity, and 4-BC impurity were incompliance with ICH criteria requirement. The presence of degradation chemicals or contaminants in drug can impair their efficacy and safety (Fig. 8).

Conclusion

The drug products quality and safety are effected by the impurities as well as the manufacturing process used and the toxicological characteristics of the active ingredients. Consequently, a thorough assessment of impurities is crucial for the regulating the quality of the drug product. The present research includes development of a simple, accurate and specific RP-HPLC method for the separation of Dapagliflozin and its associated impurities and stability studies of Dapagliflozin also. The stability studies on DPF were carried out under ICH-recommended settings to investigate its degradation profile and characterise the structures of the degradants. Our findings suggested that the method suggested in this study can be used to evaluate the quality of DPF samples.

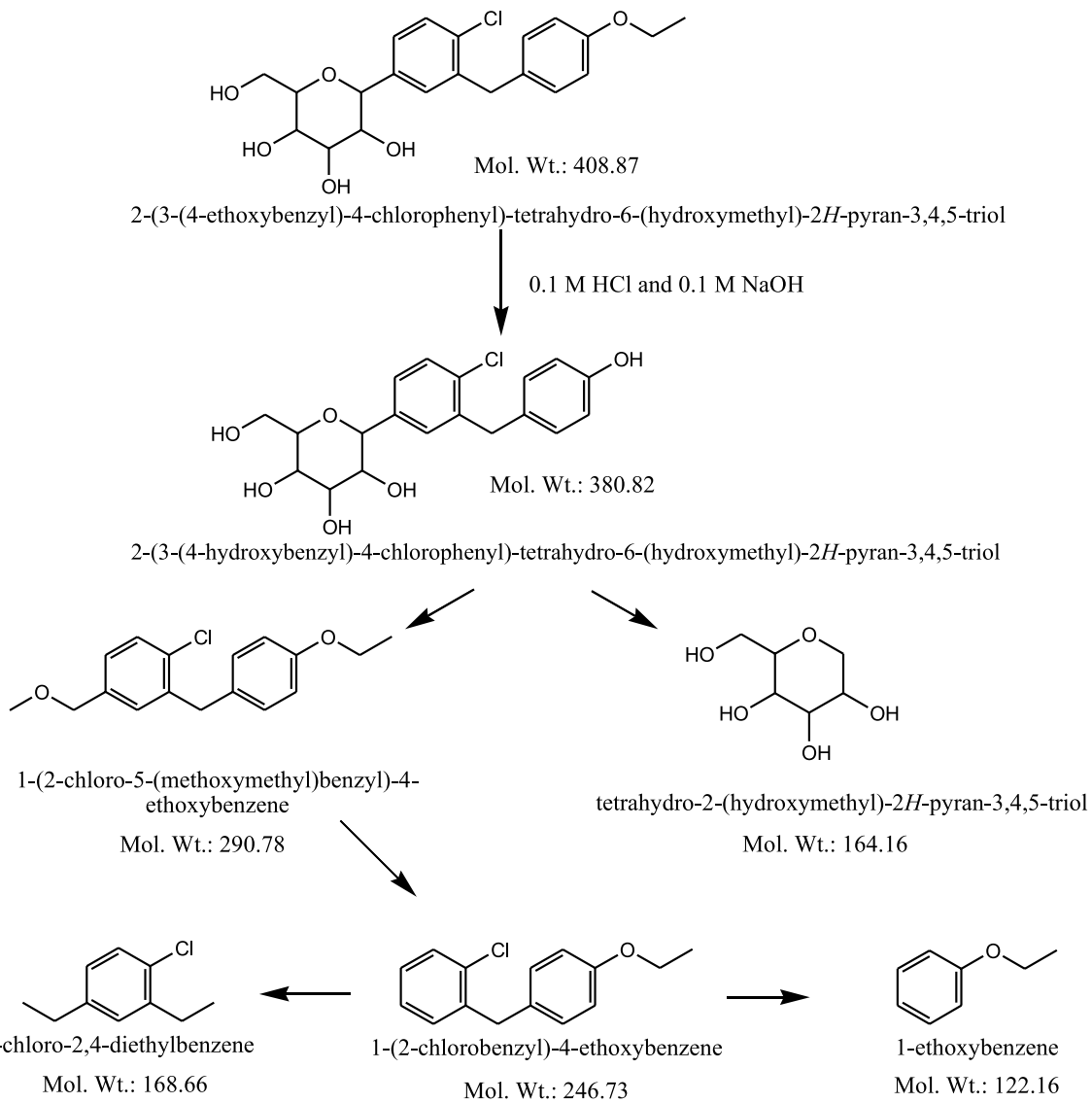

Fig. 8 The suggested fragmentation paths of DPF degradation products under acid and base hydrolysis

Abbreviations

DPF	Dapagliflozin
RT	Retention time
LOD	Limit of detection
LOQ	Limit of quantification
PPM	Parts per million
RSD	Relative standard deviation
PDA	Photodiode array
MeOH	Methanol
TFA	Trifluoroacetic acid

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Author contributions
All the authors contributed equally in carrying out his work, analysing the data and in the preparation of this manuscript. All the authors have approved this manuscript.

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Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
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Competing interests
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