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Isolation, characterization and cumulative quantification approach for nine organic impurities in empagliflozin and linagliptin drugs

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The aim of the study is to identify impurities in empagliflozin and linagliptin by using ^{1}H & ^{13}C NMR, LC-MS, preparative, and analytical HPLC. To identify and quantify contaminants, a novel HPLC method has been validated. A gradient programme has been used to separate four linagliptin impurities and five empagliflozin impurities, process-related and degradation impurities that produced under stress, on the ACE PFP C18 column. Mobile phase A is acetonitrile and 0.1% orthophosphoric acid. As the mobile phase B, methanol and acetonitrile are combined in a 10:90%v/v. Pump flow is adjusted to 1.0 mL/ min and 230 nm wavelength was chosen at 40 °C column oven temperature. During stress conditions, acid, humid, base, ultraviolet light, thermal, and oxidative processes are carried out. The separation is achieved between the peaks and degradation products. The method is validated with respect to current ICH guidelines for solution stability, specificity, linearity, precision, accuracy, and robustness. Preparative HPLC is used to isolate two unknown impurities, which were then identified using LC-MS, ^{1}H & ^{13}C NMR, based on literature, This is the first research study to propose nine impurities in mixed dosage forms of empagliflozin and linagliptin with quantitative method.

Keywords: Empagliflozin, Force degradation, HPLC, Impurity, Linagliptin

Introduction

The fixed dose combination of two active pharmaceutical ingredients empagliflozin and linagliptin is called Glyxambi. The first combo medication to treat type 2 diabetes has a dual mechanism. Empagliflozin is available in two dosage strengths (10 mg and 25 mg), whereas linagliptin is available in a single dose (5mg). Linagliptin is an oral, xanthine-based DPP-4 inhibitor that is selective. Linagliptin lowers blood sugar levels similarly to other DPP-4 inhibitors by extending the half-life of the hormone GLP-1, also known as glucagon-like peptide-1, which is secreted in response to eating. The renal proximal tubules express empagliflozin, a strong and specific SGLT-2 inhibitor that accounts for around 90% of renal glucose reabsorption. SGLT-2 inhibition decreases blood glucose levels by increasing excretion of glucose in urine and decreasing renal reabsorption of glucose[1,2].

Empagliflozin is sodium glucose co-transporter 2 (SGLT2) inhibitors that provides a platform for lowering blood sugar levels that is independent of insulin. An effective SGLT2 inhibitor is empagliflozin[3-5]. Linagliptin is xanthine-based DPP-4 inhibitor[6-9]. Review of literature demonstrates that empagliflozin and linagliptin can be calculated either individually or in combination with another active pharmaceutical ingredient by many methods, which include by spectrophotometric[10-15], spectrofluorometric method[16,17], mass spectroscopic method[18-22], high performance thin layer chromatography[23-25], electrochemical and amperometry methods[26] and the HPLC method[27-32]. However, there is some information available on the degradation behaviour of linagliptin and the isolation of impurities[33-37].

None of the literature shows the organic volatile impurities in empagliflozin and linagliptin combined dosage form, none of the analytical methods were reported. Based on literature survey, we conclude that the method is not reported for organic volatile impurities of empagliflozin and linagliptin combined dosage forms (i.e., tablets). The goal of this work was to create a single, efficient HPLC method for separating empagliflozin and linagliptin, their potential impurities, and degradation impurities during acid hydrolysis, oxidative degradation, alkali hydrolysis, photolytic and thermal, stress conditions studies. There are five potential impurities that are possible in empagliflozin, like PTSA (p-toluenesulfonic acid), methoxy empagliflozin, condensed phenol, and furanose impurities (Fig. 1). There are four potential impurities: N-formyl

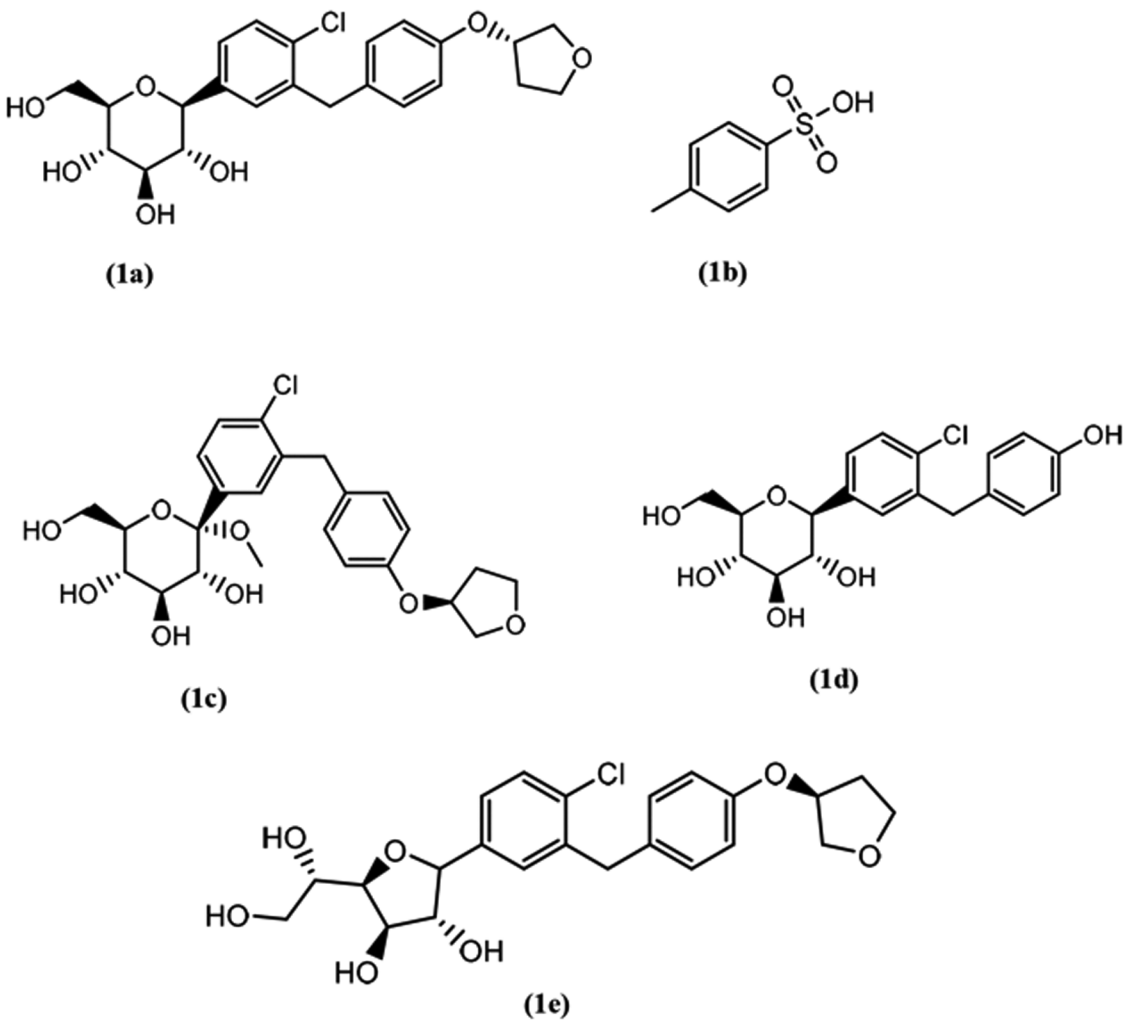

Fig. 1 — Empagliflozin drug and it's impurities structure: (1a) Empagliflozin Drug, (1b) Para toluene sulphonic acid impurity, (1c) Methoxy Empagliflozin impurity (1d) Empacondensed phenol impurity and (1e) Furanose impurity

linagliptin, N-aminoacyl linagliptin, and N-acetyl linagliptin impurities (Fig. 2). A total of nine compounds, including all potential impurities, empagliflozin and linagliptin, are quantified parallelly using this developed organic volatile impurities method.

Experimental Section

Chemical and Reagent

Zydus Life Science Limited, Gujarat, India, provided the medicinal ingredients empagliflozin and linagliptin, as well as any potential contaminants. HPLC grade water, gradient grade acetonitrile, methanol, orthophosphoric acid, hydrogen peroxide, hydrochloric acid, and sodium hydroxide were acquired from Merck.

Preparation of Mobile Phase and Diluent

The mobile phase A used in the gradient liquid chromatographic technique has a buffer: acetonitrile ratio of 88:12 (%v/v). The mobile phase B was used an organic blend of acetonitrile and methanol that was 90:10 (%v/v). To make the buffer, 1.0 mL of orthophosphoric acid was diluted with 1000 mL of filtered water and thoroughly mixed. The pH of the buffer solution was 2.3. As a diluent, water, methanol and acetonitrile were utilised (50:25:25 %v/v/v).

Standard preparation

30 mL of diluent, 5.04 mg of empagliflozin standard, 5.03 mg of condensed phenol of empagliflozin standard, 4.98 mg of PTSA standard, 5.05 mg of methoxy empagliflozin, 4.99 mg of furanose impurity standard and 2.5 mg of linagliptin

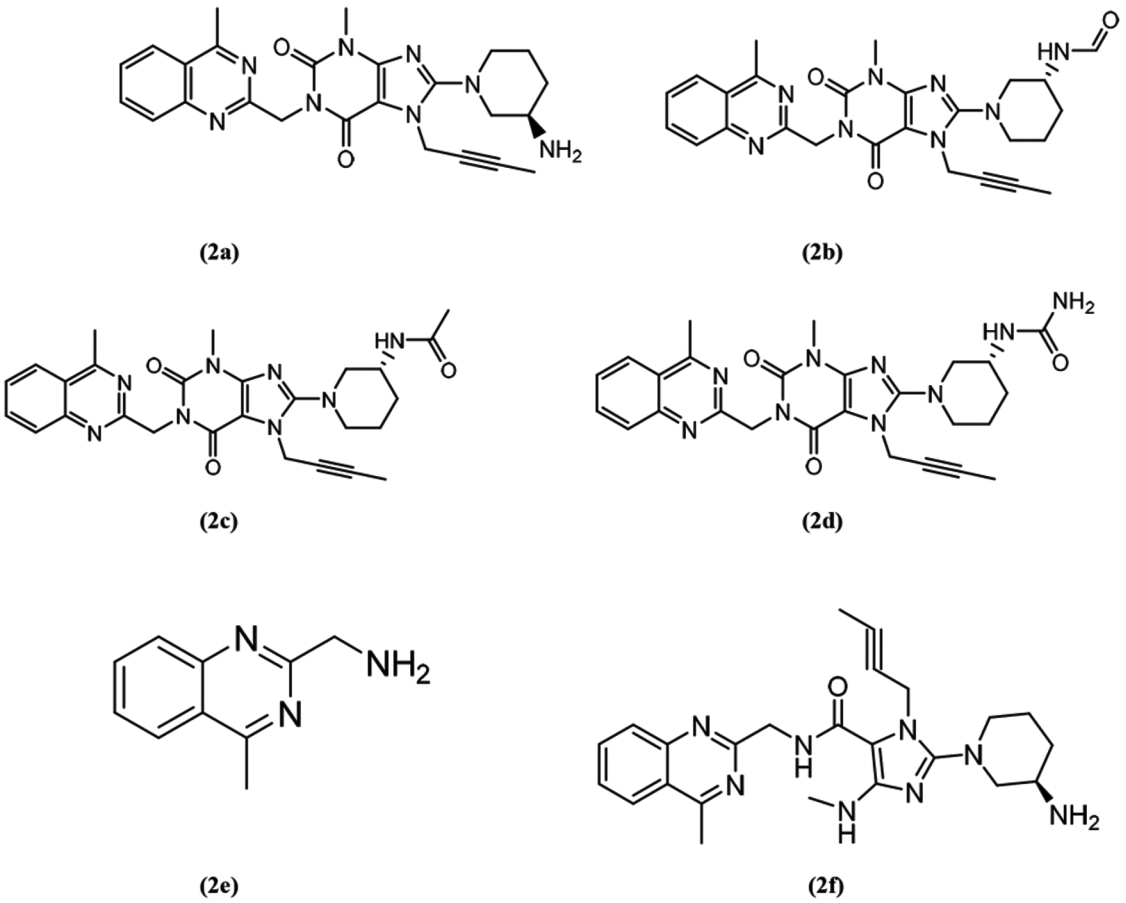

Fig. 2 — Linagliptin drug and it's impurities structure: (2a) Linagliptin Drug, (2b) N-formyl linagliptin impurity, (2c) N-acetyl linagliptin impurity, (2d) N aminoacyl linagliptin impurity, (2e) 4-methyl-2-quinazoline methanamine and (2f) (R)-2-(3-aminopiperidin-1-yl)-1-(but-2-yn-1-yl)-4-(methylamino)-N-((4-methylquinazolin-2-yl)methyl)-1H-imidazole-5-carboxamide impurity

standard, 5.10 mg of N-acetyl linagliptin, 5.12 mg of N-formyl linagliptin, 5.09 mg of N-aminoacyl linagliptin, were added to a 50 mL volumetric flask. Liquid was then sonicated to dissolve the particles. All the ingredients were mixed after increasing the amount of diluent to the desired level. To achieve concentrations of 2 ppm empagliflozin, 2 ppm condensed phenol of empagliflozin, 2 ppm PTSA, 2 ppm methoxy empagliflozin 2 ppm furanose and 1 ppm linagliptin, 2 ppm N-acetyl linagliptin, 2 ppm N-formyl linagliptin, 2 ppm N-aminoacyl linagliptin the aforementioned solution was diluted.

Sample preparation

Equivalent to 10 mg linagliptin and 20 mg empagliflozin were weighed into a 50 mL flask. 30 mL of diluent was added, and the sonicator was run for 10 min while being shaken vigorously every 2 min for 30 s and cooled at RT. The volume was made up with diluent and mixed. Solution was further filtered through MDI Nylon 0.45 µm. Collect the filtrate. The concentration of the sample was 200 ppm of linagliptin and 400 ppm of empagliflozin. In order to prepare samples for method development, all empagliflozin and linagliptin impurities were dissolved in diluent and spiked according to the specified levels. (Fig. 3).

Experiment design

To identify all the impurities smoothly, initially empagliflozin and linagliptin drugs and their impurities were developed under different stress conditions and separated on analytical HPLC. The HPLC method was developed and validated. Through a comparison of retention time (RT) with reported impurities, seven impurities were identified. Two major degradation impurities were detected at RT of 4.80 min and 8.70 min, with 4.80% area and 6.66% area, respectively, and labelled as unknown Impurity-I and unknown Impurity-II. Solution was injected into

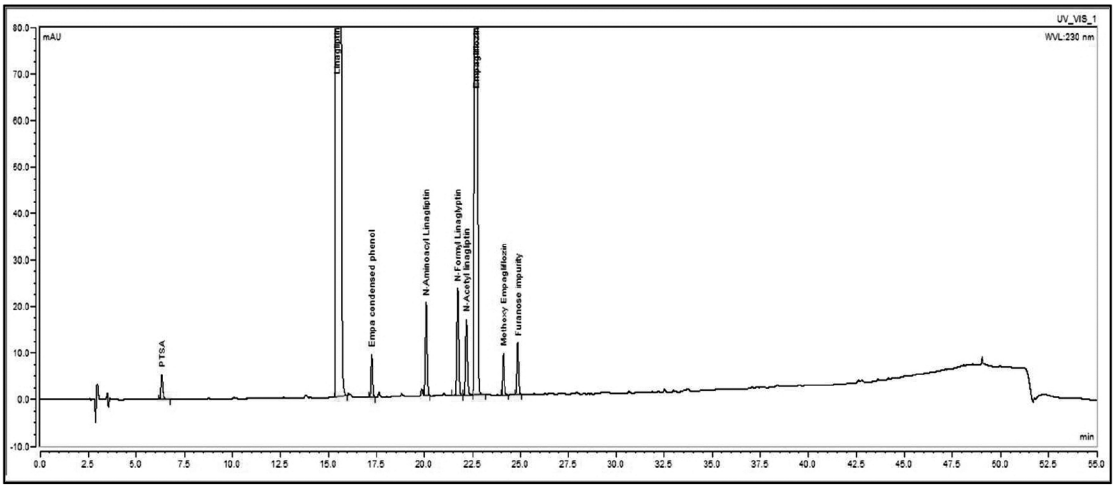

Fig. 3 — Chromatogram of the spiked sample solution

the LC-MS method to investigate the molecular weight of unknown Impurity-I and impurity -II. Through preparative HPLC, both unidentified contaminants were isolated and characterised with NMR, but unknown Impurity-I and impurity -II did not match any captured impurity or intermediate.

Chromatographic conditions optimization

The determination and quantification of the organic volatile impurities of empagliflozin and linagliptin, were performed using the Dionex Ultimate 3000 UHPLC system. It was equipped with a degasser, quaternary pump, autosampler and PDA detector. The Chromeleon software was used to measure the output signal and processing.

The creation of chromatographic techniques attempts to distinguish known impurities of both analytes from potential impurities that may have been created during the force degradation research from the peaks of empagliflozin and linagliptin. To achieve the separation, trials were started with polar columns such as cyano, and phenyl hexyl. In the phenyl hexyl column, N-amino acetyl linagliptin peak shape was not proper and N-amino acetyl linagliptin and N-formyl linagliptin eluting very close to each other. Development trials were also carried out in cyano column. In this study, separation was not found satisfactory as few peaks were merged. So, it was concluded that separation and peak shape were probably difficult in polar columns. Development trials were initiated with non -polar column. In the C8 column, N-formyl linagliptin, N- acetyl linagliptin, and empagliflozin were eluted very closely. Resolution was very poor. In the PFP C18 column, orthophosphoric acid in a 0.1% aqueous solution is present in mobile phase A, whereas acetonitrile is present in mobile phase B. Two impurities N-formyl linagliptin and N-acetyl linagliptin were co-eluted with empagliflozin. Further, trials were conducted with the help of organic modifiers such as methanol. For more separation 10%methanol was added in the mobile phase B. Two impurities N-formyl linagliptin and N-acetyl linagliptin were slightly separated from empagliflozin. 10% acetonitrile was added in mobile phase A. All peaks are separated from each other. For more separation again 2% more acetonitrile was added in mobile phase A and resolution were good. Based on results of development trials, chromatographic conditions were finalized.

Equipment and chromatographic conditions

Separation was accomplished on ACE 3C18 PFP (250 x 4.6 mm), 3 µm. The mobile phase A used in the gradient liquid chromatographic technique was a buffer: acetonitrile ratio of 88:12 (%v/v).The mobile phase B used was an organic blend of acetonitrile and methanol that was 90:10 (%v/v). To make the buffer, 1.0 mL of orthophosphoric acid was diluted with 1000 mL of filtered water and thoroughly mixed. The pH of the buffer solution was 2.3.Utilising the gradient programme (min/B%), the following gradient values were used: 0.0/0, 30.0/45.0, 40.0/70.0, 45.0/95.0, 48.0/95.0, 50.0/0, and 55.0/0. The column oven was kept at 40 °C. Injection volume and flow rate were 10µLand 1.0 mL/min respectively. The analysis was carried out at 230 nm wavelength. As a

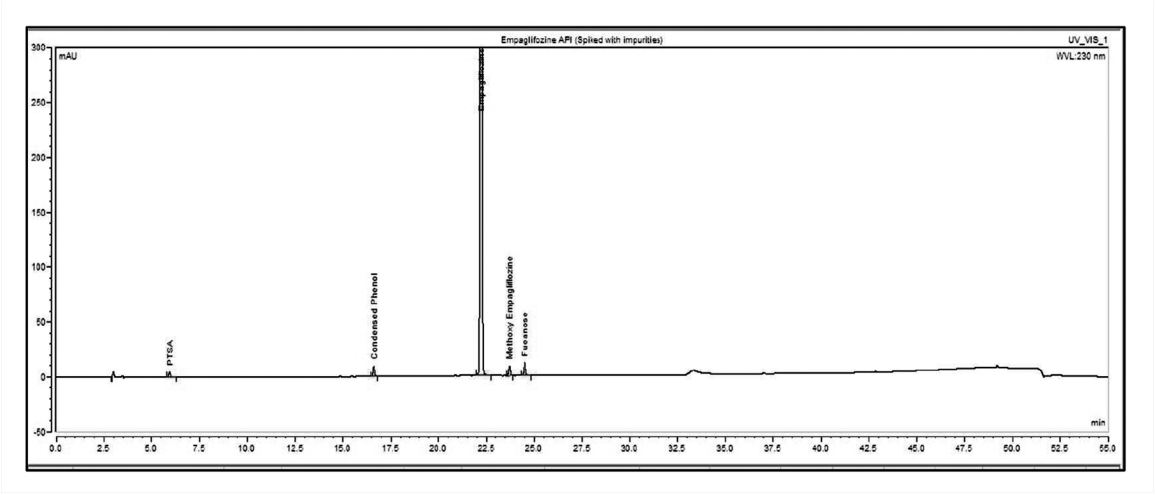

Fig. 4 — Chromatogram of the Empagliflozin API (spiked)

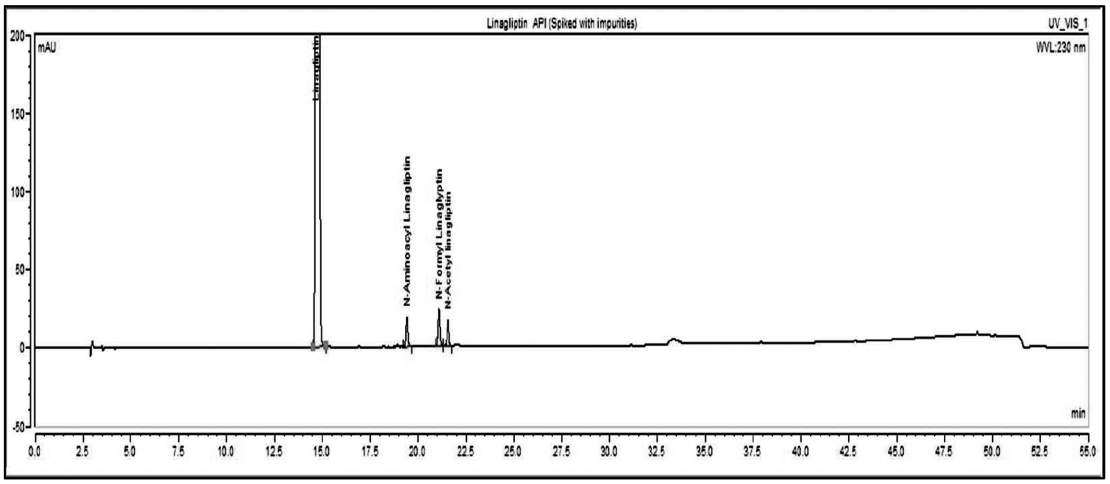

Fig. 5 — Chromatogram of the Linagliptin API (spiked)

diluent, water, methanol, and acetonitrile were utilised (50:25:25 %v/v/v).Individual impurity and drug substance standards were injected to identify the peaks. Empagliflozin and their impurities were spiked at specification level (Fig. 4). Linagliptin and their impurities were spiked at specification level (Fig. 5).

Preparative liquid chromatography conditions for isolation of unknown impurities

To isolate the impurity, a Knauer Preparative HPLC (made in Germany) equipped with a PUMP 1800 module and a Detector (S-2500) UV detector was used. Clarity chrome Software was used to analyse the data. To achieve chromatographic separation, a YMC ODS AQ C18 (250 x 50 mm, 10 µm) column (Japan) was used. In diluent, a sample concentration of 350 mg/mL was produced. As mobile phases A and B, aqueous trifluoroacetic acid (0.1%) and acetonitrile were used. Mobile phases A and B were used as the diluent in a 90:10, %v/v. At 230 nm, the column eluent was measured. The fractions were gathered, combined, and lyophilized using a lyophilizer for further characterization of isolated impurities, LCMS and NMR instrument was used.

LC-MS conditions

MS studies were carried out on an AB Sciex QTRAP mass spectrophotometer with Analyst 1.7.2 software. The electrospray ionisation source (positive ion mode) was used, with a source voltage of 5500 volts and a temperature of 550 °C. The chromatographic separation was performed on an Agilent 1200 (USA). HPLC outfitted with a

photodiode array detector and an ACE C18 PFP (250 x 4.6mm), 3µm column at 40 °C. The components were eluted using mobile phases A (0.1% methanoic acid and acetonitrile in a 85:15 %v/v) and B (acetonitrile and methanol in a proportion of 90:10 %v/v). Separation was achieved by a gradient plan (Tmin/A:B) T0/100:0, T25/55:45, T35/30:70, T40/5:95, T43/5:95, T45/100:0, T50/100:0 with a flow rate of 1.0 mL/min. To gather statistics, the eluent was put through an MS analyzer.

Nuclear magnetic resonance spectroscopy (NMR)

Using deuterated dimethylsufoxide (DMSO-d_6) as the solvent, 1D NMR (^{1}H &^{13}C NMR studies) were conducted on Bruker 400 MHz (Bruker, Fallanden, Switzerland), and the corresponding chemical shift values were assigned. Unknown impurity-I and II which was degradant isolates (3–20 mg) were individually treated in DMSO-d_6 (0.2–0.5 mL) for NMR analysis.

Evaluation of developed method

The established analytical technique was verified in accordance with the most recent ICH Q2(R1) guideline. According to ICH guideline, the validation parameters were put into practice such as specificity, linearity, accuracy, limit of detection, limit of quantification, precision, solution stability and robustness[38-39].

Specificity

The impact of additives and excipients found in the combined tablet dosage form of empagliflozin and linagliptin was examined under ideal circumstances. Specificity of method was established by introducing both placebo and blank solution into the system. All the impurities of empagliflozin and linagliptin were introduced in the test solution at their target level concentrations in order to determine the purity and sameness of each analyte using Diode-Array Detection and the Chromeleon software.

Linearity

By examining the composite standard solutions at seven different concentration levels, a linearity study was carried out. The solutions of linearity were generated from the LOQ level to 200% of their specification limit. The calibration's %bias, slopes, y-intercepts, and correlation coefficient(r) were noted.

LOD and LOQ

The limit of detection (LOD) should be determined by injecting the smallest amount of substance that can be reliably sensed. The limit of quantification (LOQ) is the smallest concentration of an analyte that can be measured in a sample. LOQ, LOD for empagliflozin and linagliptin and all their impurities were established (Fig. 6). Matching signal-to-noise ratios of 3:1 and 10:1. Precision study and accuracy at the LOQ level were also performed by injecting separate preparations of empagliflozin and linagliptin and their impurities, and figuring out the impurity peak area's %RSD.

Precision

The degree of agreement between replicate measurements of an analyte is known as precision. Six samples were prepared in accordance with the test procedure, and impurities of empagliflozin and linagliptin were spiked at the specified quantity in each sample to find out the method's precision. Fresh six sample solutions were made for the daily precision investigation and injected by another column, another instrument, another day, and different analysts. The %relative standard deviation was used to calculate the method's accuracy.

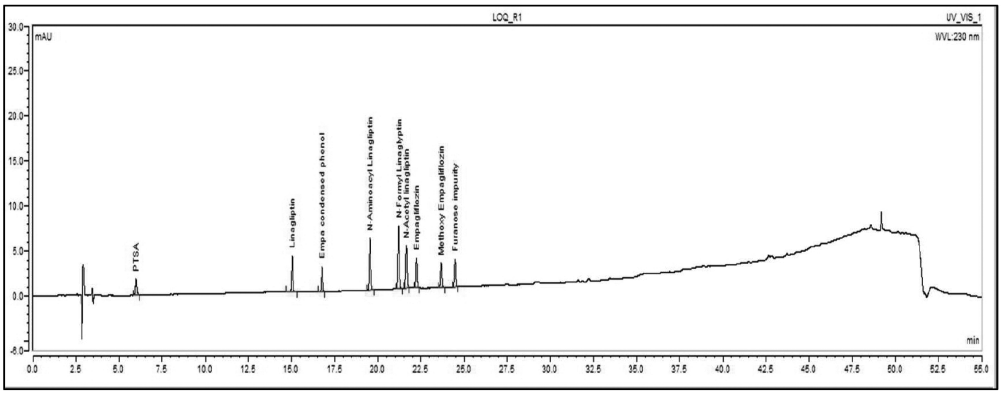

Fig. 6 — Chromatogram of the quantitation limit

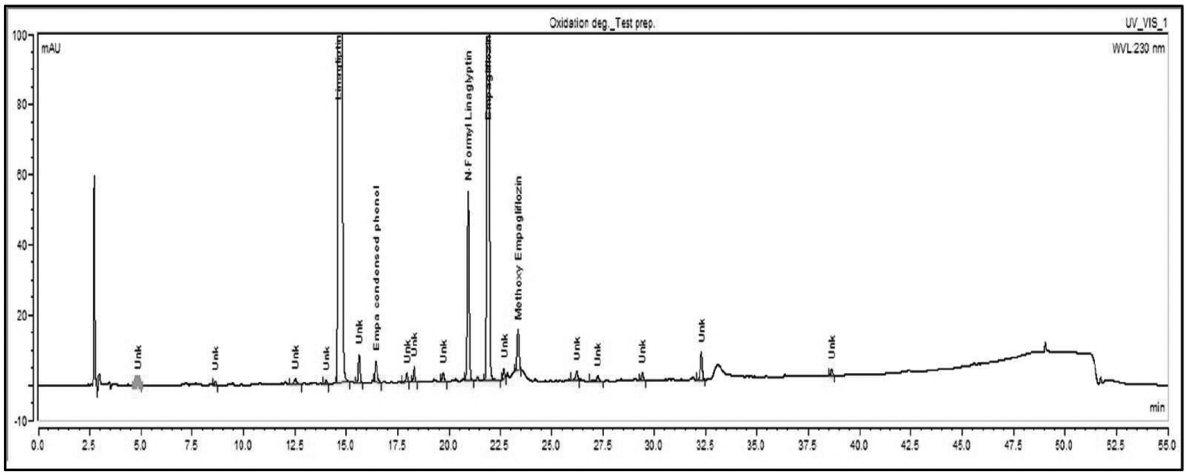

Fig. 7 — Chromatogram of combine dosage form for peroxide degradation study

Accuracy

Experiment was performed by adding known amount of empagliflozin and linagliptin impurities to the pre-analysed samples with LOQ, 50, 100, 120, and 200% levels of the targeted concentration. Analysis was performed in triplicate.

Robustness

To determine if the technique results are affected or not, method parameters were purposefully changed during a robustness study. In liquid chromatography (LC), common cases of modifications include a flow rate (1.0 mL/min), buffer and acetonitrile were mixed at different ratios in mobile phase A (86:14 %v/v and 90:10 %v/v). Changes were made to the acetonitrile and methanol ratios in mobile phase B (88:12 %v/v and 92:08 %v/v, respectively), temperature of the column compartment (35 °C and 45 °C), and others. In every one of the adjusted conditions, the selectivity as well as the separation between each closely spaced peaks were examined.

Solution stability

At various time intervals, the solution steadiness of the standard and the examination solution was assessed. For each impurity response in the cumulative RSD, a %RSD was generated to assess consistency. Standards and sample preparation were held constant throughout this study.

Force degradation

Force degradation study of drug products and drug substances can guide us to know degradation products, which can facilitate to set up the degradation route and inherent stability of the compound. It works well as a tool for figuring out to establish the stability of the product, an analysis of force degradation was carried out employing acid hydrolysis, peroxide oxidation, alkali degradation[40,41], ultraviolet radiation, thermal and humidity, which might help prove the method's specificity in terms of drug degradation. A 5 mL solution of 2 N hydrochloric acid heated to 80 °C for an hour was used to perform an acid degradation; the test solution was then neutralised and diluted to the required concentration. A study on alkali deterioration was conducted with a 1 N sodium hydroxide solution. 5 mL solution was used for degradation study and degradation sample solution was heated for 1 h at 80 °C, followed by neutralisation and diluted as per procedure. In a photolytic chamber, the samples were exposed to UV radiation and light at 1200000 Lux h and 200 W h m^2. Samples were exposed to 1 mL of 1.0% hydrogen peroxide solution at 80 °C for 1 h under the oxidative condition. With regard to thermal deterioration, sample solution was diluted to the target sample concentration after being stored at 90 °C for 7 days. (Fig. 7-9).

Results and Discussion

Specificity

The diluent blank and Placebo exhibited no significant interference at the retention times of impurity standards, empagliflozin and linagliptin as shown in (Fig. 10), demonstrating the suggested approach's specificity. (Fig.10). PDA detector was employed to check and ensure the homogeneity and purity of each analyte peak and peak purity found passing.

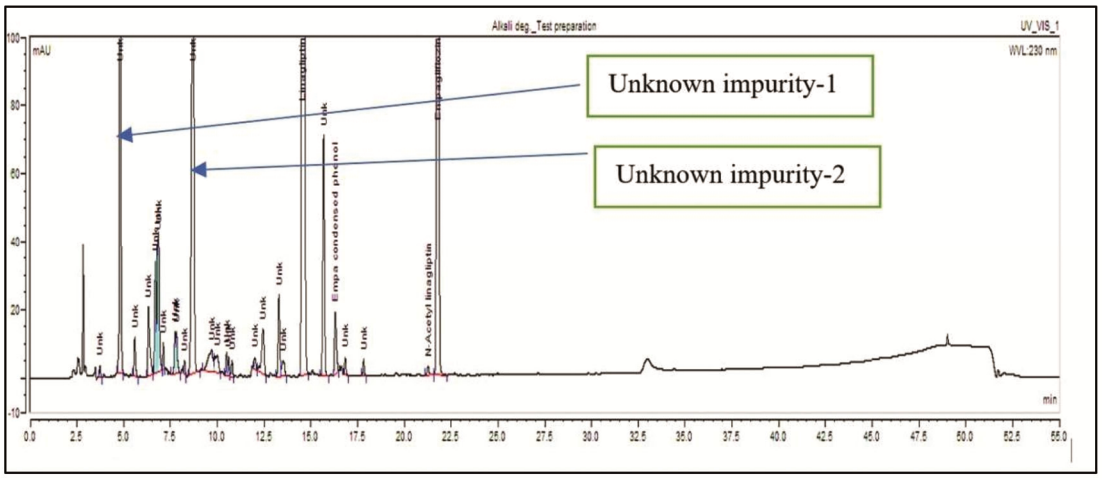

Fig. 8 — Chromatogram of two unknown impurities present in alkali hydrolysis

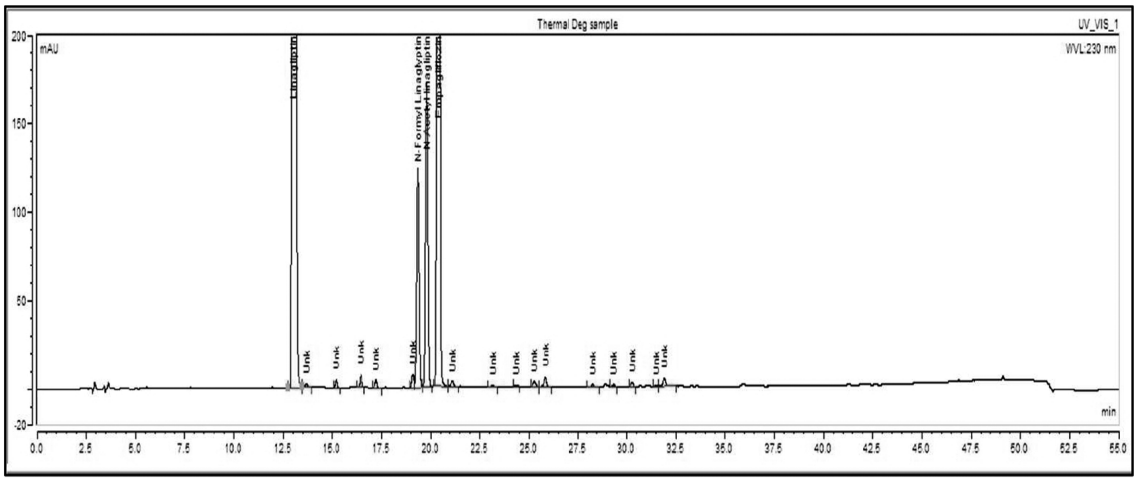

Fig. 9 — Chromatogram of combine dosage form for thermal degradation

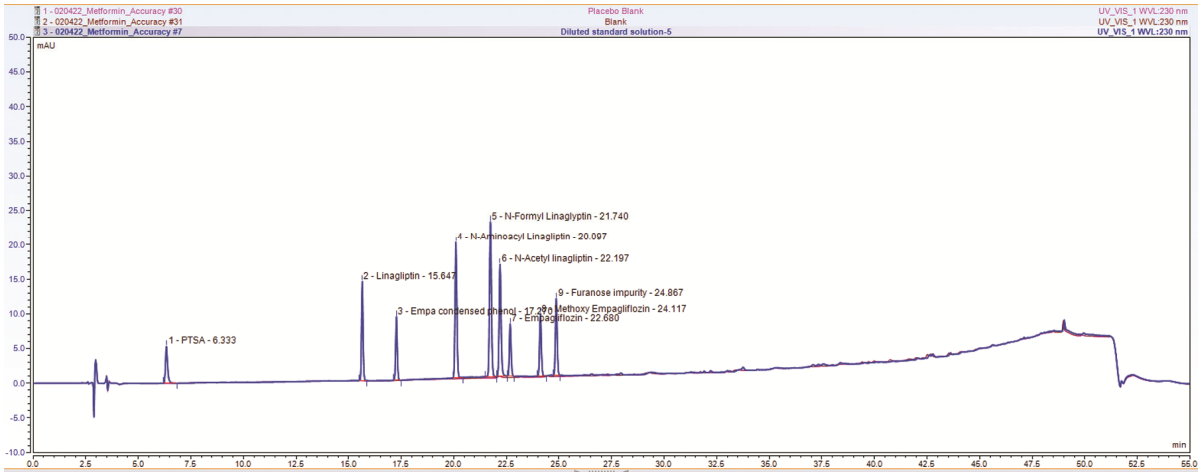

Fig. 10 — Overlay chromatogram of diluent bank, placebo and standard

Table 1 — Summary data of correlation coefficient (r) and precision and intermediate precision

Name of the impurity	Parameter								
	LOD ppm	LOQ ppm	Correlation coefficient	%RSD of Intraday precision	%RSD of Inter-day precision	Linearity range (ppm)	Intercept	slope	%Bias
PTSA	0.197	0.597	0.9996	2.69	0.00	0.597-3.980	0.020	17.823	0.060
Empa condensed phenol	0.198	0.601	0.9990	0.00	0.00	0.601-4.004	0.121	23.359	0.261
N-aminoacyl linagliptin	0.200	0.606	0.9990	0.80	1.04	0.606-4.039	0.774	54.624	0.713
N- formyl linagliptin	0.202	0.611	0.9999	0.00	0.89	0.611-4.071	1.547	68.466	1.093
N-acetyl linagliptin	0.201	0.608	0.9998	1.14	1.47	0.608-4.051	0.103	46.463	0.108
Methoxy empagliflozin	0.200	0.605	0.9995	0.00	0.00	0.605-4.032	0.157	24.535	0.322
Furanose impurity	0.197	0.596	0.9989	0.00	1.94	0.596-3.972	0.225	28.981	0.399
Acceptance criteria	-	-	0.980	<10	<10	-	-	-	-5 to+5

Linearity

For known impurities, linearity was performed at least seven distinct concentration levels, ranging from 200% to LOQ. Each impurity in the linear regression equation's y-intercept, correlation coefficient (r), slope and %bias was examined and observed to be greater than 0.990, as exposed in Table 1.

LOD and LOQ

The lowest amount should be injected to define the visual method's detection limit. Analyte detection was reliable at that concentration. All seven of their impurities, as well as the LOQ and LOD for empagliflozin and linagliptin, were investigated at 10:1 and 3:1 signal-to-noise ratios, respectively. There was also research done on accuracy and precision at the LOQ level computing the impurity peak areas' % RSD. (Table 1)

Precision

Using injections of six various treatments (n = 6) of the tablets empagliflozin and linagliptin were spiked with a known concentration of each contaminant within its permitted range. The associated substance method's intraday precision study was calculated. Table 1 provides a summary of the % RSD for each impurity. Within 5% of the %RSD of area, the difference between intraday precision and intermediate precision was identified. The Intermediate precision analysis was carried out utilising different columns, analysts, and HPLC systems (Waters e2695 Separation Module) on different days to be able to demonstrate the method's reliability. Based on the data, the %RSD was computed and found to be within the tolerances (5%). Table 1 provides the precision results.

Accuracy

The findings were confirmed by adding known quantities of empagliflozin and linagliptin impurities to the pre-analysed samples in triplicate at five distinct concentration levels, i.e., at 200, 120, 100, 50, and LOQ of the prescribed limit. Except for at the LOQ level, the average recovery result was found to be between 90 to 110% for each contaminant. The RSD values were less than 5%, which shows that the measured value and true value agree well (Table 2).

Robustness

The recently developed method's robustness was evaluated by changing the chromatographic parameters. The pump flow rate was adjusted to 1.0 mL/min. Impact of flow rate can be seen via changed to increase 0.10 mL/min flow and by decrease 0.10 mL/min flow. To determine the effect the column compartment temperature at 40 °C was changed by increase 5 °C units to 45 °C and decrease to 5 °C units to 35 °C. The makeup of the buffer and organic phase composition was altered. To assess the suitability of the system parameters variation was done in mobile phase A [Acetonitrile and buffer (14:86 %v/v,10:90 %v/v)]. Variation was also done in organic composition of mobile phase B. [Methanol and acetonitrile (12:88 %v/v, 08:92% v/v)]. In all varying situations, each peak was clearly differentiated from its neighbouring summits (Buffer ratio, flow rate, organic ratio, and column section temperature), indicating resolution greater than 2.0, and elution order did not alter, indicating the method's reliability.

Solution stability

According to the results of the experiments on solution stability, the solution of standard which was prepared in the diluent was stable for up to 24 h at the 10 °C sampler cooler temperature. The solution of sample is found stable for 24 h at the 10 °C sampler cooler temperature based on data.

Table 2 — Outcomes of the accuracy study at different level

% of recovery[a]	Recovery level[b]				
	LOQ	50%	100%	120%	200%
PTSA	102.91±0.28(0.27)	99.90±0.60(0.60)	99.10±0.78(0.79)	99.98±0.60(0.60)	99.42±0.17(0.17)
Empa condensed phenol	110.84±0.82(0.74)	104.22±0.31(0.30)	99.89±0.14(0.14)	101.06 ±0.17(0.17)	100.20±0.51(0.51)
N-aminoacyl linagliptin	99.66±0.42(0.43)	97.02±0.09(0.10)	97.56±0.09(0.09)	97.28±0.29(0.30)	97.83±0.22(0.23)
N- formyl linagliptin	106.30±0.51(0.48)	100.21±0.86(0.86)	100.37±0.16(0.16)	100.19±0.19(0.19)	99.59±0.26(0.26)
N-acetyl linagliptin	109.99±0.68(0.62)	103.77±0.23(0.22)	100.68±0.16(0.16)	101.35±0.14(0.14)	100.10±0.18(0.18)
Methoxy empagliflozin	104.79±0.35(0.34)	100.59±0.28(0.27)	99.72±0.14(0.14)	99.97±0.26(0.26)	99.28±0.24(0.24)
Furanose impurity	105.62±0.40(0.37)	101.56±0.02(0.02)	100.24±0.12(0.12)	100.77±0.18(0.17)	100.16±0.21(0.20)

[a]Threeobservations at each level, Mean±SD (%RSD)
[b]Impurity levels increased significantly compared to the standard level

Table 3 — Forced degradation study outcomes and specificity

Impurity	Condition of the experiment					
	Acidic condition	Basic condition	Oxidative condition	Heat (Thermal) condition	Humidity Degradation	UV condition
	5 mL solution of 2 N HCl heated for 1 h to 80°C (% w/w)	5 mL solution of 1 N NaOH heated for 1 h to 80°C (% w/w)	1 mL of 1.0% H_2O_2 solution at 80°C for 1h (%w/w)	7 days at 90°C (%w/w)	Ten days at 40°C±2/75±5 % RH (% w/w)	1200000 Lux h and 200 W h m^2 (% w/w)
PTSA	-	-	-	-	-	-
Empa condensed phenol	2.11	-	0.93	-	-	-
N-aminoacyl linagliptin	0.03	-	-	-	0.03	1.88
N- formyl linagliptin	-	-	4.94	6.21	0.08	0.81
N-acetyl linagliptin	0.04	0.17	-	12.67	-	0.11
Methoxy empagliflozin	-	-	1.54	-	-	-
Furanose impurity	-	-	-	-	-	-
Major unknown -I	-	7.17	-	-	-	-
Major unknown -II	-	9.97	-	-	-	-

Force degradation

During thermal degradation N-formyl linagliptin and N-acetyl linagliptin was formed. Under oxidative degradation N-formyl linagliptin was formed. In alkali degradation two unknown peaks were formed. During acid degradation, and UV degradation compounds are found stable. The PDA detector was used to determination and confirmation of the sameness. Peak purity of each analyte shows more than 990 (Chromeleon software). Means complies the requirement of peak purity. Table 3 provides an overview of the results of the forced degradation experiment. Fig. 7-9 display the resolution recorded during the forced degradation experiment.

Impurity-I and II's proposed structural elucidation by NMR and ESI-MS

Seven impurities were identified through analytical HPLC. Through preparative HPLC, the remaining two unidentified contaminants were separated and subsequently characterised.

NMR Interpretation of impurity-I

NMR spectroscopic analysis was anticipated to confirm structure of unknown impurity-I. We did not detect any appreciable variation in the chemical shift values in the impurity-I in ^{1}H NMR spectra. The suggested structure of unknown impurity-I was proven to be 4-methyl-2-quinazolinemethanamine based on the spectroscopic study.

The unknown impurity – I was identified as 4-methyl-2-quinazolinemethanamine. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.97 (s, 3H, H-1), 4.44 (s, 2H, H-10), 7.77-7.81 (m, 1H, H-5), 8.00-8.08 (m, 2H, H-4&H-6), 8.35 (d, J = 8.0 Hz, 1H, H-7), 8.56 (s (br), 2H, H-11) ppm. ^{13}C NMR (400 MHz, DMSO-d_6): δ 22.02 (C-1), 43.96 (C-10), 123.15 (C-3), 126.60 (C-4), 128.15(C-5),128.48(C-7), 135.33 (C-6), 149.01 (C-8), 158.97 (C-2), 170.13 (C-9) ppm; ESI-MS (positive mode) m/z: 174.1(Fig. 11).

NMR Interpretation of impurity-II

Nuclear magnetic resonance spectroscopic profiles like ^{1}H &^{13}C NMR analysis were anticipated to

confirm the structure of unknown impurity -II. We did not detect any appreciable variation in the chemical shift values in the ^{1}H NMR spectra of impurity-II. The predicted structure of unknown Impurity II was confirmed to be (R)-2-(3-aminopiperidin-1-yl)-1-(but-2-yn-1-yl)-4-(methylamino)-N-((4-methylquinazolin-2-yl)methyl)-1H-imidazole-5-carboxamide based on the spectroscopic study.

The unknown impurity - II ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.76-1.83 (m, 8H, H-23,H-18, H-19 and H-20); 2.91(s, 5H, H-8, H-24); 3.02-3.1 (s, 5H, H-10, H-27); 3.23-3.25 (d, J = 3 Hz, 1H, H-15); 4.68-4.69 (m, 3H, H-16); 5.44 (s, 2H, H-22); 6.63 (s, 1H, H-21);7.68-7.72 (t, 1H, H-2, J=16Hz,);7.92-8.00 (m, 2H, H-1, H-4); 8.25-8.27 (d, 1H, H-3, J=8Hz); 9.25 (s, 1H, H-11) ppm.^{13}C NMR (100 MHz, DMSO-d_6): δ 3.56 (Alyne-CH_3); 22.06 (pyrimidine ring-C); 22.67, 22.95 (piperidine ring-C); 33.00 (Alkyne-CH_3); 34.79 (NH-CH_3); 46.95 (CH_2-NH); 47.45, 51.78, 56.69 (piperidine ring-C); 74.52, 81.57 (Alkyne-C); 122.91, 126.26, 127.72, 128.29, 134.59,139.93, 147.72, 149.42, 155.68 (aromatic ring-C); 162.80 (amidic- C), ppm; ESI-MS (positive mode) m/z : 447.3 (Fig. 12).

Based on literature search three impurities such as empacondensed phenol impurity, furanose impurity, methoxy empagliflozin impurity in empagliflozin drug substance was reported by Chen et al. Impurities were isolated, and their structures were clarified by MS analysis. Isolation and identification were carried out using UPLC-PDA-MS and NMR analysis[33-37]. An empacondensed phenol impurity was found in combination empagliflozin of and linagliptin tablets by Jagadabi et al. and for impurity quantification, they used a stability-indicating RP-UPLC technique[17]. Yadav et al. reported that three DPs were produced during basic hydrolysis (B1, B2, and B3) on LC-MS which is a costly instrument compared to HPLC[35].

Instead of reported work, developed method is significantly more sensitive. For the combination of empagliflozin and linagliptin tablets, nine impurities, including those linked to the manufacturing process and those associated to degradation, were separated

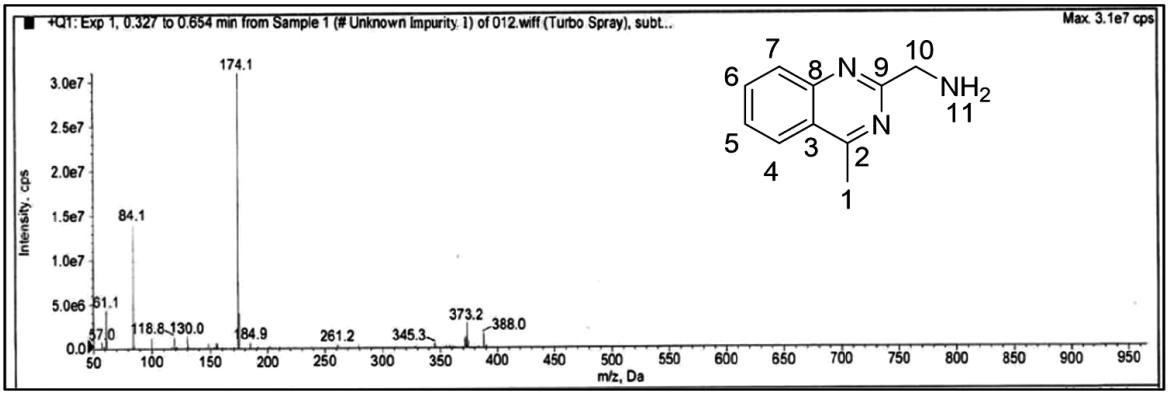

Fig. 11 — Mass spectrum of unknown impurity -I

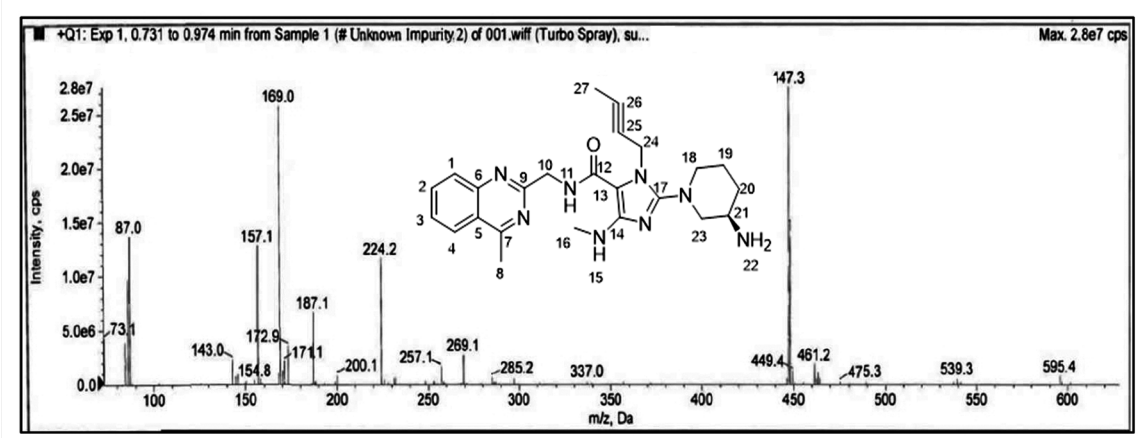

Fig. 12 — Mass spectrum of unknown impurity -II

using HPLC with a PDA detector. HPLC technique developed was unique. Rest of impurities were isolated and unique method was developed. The recommended method has additional benefits, including the ability to identify process and degradation contaminants in formulation product using a single, rapid, and reproducible RP-HPLC method. A one-step, rapid, and repeatable RP-HPLC approach to identify nine contaminants in product composition was reported. It can be used by quality controlled laboratory for release analysis activities. As a result, this method can be widely used in industries for commercial purposes because to its exceptional accuracy, precision, and recovery.

Conclusion

Process-related and degradation impurities have been identified in empagliflozin and linagliptin formulated product. LC-MS, ^{1}H & ^{13}C NMR were used to isolate and identify these contaminants as PTSA (p-toluenesulfonic acid), methoxy empagliflozin impurity, empacondensed phenol impurity, Furanose impurity. N-formyl linagliptin impurity, N-acetyl linagliptin impurity, N-aminoacyl linagliptin impurity, 4-methyl-2-quinazoline methenamine, (R)-2-(3-aminopiperidin-1-yl)-1-(but-2-yn-1-yl)-4-(methylmino)-N-((4-methylquinazolin-2-yl)methyl)-1H-imidazole-5-carboxamide impurity. For the combined dose form of empagliflozin and linagliptin, a related substance method was created and validated in accordance with the most recent ICH recommendation. The study's findings shows that all of the degradation products are extremely clearly separated, proving the technique's suitability as a stability indicator. Impurity analysis was performed on same batch of drug material and combination dosage forms using the established method. The recognised contaminants are found at levels below quantitation. The suggested method can be used to analyse empagliflozin and linagliptin in mixed dose forms for quality control. The developed method can be used for its intended purpose in pharmaceutical industry.

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