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Simultaneous estimation of dapagliflozin and linagliptin using reverse phase-HPLC with photo diode array (PDA)

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Abstract: Dapagliflozin inhibits selective sodium–glucose co-transporter-2 and Linagliptin competitively and reversibly inhibits dipeptidyl peptidase-4 in fixed dose Combination (1:1) is used in the treatment of Type 2 Diabetes Mellitus. For estimation Dapagliflozin and Linagliptin in bulk and Tablet formulation, an accurate and precise method using RP-HPLC was developed and validated. In the method being discussed here was optimized using Hypersil C18 (250 × 4.6 mm, 5 µm) column as Stationary Phase, Mobile Phase being used is Acetonitrile: Water (90:10) adjusting pH 3 using Ammonium Acetate. The Flow Rate was adjusted to 1mL/min. Both Dapagliflozin and Linagliptin (1:1) sample was detected at analytical wavelength of 244nm using Photo diode array detector. The Linearity Range was between Concentration of 0.1µg/mL to 20µg/mL with correlation Coefficient of 0.995 and 0.999 for Dapagliflozin and Linagliptin Respectively.

Keywords: Diabetes mellitus; dapagliflozin; linagliptin; RP-HPLC; method validation.

1. Introduction

The IUPAC name of Dapagliflozin is (2S,3R,4R,5S,6R)-2-[4-chloro-3-[(4-ethoxyphenyl) methyl] phenyl]-6-(hydroxymethyl) oxane-3,4,5-triol is the chemical name of Dapagliflozin (Figure S1 in supporting information). It has a molecular weight of 408.9 g/mol with molecular formula of $C_{12}H_{25}ClO_6$. Its log P value is 2.7 and pKa value is 12.6. Its mechanism is to inhibit sodium Glucose Co-transporter 2 (SGLT2) which is located at proximal tubule site of the Nephron. It belongs to Anti-Diabetic Category as they lower the level of sugar in blood by inhibiting reabsorption of glucose by the kidney. The IUPAC name of Linagliptin is 8-[(3*R*)-3-aminopiperidin-1-yl]-7-but-2-ynyl-3-methyl-1-[(4-methylquinazolin-2-yl) methyl] purine-2,6-dione (Figure S2, in supporting information) with molecular weight of 472.5 g/mol and molecular formula of $C_{25}H_{28}N_8O_2$. It has log P value of and pKa value of 8.6. It inhibits dipeptidyl peptidase 4 leading to increase in insulin secretion and glucagon release is suppressed causing Anti-glycemic action.

Both the drugs are complementary in action to one another Hence, their combination is beneficial and Advantageous in the treatment of Diabetes Mellitus Type 2 [1-3]. The thorough literature review was done and it showed that there are several methods for estimation of Dapagliflozin in bulk

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and dosage form such as UV Spectroscopy, RP-HPLC method or in combination with other drugs like Metformin and Saxagliptin using analytical methods such as RP-HPLC, HPTLC, UPLC etc.,

Same goes with Linagliptin there are several methods for estimation of Linagliptin in bulk and dosage form such as UV Spectroscopy, RP-HPLC method or in combination with other drugs like Metformin and Empagliflozin using analytical methods such as RP-HPLC, HPTLC [4-23].

From the extensive literature survey, came to knew that there is one RP- HPLC method on this Combination published [24] and as compared to published method proposed method is sensitive in context to linearity range and mobile phase used in published method very complex as compared to mobile phase used in proposed method. This led to the development of accurate, sensitive, repeatable, and precise RP-HPLC method for the 1:1 fixed dose combination of Dapagliflozin and Linagliptin in Bulk and Pharmaceutical dosage form.

HPLC equipment are a great tool for tracking variability across different manufacturing batches since they can separate and even detect minute levels of contaminants and degradants in your raw materials and finished items. Compared to other analytical methods like gas chromatography (GC) and thin-layer chromatography (TLC), HPLC has several advantages. Among them are high sensitivity, analyzing trace quantities of analytes is possible because of HPLC's high sensitivity and detection limits.

2. Experimental work

2.1. Materials and Chemicals

Dapagliflozin and Linagliptin API was provided by a reputed pharmaceutical company with purity of 99.40 % and 99.75 %, respectively. The 5mg tablets of Dapagliflozin and Linagliptin marketed as Dapagold 5 and Linares respectively was used. Acetonitrile used was of HPLC grade and Ammonium Acetate both provided by SRL Chemicals pvt. Ltd., Ahmedabad, India.

2.2. Selection of Wavelength

Dapagliflozin and Linagliptin individual solution of concentration 10 µg/mL was prepared using Acetonitrile as solvent. Both the solutions were scanned from wavelength 400 nm to 200 nm using UV-Visible double beam spectrometer. After scanning the iso-absorptive point was found to be 224 nm.

2.3. HPLC System

The instrument used to develop the method was RP-HPLC Waters system which consists of a Hypersil C18 column and Empower software and Photo diode array detector. The Hypersil C18 column was used as a stationary phase. Mobile phase used is Acetonitrile: Water (90:10) and Ammonium Acetate was used to adjust pH 3 of the mobile phase. The flow rate was adjusted to 1mL/min. Analytical Wavelength was used to perform the Detection.

2.4. Preparation of Standard Solutions

10 mg of Dapagliflozin and 10 mg of Linagliptin was weighed and transferred in individual 10 mL flask and dissolved with small quantity of Acetonitrile. The acetonitrile is added to make up the volume up to 10 mL. The concentration of solution prepared was 1000 µg/mL. From this stock solution a working solution of 100 µg/mL was prepared using proper dilutions.

2.5. Determination of Calibration Curve

From the working solution of 100 µg/mL the aliquot of 0.01 mL, 0.05 mL, 0.1 mL, 0.5 mL, 1 mL, 2 mL were taken was transferred to different volumetric flack and volume was adjusted to 10mL using mobile phase.

2.6. Validation

As per the Q2(R2) guideline of International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) validation of RP-HPLC method was carried out [25].

2.6.1. Linearity

To obtain the linearity of Dapagliflozin and Linagliptin in Fixed dose ratio (1:1) five calibrations were taken in concentration range of 0.1 µg/mL to 20 µg/mL. Peak Area v/s Concentration was plotted in the graph to obtain the calibration cure with a straight-line equation.

2.6.2. Precision

Precision is the closeness of the values or measurements obtained from homogeneous sample. To conduct the repeatability study middle concentration of the linearity range (5µg/mL) was injected six times. To conduct intermediate precision two studies were performed namely intraday and interday. Intraday was performed on the same day while interday was performed on individual days. Three concentrations were chosen to determine the intermediate precision i.e., Lowest (0.1 µg/mL), Middle (0.5 µg/mL) and Highest (20 µg/mL). Mean of Area and %RSD was calculated from the peak ages determined.

2.6.3. Accuracy

Accuracy is the closeness of the observed value with that of the true value. To perform the accuracy studies standard spiking method was used. Based in this method the Dapagliflozin and Linagliptin sample solution was spiked with the standard solution at 80%, 100% and 120% levels and the concentration of 5 µg/mL sample solution was used for the spiking purpose. 3 injections of each spiked sample were given. Mean Area and percentage recovery was calculated.

2.6.4. Limit of Detection and Limit of Quantification

The lowest concentration at which the technique can reliably identify the analyte inside the matrix (but not measure it). Limit of Quantification refers to smallest quantity or lowest concentration of a material that may be ascertained using a particular analytical technique while maintaining the specified levels of accuracy, precision, and uncertainty. Both can be calculated using the following formula.

$$\text{LOD} = 3.3\times \frac{\sigma}{s} \text{ and LOQ} = 10\times \frac{\sigma}{s}$$

Where σ is the standard deviation of y-intercepts of regression lines and S is the average slope of the calibration curves.

2.6.5. Robustness

The assessment of an analytical method's robustness is whether the outcomes are consistent even under slightly different conditions. It the capacity of a method to continue working even when minor adjustments are made. The minor changes that were made to evaluate robustness of the method included change in flow rate, change in analytical wavelength and change in mobile phase composition. Middle concentration of 5µg/mL was selected to perform the robustness analysis. Mean area and %RSD were than calculated from the areas obtained after the injection.

2.6.6. System Suitability

System suitability parameter for Dapagliflozin and Linagliptin (1:1) was determined using the middle concentration (5 µg/mL).

2.6.7. Specificity

The specificity study was carried out by commonly used excipients present in selected tablet formulation. The appropriate quantities of excipients were mixed with a pre weighed quantity of drug mixture DAPA (10.0 mg) and LINA (10.0 mg) transferred it in 10 mL volumetric flask. A few mL of acetonitrile was added to the above flask and flask was sonicated for 5 min. The solution was filtered through Whatman filter paper (No. 42) into another 10 mL volumetric flask, the residue was washed twice with a few mL of acetonitrile, filtrate and washing combined. The volume was adjusted to the mark with the acetonitrile. Aliquot (0.05 mL) of the above solution was transferred into 10 mL volumetric flask and diluted with mobile phase to obtain a solution containing of 5 µg/ mL of DAPA and 5 µg/ mL of LINA. The solution was analyzed by proposed method and chromatogram recorded. The amount of DAPA and LINA were computed using regression equation.

2.6.8. Assay of Marketed Formulation

The Dapagold 5 Tablet of Dapagliflozin and Linares Tablet of Linagliptin were weighted accurately to 5mg in the individual flask on 10mL and dissolved in small quantity of acetonitrile and then make up the volume up to 10mL to produce solution of 500 µg/mL. from these solutions take out 1mL in the individual flak and makeup the volume up to 10 mL this produces solutions of 50µg/mL. Again, take out 1mL and transfer in the common flask and makeup the volume 10mL this would produce a sample of Dapagliflozin and Linagliptin (1:1) Solution of 5 µg/mL. To analyze this sample the developed RP-HPLC method was used. The amount of Dapagliflozin and Linagliptin in the solution was then calculated using the regression equation.

3. Results and Discussion

3.1. Selection of Wavelength

Dapagliflozin and Linagliptin 10µg/mL solution was produced by dissolving in acetonitrile and both the solutions were scanned using Double beam UV-Visible spectrometer from 400 nm to 20 0nm. The considerable absorption was found to be 224 nm which was selected as analytical wavelength as shown in Figure S3 in supporting information document.

3.2. Mobile Phase Optimization

Mobile phase that produced sharp and selective peak for dapagliflozin at R.T 3.01 minute and Linagliptin at R.T at 6.89 minute was Acetonitrile: Water (90:10) using Ammonium Acetate to adjust pH 3.0.

3.3. Validation of Method

As per the Q2(R2) guideline of International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) validation of RP-HPLC method was carried out. The results obtained are as follows.

3.3.1. Linearity

The Correlation coefficient of 0.995 and 0.999 for Dapagliflozin and Linagliptin was found to be linear in the given concentration range of 0.1µg/mL to 20µg/mL for the developed RP-HPLC method. The Overlay of the Chromatogram is depicted in Figure 1. Table 1 depicts the value of regression analysis

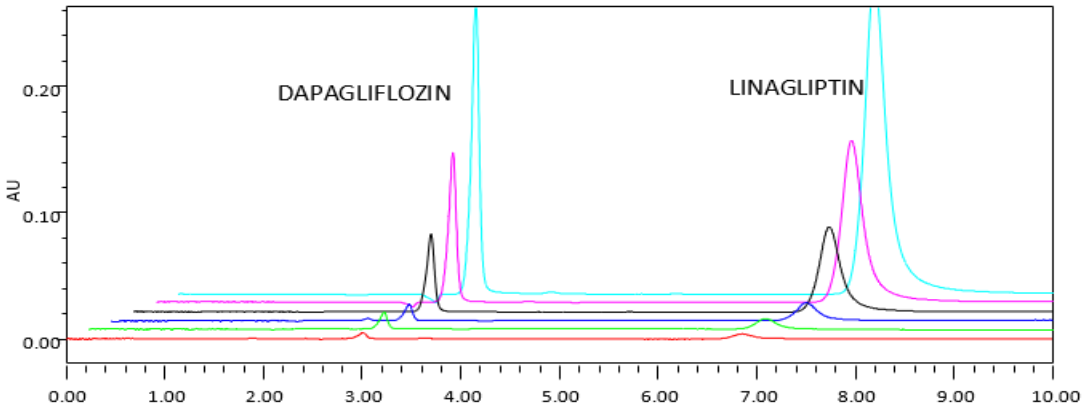

Figure 1. Chromatogram of Dapagliflozin and Linagliptin (0.1–20 µg/mL) combination (1:1)

Table 1. Regression analysis of Dapagliflozin and Linagliptin

Parameters	**Dapagliflozin**	**Linagliptin**
Linearity Range (µg/mL)	0.1-20	0.1-20
Regression Coefficient (R^2)	0.995	0.999
Slope of regression equation	59390	161709
Standard deviation of slope	120.40	1079.9
Intercept of regression	37892	42505
Standard deviation of intercept	734.75	5994.81

3.3.2. Precision

That value of Interday precision for Dapagliflozin and Linagliptin ranges from 0.80%-1.24% and 1.10-1.54% respectively while for Intraday Precision the Value ranges from 0.32%-1.89% and 0.76-1.93 for Dapagliflozin and Linagliptin respectively. The result of precision shows that there is closeness between the values when the measurements are taken intraday and intraday. The Result of precision analysis is shown below in Table 2.

Table 2. Result of validation parameters of RP-HPLC method for Dapagliflozin and Linagliptin

Parameters	Dapagliflozin	Linagliptin	Acceptance criteria
Linearity Range (µg/mL)	0.1 – 20	0.1-20	-
Retention Time (min)	3.01	6.89	-
Detection Limit (µg/mL)	0.052	0.094	-
Quantitation Limit (µg/mL)	0.15	0.28	-
Accuracy (% Recovery)	98.23-101.16	98.92-101.23	> 98% - <102%
Specificity	Specific	Specific	-
Robustness	Robust	Robust	%RSD value <2%
Precision (%RSD)			
Interday Precision (n=3)	0.80 – 1.24	1.10-1.54	%RSD value <2%
Intraday Precision (n=3)	0.32-1.89	0.76-1.93	%RSD value <2%
Repeatability Study (%RSD)			
Injection Repeatability (n=6)	1.49	1.56	%RSD value <2%
Assay (% Recovery)	100.6	98.5	> 98% - <102%

RSD is Relative Standard Deviation and "n" is number of determinations.

3.3.3. Accuracy

To perform the accuracy studies standard spiking method was used. % Recovery of Dapagliflozin and Linagliptin was calculated from the regression equation. % Recovery was found to be 98.23%-101.16%w/w and 98.83%-101.41%w/w for Dapagliflozin and Linagliptin respectively. (Table 2) The method is considered Accurate as the values are within the limits of accuracy, i.e., 98%-102%.

3.3.4. Limit of Detection and Limit of Quantification

The LOD and LOQ for Dapagliflozin was found to be 0.052 µg/mL and 0.15 µg/mL respectively while for Linagliptin it was found to be 0.094 µg/mL and 0.28 µg/mL which shows that the method is highly sensitive to detection and quantification.

3.3.5. Robustness

To conduct the robustness study some of the intentional changes were made in the test parameters. The changes included change in flow rate from 1mL/min to 0.9mL/min and 1.2mL/min, change in analytical wavelength from 224nm to 222nm and 226nm, change in Mobile Phase composition from Acetonitrile: Water (90:10) to 95:5 and 85:15. The results are shown below in the Table 3 and Table 4. The mean area and %RSD were calculated and %RSD was to be ≤ 1. This shows that with the slight changes in the test parameters Dapagliflozin and Linagliptin in 1:1 ratio can be evaluated using this method.

Table 3. Robustness Results for Dapagliflozin (5µg/mL)

Parameters	Optimized Condition	Change in Condition	Mean area ± SD (n=3)	% RSD	Retention Time ± SD (n=3)	% RSD
Change in Wavelength	224 nm	222 nm	453097.5± 1221.173	0.22	3.01±0.013	0.44
		226 nm	478304± 2117.078	0.24	3.00±0.010	0.34
Change in Flow Rate	1 mL/min	0.9 mL/min	447282± 15598.78	0.31	2.99±0.031	1.02
		1.1 mL/min	417655± 4556.596	1.00	3.01±0.015	0.51
Change in Mobile Phase Composition	90:10 v/v	95:5 v/v	485811± 14721.96	0.86	3.00±0.012	0.39
		85:15 v/v	407086± 2609.224	0.64	3.01±0.014	0.46

Table 4. Robustness Results for Linagliptin **(**5µg/mL)

Parameters	Optimized Condition	Change in Condition	Mean area ± SD (n=3)	% RSD	Retention Time ± SD (n=3)	% RSD
Change in Wavelength	224 nm	222 nm	1086651 ± 4985.81	0.45	7.01±0.011	0.15
		224 nm	1075489± 710.6423	0.06	7.00±0.026	0.37
Change in Flow Rate	1 mL/min	0.9 mL/min	1034681± 5644.126	0.54	6.97±0.060	0.86
		1.1 mL/min	1057866± 831.5576	0.70	7.01±0.011	0.16
Change in Mobile Phase Composition	90:10 v/v	95:5 v/v	1048424± 4666.905	0.44	6.98±0.065	0.93
		85:15 v/v	1050560± 7686.958	1.07	7.00±0.020	0.28

3.3.6. System Suitability

The results of the system suitability parameters are shown in Table 4, and the numbers for the retention duration, theoretical plate (which must be greater than 2000), tailing factor (which must be less than 2), and resolution were taken straight from the Empower program. All the numbers that were obtained were within the recommendations standard value range.

Table 4. Result of System Suitability

Parameters	Dapagliflozin	Linagliptin
Retention Time (min)	3.01	6.89
Theoretical Plate	7131.5	5598.65
Tailing Factor (T_f)	1.508	1.16
Resolution	7.85	-

3.3.7. Specificity

The specificity study was carried out to check the interference from the excipients used in the formulations by preparing synthetic mixture containing both the drugs and excipients. The chromatogram showed peaks for both the drugs (DAPA and LINA) without any interfering peak and the estimation of both the drugs were found to be satisfactory.

3.3.8. Assay of Marketed Formulation

The recommended approach was applied to analyze the marketed tablet formulation for Dapagliflozin and Linagliptin. The findings showed a drug recovery percentage of 100.6 %w/w and 98.5%w/w, showing the applicability of the method. The overlay chromatogram, selectivity, and specificity of the Dapagliflozin as well as Linagliptin standard and formulation are shown in Figure 2.

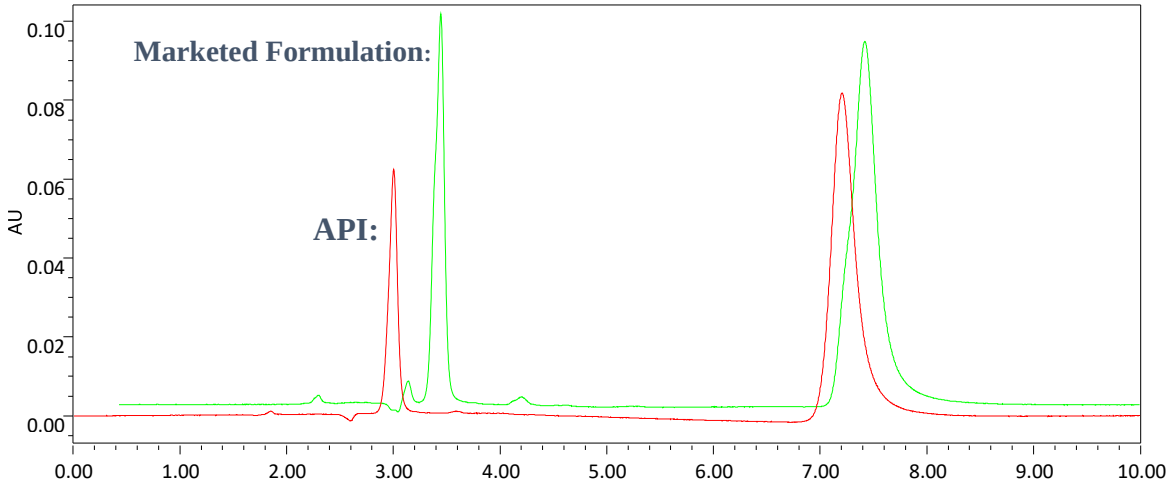

Figure 2. Overlay chromatogram of standard and marketed formulation of Dapagliflozin and Linagliptin (5µg/mL)

3.4. Uncertainty Assessment

The uncertainty assessment for method was done as per the EURACHEM/CITAC guide and the corresponding literatures [17-21]. We have reported the combined uncertainty ($u_{combined}$) and expanded uncertainty ($U_{Expanded}$), calculated from the uncertainty in standard preparation ($u_{standard}$), uncertainty associated with the slope of calibration curve ($u_{calibration}$), uncertainty of recovery ($u_{recovery}$) and uncertainty of repeatability ($u_{repeatability}$) using following equation 1.

$$u_{combined} = \sqrt{(u_{standard})^2 + (u_{calibration})^2 + (u_{recovery})^2 + (u_{repeatability})^2} \quad (1)$$

$u_{standard}$ of analyte was calculated from the % purity provided by the supplier using equation 2.

$$u_{standard} = 100 - \%\text{Purity}/\sqrt{3} \quad (2)$$

$u_{calibration}$ was calculated for analyte from the standard error of slope and slope value for the calibration curve using equation 3.

$$u_{calibration} = (\text{Standard Error of Slope} * 100)/\text{ Slope} \quad (3)$$

The mean relative standard deviation (RSD) associated with the recovery studies was considered as $u_{recovery}$ while that of repeatability studies was considered as $u_{repeatability}$ for the analyte. Expanded uncertainty at a 95% confidence interval is calculated by multiplying combined uncertainty with the coverage factor (k = 2) The uncertainty profile for the present method is given in Table 5.

Table 5. Uncertainty Assessment of Reverse Phase-HPLC method

Uncertainty (U)	DAPA	LINA
$u_{standard}$	0.09	0.015
$u_{calibration}$	2.83	1.85
$u_{recovery}$	0.76	0.77
$u_{repeatability}$	0.52	0.073
$u_{combined}$	2.03	1.65
$U_{expanded}$	4.06	3.29

$U_{Expanded}$: k=2 95 % confidence level; U % values reported

4. Conclusion

Dapagliflozin and Linagliptin can be quantified using a reverse phase high performance liquid chromatographic method with Acetonitrile: Water (90:10 v/v) as the mobile phase and C18 Hypersil (250 x 4.6 mm, 5µm) as the stationary phase. The proposed technique for Dapagliflozin and Linagliptin combination (1:1) was shown to be linear with a retention time of 3.01 minutes and 6.89minutes for Dapagliflozin and Linagliptin in the range of 0.1-20 µg/mL. 101–102% was found to be Dapagliflozin's recovery percentage. While for Linagliptin it was found to be 98%-102%. Low quantitation and detection limit concentration results reflect the sensitivity of the approach to identify Dapagliflozin and Linagliptin in trace levels. The method was proven to be reliable, reproducible, selective, accurate, and precise for routine Dapagliflozin and Linagliptin in 1:1 combination analysis. Consequently, the approach presented here can be utilized to quantify Dapagliflozin and Linagliptin in both bulk and tablet dosage without interference from excipients.

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