Research Article

DEVELOPMENT AND VALIDATION OF REVERSE PHASE-HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY-DAD STABILITY INDICATING ASSAY METHOD FOR THE SIMULTANEOUS QUANTITATIVE ESTIMATION OF EMPAGLIFLOZIN AND LINAGLIPTIN IN A COMBINED FIXED-DOSE TABLET DOSAGE FORM

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ABSTRACT

Objective: A simple, specific, accurate and precise, validated reverse phase high performance liquid chromatographic stability indicating method has been developed for the quantitative determination of empagliflozin and linagliptin in a tablet dosage form, simultaneously in the presence of their degradation products. **Methods:** The isocratic elution of two therapeutic agents was achieved on a octadecyl silane C_{18} (150 × 4.6 mm × 5 µm) column maintained at ambient temperature, using a mobile phase comprising buffer and acetonitrile in the proportions of 30:70 (v/v) with a flow rate of 1.1 ml/min and eluents are separated, monitored by photodiode array detector at 245 nm in a run time of 8 min. Forced degradation studies were conducted under acidic, basic, neutral oxidative, thermal, humid, and photolytic conditions as per International Conference on Harmonization (ICH) guidelines. **Results:** Empagliflozin and linagliptin was eluted at 2.29 and 5.06 min and permitted the quantification over a linear concentration range of 10-60 and 5-30 µg/ml, respectively. The % recoveries are ranging 99.57-101.5% for empagliflozin and 100.15-100.85% for linagliptin, respectively. The relative standard deviation (%) of intra- and interday is calculated within the acceptable limit (≤2%). The method was validated in terms of specificity, linearity, limit of detection, limit of quantification, accuracy, precision, and robustness as per ICH guidelines and the outcome of the validation study was within the acceptable limits. **Conclusion:** The developed method is simple, specific, rugged, robust and stability indicating, which may probably be suitable for routine laboratory analysis.

Key Words: Stability, empagliflozin, isocratic, linagliptin, peak purity

INTRODUCTION

Empagliflozin, chemically it is (2S, 3R, 4R, 5S, 6R)-2-[4-chloro-3-[[4-[(3S)-oxolan-3-yl] oxy phenyl] methyl] phenyl]-6-(hydroxyl methyl) oxane-3, 4, 5-tirol [Figure 1a]. It belongs to the gliflozin category and intended for the treatment of Type II diabetes.[1] It inhibits sodium glucose co-transporter-2 in kidneys, thereby excreting glucose through urine.[2-4]

Linagliptin, chemically it is, 8-[(3R)-3-aminopiperidin-1-yl]-7-but-2-ynyl-3-methyl-1-[(4-methylquinazolin-2-yl)methyl] purine-2,6-dione [Figure 1b] and vanish the glucagon-like peptide-1 and glucose-dependent insulinotropic polypeptide by inhibiting dipeptidyl peptidase-4. It may be used alone or with combination of antidiabetic medicines.

As per literature review, no method was reported for the estimation of empagliflozin either individually and combination with other therapeutic agents, while fewer methods were reported for the estimation of linagliptin by ultraviolet (UV) spectrophotometric method,[5,6] estimation in pharmaceutical dosage form using high-performance liquid chromatography (HPLC),[7-17] establishment of stability indicating assays using HPLC,[18-22] estimation using high performance thin layer chromatography,[23] estimation in human plasma using HPLC,[24,25] using Liquid chromatography–tandem mass spectrometry,[26] estimation using ultra-performance liquid chromatography.[27]

Since lack of a suitable method, the efforts were made to establish a validated technique for the simultaneous determination of empagliflozin and linagliptin in tablet dosage form.

EXPERIMENTAL

Materials

The standards of empagliflozin and linagliptin were obtained as gift sample from a reputed pharmaceutical company (Hyderabad, India), and the same fixed dose pharmaceutical product, Glyxambi, with a label claim of 10 and 5 mg was procured from local pharmacies. All the chemicals were used of HPLC grade purchased from Merck Specialties, Private Ltd., Mumbai, India.

Instrumentation

Water's Alliance HPLC system 2695, comprising a quaternary pump, autosampler, photo diode array detector (PDA, model 2996), and thermostatic column operated with empower software is used for the study.

Optimized chromatographic condition

Isocratic elution of mobile phase comprising 0.1% orthophosphoric acid and acetonitrile in the proportions of 30:70 v/v pumped onto reverse phase (RP) intersil octadecyl silane column C_{18} (150 × 4.6 mm ID; 5 µ) maintained at 30°C, with a flow rate of 1.1 ml/min. The run time was set at 8 min, and the volume of injection is 10 µl. The eluents were monitored at 245 nm using a diode array detector; data were acquired, stored, and analyzed by Empower software.

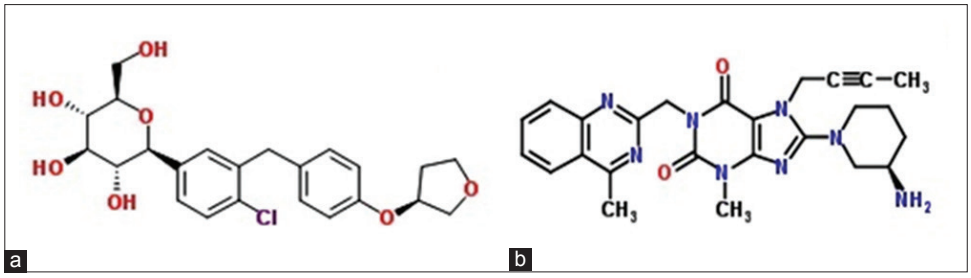

Figure 1: (a and b) Chemical structures of empagliflozin and linagliptin

Mobile phase preparation

Pipette 10 ml of concentrated orthophosphoric acid into a 100 ml volumetric flask, diluted with water (0.1%) and mix with acetonitrile in the proportions of 30:70. Methanol and water in the proportion of 50:50 v/v were used as diluent. All the solutions filtered through a 0.45 µm nylon filter and degassed.

Standard solution preparation

Accurately weighed quantities of 10 mg of empagliflozin and 5 mg of linagliptin were placed in two different 100 ml clean, dried volumetric flasks, dissolved by adding diluent, subsequently diluted up to the volume with diluent (Stock solution) to attain the final concentration of 100 and 50 µg/ml, respectively.

Construction of linearity

From above stock solutions, pipette 1, 2, 3, 4, 5, and 6 ml of solutions into a clean, dried 10 ml volumetric flasks, diluted with diluent and filtered through a 0.45 µ nylon filter to acquire 10, 20, 30, 40, 50, and 60 µg/ml solutions of empagliflozin and 5, 10, 15, 20, 25, and 30 µg/ml of linagliptin, respectively. Inject 10 µL of each concentration six times and the responses were recorded. Calibration curves were linear in the concentration range of 10-60 µg/ml for empagliflozin and 5-30 µg/ml for linagliptin.

Sample preparation

About 20 tablets were crushed and the powder equivalent to 10 and 5 mg of empagliflozin and linagliptin was placed into a clean dried 100 ml volumetric flask, 30 ml of diluent was added, dissolved by sonication for 30 min and diluted to volume. Filtered through a 0.45 µ nylon filter and 1 ml of solution diluted to 10 ml.

Stability of solutions

To demonstrate the stability of the solutions during the analysis, they were analyzed for 24 h at room temperature, and the responses were recorded. No significant degradation was observed during this period, and hence, the solutions were stable, resulted no deviation in the responses.

FORCED DEGRADATION STUDIES

The stress studies of empagliflozin and linagliptin were performed by treating them with an acid (2N HCl), alkali (2N NaOH), aqueous (water), oxidizing agent (20% hydrogen peroxide) and exposing them to light (200 W UV-C Lamp), temperature (hot air oven), and humid environment (desiccator, 97% RH at 25°C).

Stock solution

Accurately weighed quantities of 10 mg of empagliflozin and 5 mg of linagliptin were placed in two different 100 ml clean, dried volumetric flasks, dissolved by adding diluent, subsequently diluted up to the volume with diluent.

Degradation by acid

To 1 ml of above solution was added to 1 ml of 2 N hydrochloric acid and refluxed on a heating mantle at 60°C for about 6 h. Further, it was neutralized by 2 N NaOH, diluted up to the level 10 ml with diluent and cooled.

Degradation by base

To 1 ml of above solution was added to 1 ml of 2 N sodium hydroxide and refluxed on a heating mantle at 60°C for about 6 h. Then, it was neutralized by 2 N hydrochloric acid, diluted up to the volume 10 ml with diluent and cooled.

Oxidative degradation

To 1 ml of above solution was added to 1 ml of 20% hydrogen peroxide and refluxed on a heating mantle

at 60°C for about 6 h, diluted up to 10 ml with diluent and cooled.

Neutral degradation

To 1 ml of above solution was added to 1 ml of HPLC grade water and refluxed on a heating mantle at 60°C for about 6 h, diluted up to 10 ml with diluent and cooled.

Thermal degradation (dry heat degradation)

The stock solution was kept in hot air oven at 105°C for about 6 h, diluted 1-10 ml with diluent and cooled.

Photolytic degradation (UV degradation)

About 10 and 5 mg of empagliflozin and linagliptin were spread over on a glass Petri dish as layer with <3 mm thickness and kept in a UV light cabinet at 30 cm distance from the UV lamp. The cover of the Petri dish was removed and out in the open to a 200 W/h/m^2 UV-C lamp at 100-280 nm, for 7 days. Further, the contents were dissolved in diluent and diluted to 100 ml with the same. Pipette 1 ml of solution and diluted to 10 ml with diluent.

Humidity degradation

The tablet powder equivalent to 10 and 5 mg of empagliflozin and linagliptin was kept in a glass desiccator at 25°C/97% RH, using a saturated solution of potassium sulphate and were analyzed after 7 days. The mixture was dissolved and diluted to volume 100 ml by diluent. Pipette 1 ml of solution and diluted to 10 ml with diluent.

Solution filtration

All above solutions were prepared filtered through a 0.45 µm nylon filter and 10 µl was injected to record responses.

RESULTS AND DISCUSSIONS

Method development and optimization of chromatographic conditions

Various attempts were made to set up the optimized chromatographic conditions pertaining to the proposed method. Optimization will be done to accomplish excellent resolution with suitable selectivity factor, considerable theoretical plates, appropriate peak symmetry for two components devoid of coeluting risks and interference from their degradants and excipients. At this point of view, the studies were performed for the selection of solvent, analytical column, mobile phase composition, flow rate, and wavelength of detection. As the both drugs are liberally soluble in methanol, the mixture of methanol and water (50:50 v/v) was used as diluent. Diverse kinds of C_{18} and C_8 columns were tested, finally, intersil C_{18} column (150 mm × 4.6 mm; 5 µm) was selected as it was satisfied required criteria. As the two components are having polar functional groups and a significant number of carbons, the acidic pH ≤3 is most apt for their separation. Thus, we had chosen 0.1% phosphoric acid as a buffer. Acetonitrile is best matched for high sensitivity analysis at short UV wavelengths as compared to methanol; hence, it was elected as an organic modifier. The buffer and acetonitrile in the proportions of 50:50, 40:60, 35:65, 30:70, and 25:75 (v/v) were verified; finally, the 0.1% orthophosphoric acid and acetonitrile in the proportion of 30:70 (V/V) was preferred as mobile phase as it gave satisfactory results. PDA detector detector was opted as it is useful for peak purity tests instability indicating assays. The wavelength of detection confirmed as 245 nm by spectral scanning.

Forced degradation

The studies demostrated that slight degradation was seen in acid, alkali and remains intact at peroxide, aqueous, humidity, thermal, and photolytic environments. Peak purity angle and threshold of the peaks revealed that

the peaks were pure, homogeneous, symmetric and without coelution risks with the degradation products.

Validation

Stability of solution

The responses and retention times at different time intervals of 0, 4, 8, 16, 20, and 24 h were recorded and indicating no degradation was observed. The solutions were stable for 24 h, which was adequate for the total analytical development [Table 1] as the % relative standard deviation (RSD) ≤2.

System suitability

About 10 and 5 µg/ml of empagliflozin and linagliptin injected about 6 times, and different parameters were computed along % RSD. The results were in the acceptable limit and summarized in Table 2.

Specificity

No peaks were observed in retention times of empagliflozin and linagliptin, and the chromatograms of forced degradation showed excellent peak purity, homogeneity, without coeluting risks from their degradants, representing the stability indicating capability of the method.

Linearity

It was evaluated by measuring the responses of different control solutions of empagliflozin and linagliptin in the specified limit of 25-150%. The standard plots were

Table 1: Stability of solutions

Time (h)	Retention time		Peak area		Theoretical plates		Tailing factor	
	Empagliflozin	Linagliptin	Empagliflozin	Linagliptin	Empagliflozin	Linagliptin	Empagliflozin	Linagliptin
0	2.298	5.067	511,032	667,832	4782	6549	1.4	1.19
4	2.295	5.021	516,766	662,693	4708	6691	1.41	1.18
8	2.297	5.036	529,398	676,728	4730	6576	1.45	1.16
12	2.297	5.04	521,808	656,725	4633	6671	1.42	1.18
16	2.299	5.046	514,498	677,044	4764	6500	1.41	1.15
20	2.301	5.054	516,014	649,940	4699	6483	1.42	1.18
24	2.302	5.067	517,371	658,885	4781	6370	1.44	1.17
Mean	2.298	5.047	518,127	664,264	4728	6548.6	1.42	1.17
SD	0.0024	0.017	5930	10,205	53.68	111.49	0.018	0.014
% RSD	0.11	0.33	1.14	1.5	1.14	1.70	1.25	1.18

SD: Standard deviation, RSD: Relative standard deviation

Table 2: System suitability findings

Factor	Value	
	Empagliflozin	Linagliptin
Retention time (min)	2.29	5.06
Theoretical plates	6537	4698
Linearity range (µg/mL)	5-30	10-60
LOD (µg/mL)	0.02	0.08
LOQ (µg/mL)	0.05	0.25
Resolution	-	14.24
Tailing value	1.08	1.32
Regression equation	y=26280x+385.1	y=11,198x+220.1
Correlation coefficient (R^2)	0.999	0.999
RSD of peak area (n=6)	0.9	0.39
Relative standard deviation of retention time	0.146	0.115

LOD: Limit of detection, LOQ: Limit of quanitification, RSD: Relative standard deviation

established by plotting mean peak area against the corresponding concentrations and R^2-value, y-intercept, and slope of the regression line were measured.

Precision (system, method and intermediate)

Tested for repeatability, reproducibility and intermediate precision and % RSD of each study was calculated as a result of six replicate injections of 10 and 5 µg/ml of empagliflozin and linagliptin and it was found ≤2%, indicating that it was more precise. The results were tabulated in Table 3.

Accuracy

Recovery studies are conducted at three different levels of 50%, 100%, and 150% for empagliflozin and linagliptin by spiking known amount of the reference drug to preanalyzed sample. The mean percentage recoveries and corresponding % RSD were calculated and were found in the array of 98-102%, indicating the reliability of the method [Table 4].

Robustness

The effect of deliberate change in the optimized chromatographic conditions on established method was screened. Flow rate, temperature of the column and mobile phase composition are varied, and the responses were measured. The results were in the acceptable limits and furnished in Table 5.

Detection and quantification limit

These were calculated using signal-to noise ratios 3:1 and 10:1 for limit of detection and limit of quantification, respectively, and were found be 0.08 and 0.25, 0.02 and 0.05, 0.07 and 0.23, and 0.09 and 0.33 for empagliflozin, linagliptin, degradant (acidic condition), and degradant (basic condition), respectively [Table 6].

Results of forced degradation studies

Empagliflozin and linagliptin showed slight degradation in acidic, basic conditions and remained unchanged and stable in peroxide, photolytic, thermal, hydrolytic

Table 3: Precision studies

S.No	System precision				Method precision				Intraday and inter-day precision					
	Empagliflozin		Linagliptin		Empagliflozin		Linagliptin		Empagliflozin			Linagliptin		
	Rt	Peak area	Rt	Peak area	Peak area	Assay recovery	Peak area	Assay recovery	% Recovery			% Recovery		
									Day 1	Day 2	Day 3[a]	Day 1	Day 2	Day 3[a]
1.	2.293	520,256	5.065	649,777	522,024	100.00	651,215	100.00	100.00	99.9	99.7	100.00	101.8	99.8
2.	2.294	519,886	5.069	658,497	517,188	99.1	640,582	98.4	99.1	101.5	99.6	98.4	103.9	101.1
3.	2.295	518,716	5.071	649,533	515,741	98.8	645,893	99.2	98.8	100.0	99.4	99.2	100.8	99.7
4.	2.295	520,671	5.078	643,310	528,716	101.3	656,926	100.9	101.3	98.6	99.8	100.9	104.0	98.8
5.	2.298	522,356	5.079	642,753	527,890	101.2	641,884	98.6	101.2	98.9	100.1	98.6	99.8	98.7
6.	2.300	516,294	5.085	647,761	516,171	98.9	650,164	99.8	98.9	99.2	98.9	99.8	101.2	99.5
Mean	2.300	519,697	5.075	648,605	521,288	99.9	647,777	99.47	99.88±1.14[b] (*n*=6)	99.68±1.05	99.58±0.41	99.48±0.94 (*n*=6)	101.92±1.67	99.6±0.87
SD	0.0026	2044.99	0.0074	5711.1	5883.3	1.13	6184.9	0.95		99.72±0.88 (*n*=18)			100.33±1.63 (*n*=18)	
% RSD	0.115	0.39	0.146	0.9	1.1	1.13	1.0	0.95						

[a]Different analyst; [b]Mean±%RSD. RSD: Relative standard deviation, SD: Standard deviation

Table 4: Accuracy data

Drug	Concentration level	Amount added	Amount present	Amount recovered	% recovery	% Mean recovery±RE
Empagliflozin	50	20	60	20.01	100.05	100.6±0.6
		20	60	20.22	101.1	
		20	60	20.13	100.65	
	100	40	80	40.17	100.42	99.57±0.7
		40	80	39.63	99.07	
		40	80	39.69	99.22	
	150	60	100	61.24	102.06	101.51±1.51
		60	100	60.79	101.31	
		60	100	60.70	101.16	
Linagliptin	50	10	30	10.003	100.03	100.15±0.15
		10	30	10.04	100.4	
		10	30	10.002	100.02	
	100	20	40	20.21	101.05	100.85±0.85
		20	40	20.164	100.82	
		20	40	20.14	100.7	
	150	30	50	30.65	102.16	100.55±0.55
		30	50	29.57	98.56	
		30	50	30.28	100.93	

RE: Relative error

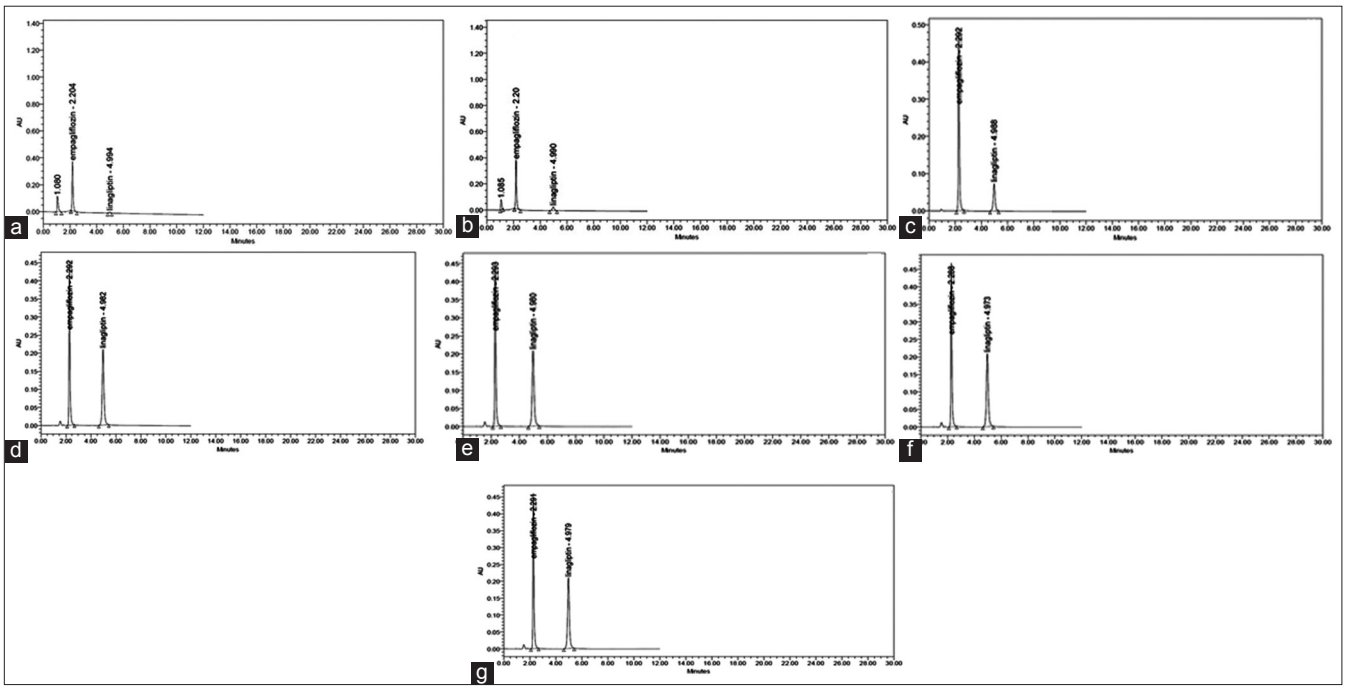

Figure 2: Chromatograms of (a) Acid, (b) base, (c) oxidative, (d) hydrolytic, (e) ultraviolet, (f) thermal, (g) humidity degradation of empagliflozin and linagliptin

and humidity conditions [Figure 2a-g]. Peak purity plots were shown in Figure 3a-g and tabulated in Table 7.

Analysis of tablet formulation

The content of empagliflozin and linagliptin in a tablet formulation was calculated by proposed method

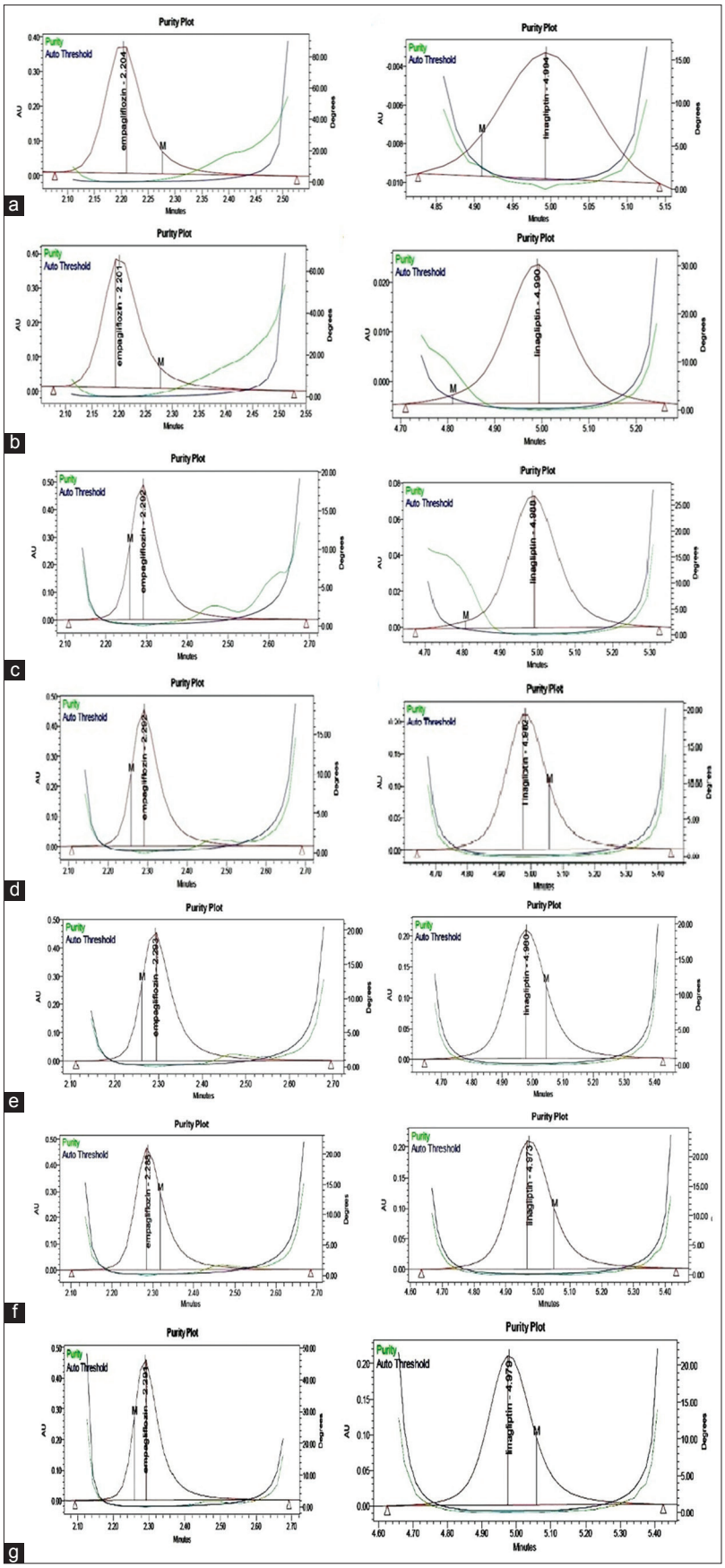

Figure 3: Peak purity plots of (a) Acid, (b) base, (c) oxidative, (d) hydrolytic, (e) ultravioelt, (f) thermal, (g) humidity degradation of empagliflozin and linagliptin

Table 5: Robustness out come

Condition	Variation	System suitability							
		Peak area*		Retention time*		Tailing factor* (<2)		Theoretical plates* (>2000)	
		Empagliflozin	Linagliptin	Empagliflozin	Linagliptin	Empagliflozin	Linagliptin	Empagliflozin	Linagliptin
Change in mobile phase composition	35:65	758,690±0.2[a]	946,145±0.2	2.335±0.13	5.231±0.15	1.34	1.08	4964	6728
	25:75	503,603±0.97	631,333±0.48	2.256±0.002	4.762±0.004	1.33	1.10	4973	6679
Change in flow rate	1.3 ml	682,747±0.13	853,991±0.31	2.099±0.17	4.546±0.39	1.36	1.08	4684	6428
	0.9 ml	551,518±1.08	685,139.5±0.55	2.514±0.07	5.454±0.16	1.38	1.10	5058	7042
Change in temperature	35^{0}C	693,219±0.24	872,494.7±0.74	2.099±0.17	4.546±0.39	1.38	1.11	4665	6377
	25^{0}C	565,229±1.17	710,668±0.83	2.514±0.07	5.454±0.16	1.43	1.16	5026	6940

*Mean of six values, [a]Mean±%RSD. RSD: Relative standard deviation

Table 6: LOD and LOQ

Formula		Empagliflozin µg/ml		Linagliptin µg/ml		Degradant (acidic condition) µg/ml		Degradant (basic condition) µg/ml	
LOD	LOQ	LOD	LOQ	LOD	LOQ	LOD	LOQ	LOD	LOQ
3.3 σ/S	10 σ/S	0.08	0.25	0.02	0.05	0.07	0.23	0.09	0.33

LOD: Limit of detection, LOQ: Limit of quanitification

Table 7: Forced degradation data

Type of degradation	Degradation medium and time (h)	Assay (mg/tab)		% Drug remained		% of degradation		Peak purity angle		Purity threshold		Number of degradants	Retention time of degradants
		Empagliflozin	Linagliptin	Empagliflozin	Linagliptin	Empagliflozin	Linagliptin	Empagliflozin	Linagliptin	Empagliflozin	Linagliptin		
Un degraded	-	9.990	4.97	99.9	99.47	-	-	-	-	-	-	-	-
Acid degradation	2 N hydrochloric acid and refluxed 6 h at 60°C	9.403	4.88	94.03	97.6	5.97	2.4	0.625	1.107	0.78	1.407	01	1.080

(Contd...)

Table 7: *(Continued)*

Type of degradation	Degradation medium and time (h)	Assay (mg/tab)		% Drug remained		% of degradation		Peak purity angle		Purity threshold		Number of degradants	Retention time of degradants
		Empagliflozin	Linagliptin	Empagliflozin	Linagliptin	Empagliflozin	Linagliptin	Empagliflozin	Linagliptin	Empagliflozin	Linagliptin		
Alkali degradation	2 N sodium hydroxide and refluxed 6 h at 60°C	9.438	4.78	94.38	95.6	5.62	4.4	0.616	0.489	0.770	0.511	01	1.085
Neutral degradation	Water and refluxed 6 h at 60°C	9.964	4.97	99.64	99.4	0.36	0.6	0.202	0.056	0.277	0.259	00	00
Oxidative degradation	20% H_2O_2 and refluxed 6 h at 60°C	9.706	4.79	97.06	95.8	2.94	4.2	0.267	0.848	0.281	0.926	00	00
Humidity degradation	Glass desiccator at 25°C/97% RH and 6 h	9.837	4.85	98.37	97.0	1.63	3.0	0.209	0.061	0.282	0.266	00	00
UV degradation	200 W h/m^2 photo stability chamber and 7 days	9.963	4.96	99.63	99.2	0.37	0.8	0.206	0.059	0.278	0.260	00	00
Thermal degradation	Placed in oven at 105°C and 6 h	9.634	4.92	96.34	98.4	3.66	1.6	0.209	0.060	0.279	0.263	00	00

UV: Ultraviolet

Table 8: Analysis of commercial product

Drug	Label claim (mg)	Mean amount found*	Content (%)	% RSD
Empagliflozin	10	9.991	99.91	1.13
Linagliptin	5	4.97	99.47	0.95

*Mean of six values. RSD: Relative standard deviation

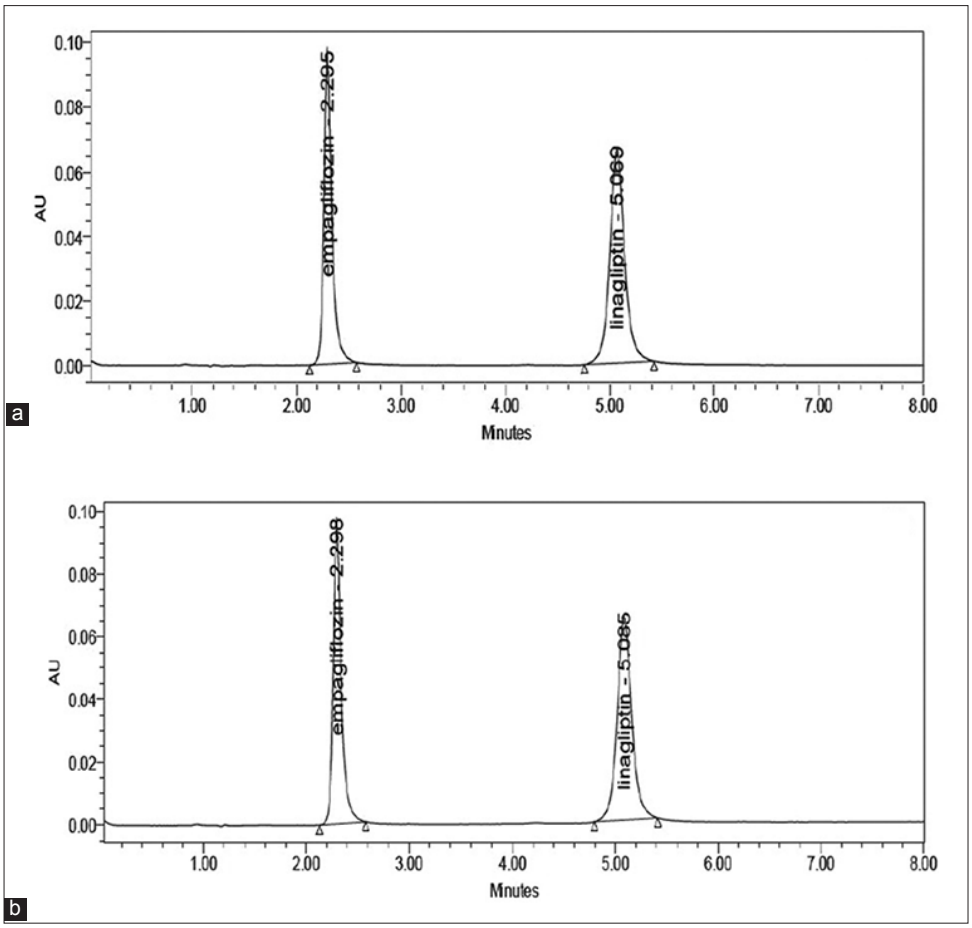

Figure 4: (a) A chromatogram of standard and (b) sample

and found as 99.91 and 99.47, respectively. The results [Table 8] and chromatograms were shown in Figure 4a and b.

CONCLUSION

A simple, specific, economic, rapid, accurate, and precise isocratic RP-HPLC stability indicating method has been developed and validated for quantification of empagliflozin and linagliptin in pure drug and tablet dosage form, which could separate the analyte from its degradants with good resolution and selectivity and can be intended for regular laboratory analysis.

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