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Research Article

Stability Indicating Method Development and Validation, Stress Degradation Studies for Empagliflozin and Sitagliptin Phosphate by Using RP-HPLC

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ABSTRACT

For the Measurement and Quantification of Empagliflozin and Sitagliptin Phosphate, A rapid, simple, sensitive, and reliable RP-HPLC technique using a Jasco HPLC System with PDA detection was designed and validated. The RP-HPLC elusion was carried out at Isosbestic point 246nm with 1 mL/min flow rate using column BDS Hypersil C18 (250*4.6mm, 5 µm) with Potassium Dihydrogen Phosphate Buffer (pH3.5): Acetonitrile (50:50 v/v) as the mobile phase. The wavelength was 246 nm, and the 1 ml/min was the flow rate. The column temperature was maintained at 25 °C. Empagliflozin and sitagliptin phosphate were shown to have retention durations of 3.5 and 5.6 minutes, accordingly. The LOD (limits of detection) & LOQ (limits of quantitation) for Empagliflozin were 5.02µg/mL and 15.23µg/mL, correspondingly, whereas for Sitagliptin phosphate they were 45.10µg/mL and 136.68µg/mL. The drug was analysed by developed method following stress conditions like; hydrolytic, photolytic, oxidative, and thermal degradation studies. The suggested process has been validated per the ICH Q2 (R1) guidelines.

INTRODUCTION

A stability-indicating assay methodology is described as: "A validated quantitative analytical approach that can detect changes over time in the physical, chemical, or microbiological features of the drug substance and drug products can be used to assess the concentration of active components and degradation products precisely and without interference."[1] For stability-indicating assay techniques the standard method for preparing samples is forced degradation/stress testing. "Testing the stability of drug substances and products under settings different from those used for accelerated stability testing" is the description

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of required degradation/stress testing.[2]. The medicine may deteriorate by 5 to 20% if it is exposed to extreme pH (NaOH or HCl solutions of several strengths) at high temperatures, UV light, hydrogen peroxide (at room temperature), as well as dry heat (in an oven) over an extended period. These experiments usually entail trial and error. A methodical approach should be aplied in place of this trial-and-error technique, that was labor-intensive, frequently costly, and time-consuming[3–4].

Empagliflozin:

Empagliflozin, a gliflozin-class drug, was authorised for treating type 2 diabetes in adults in 2014. Boehringer Ingelheim and Eli Lilly & Company[5] created it. Empagliflozin prevents SGLT-2 (sodium-glucose cotransporter-2), causing sugar in blood to be eliminated and removed in the urine. It is commonly used in type 2 diabetics to reduce blood glucose levels[2]. It can decrease the danger of cardiovascular fatalities and congestive heart failure in individuals with type 2 diabetes. Empagliflozin inhibits SGLT-2, a sodium-glucose cotransporter located mostly in the kidney's proximal tubules. About 90% of the reabsorption of glucose into the bloodstream is attributed to SGLT-2. By inhibiting reabsorption in the kidney and its excretion through urine, blocking SGLT-2 lowers blood glucose levels[6–8].

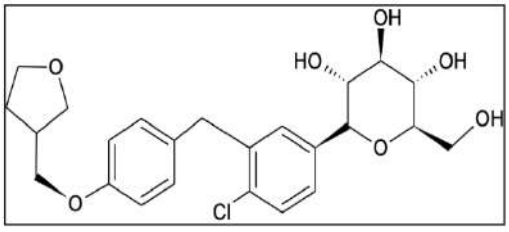

Figure 1: Structure of Empagliflozin

IUPAC Name:
(2S,3R,4R,5S,6R)-2-[4-chloro-3-({4-[(3S)-oxolan-3yloxy]phenyl}methyl)phenyl]-6-(hydroxymethyl)oxane-3,4,5-triol

Molecular Formula: $C_{23}H_{27}ClO_7$

Molecular Weight: 450.9 g/mol

Half-life:12.4 hours

Solubility: In methanol sparingly soluble; and in ethanol and ACN slightly soluble

Category: Anti-diabetic

Sitagliptin Phosphate:

Sitagliptin phosphate is a dipeptidyl peptidase-4 (DPP-4) inhibitor that is taken orally to treat hyperglycemia. This enzyme-inhibiting drugs is used to treat type 2 diabetes mellitus alone or in combination with other oral antihyperglycemic drugs. Sitagliptin works by competitively inhibiting the enzyme DPP-4. This enzyme degrades the incretins GLP-1 and GIP, gastrointestinal hormones secreted in response to a meal. They can boost insulin secretion while suppressing glucagon release by inhibiting GLP-1 and GIP inactivation. This brings blood glucose levels closer to normal[9-13].

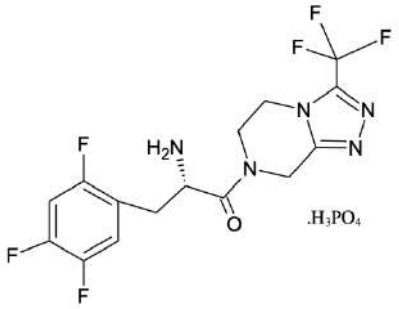

Figure 2: Structure of Sitagliptin Phosphate

IUPAC Name:
("3R)-3-amino-1-[3-(trifluoromethyl)-6,8-dihydro-5H-[1,2,4]triazolo[4,3-a]pyrazin-7-yl]-4-(2,4,5-trifluorophenyl)butan-1-one;phosphoric acid

Molecular Formula: $C_{16}H_{18}F_6N_5O_5P$

Molecular" Weight: 505.31g/mol

Half-life: 8 to 14 hours

Solubility: Water-soluble; very weakly soluble in ethanol

Category: Anti-diabetic

According to the literature review, different analytical processes have been stated for the individual analysis of Empagliflozin & Sitagliptin phosphate, as well as for their combinations with other drugs. However, for the simultaneous

estimation of Sitagliptin phosphate and Empagliflozin, only a limited number of techniques exist. For the immediate estimation of empagliflozin and sitagliptin phosphate in their combination dose form, no stability-indicating RP-HPLC technique has been created. The objective is to validate and develop a straightforward, accurate, precise, and specific RP-HPLC technique that indicates the stability for quantifying sitagliptin phosphate and empagliflozin.

MATERIALS AND METHODS:

Instrument: The absorbance of Empagliflozin and Sitagliptin phosphate was determined using a Shimadzu 1800 UV-VIS spectrophotometer. For HPLC analysis, a Jasco Extrema LC-4000 system with a PDA detector was employed, and ChromNAV software was used for data analysis.

Chemicals and Reagents: The Empagliflozin's working standard was kindly provided by Metrochem API Private Limited, Andhra Pradesh, while the Sitagliptin phosphate working standard was sourced from Morepen Laboratories Limited, Himachal Pradesh. HPLC-grade Acetonitrile and Methanol had been procured from the Merck Life Science Pvt. Ltd., and Potassium dihydrogen phosphate had been obtained from Rankem.

Chromatographic Conditions:

A Jasco Extrema LC-4000 HPLC system with a photodiode array detector was employed. ChromNAV software was used on a computer to monitor and analyze the output signal. A BDS Hypersil C18 column (250×4.6 mm, 5 µm) was used to achieve separation. Acetonitrile and potassium dihydrogen phosphate buffer were mixed 50:50 (v/v) to create mobile phase, which had a 1 mL/min flow rate. At 246nm, detection took place while the temp. of the column has been kept at 25°C. The volume of the injection was 10µL. The diluent was a 60:40 v/v ratio of methanol to water. Table 1 displays the optimal circumstances.

Table 1: Chromatographic Conditions

Column	BDS Hypersil C_{18} (250*4.6mm,5 µm)
Mobile Phase	Potassium Dihydrogen Phosphate Buffer: Acetonitrile (50:50 v/v) pH adjusted to 3.5
Wavelength	246nm
Flow rate	1mL/min
Injection volume	10 µL
Run time	10 min
Temperature	25^0C
Diluent	Methanol:Water (60:40 v/v)
Elusion mode	Isocratic

Methods:

Preparation of Potassium Dihydrogen Phosphate Buffer(0.01M): In 1000milliliters of water, dissolve 1.36grams of potassium dihydrogen phosphate. Use orthophosphoric acid to bring the pH down to 3.5.

Preparation of Standard solution: Separately weigh 100mg of sitagliptin phosphate and empagliflozin, then transfer each to a 100mL volumetric flask that has been cleaned and dried. To achieve a concentration of 1000µg/mL, add a 50:50 v/v mixture of methanol as well as water to each flask. Sonicate the solutions until they are completely dissolved. Using the same solvent, dilute 10mL of the 100mL of standard stock solution, resulting in a conc. of 100 µg/mL, to create a sub-stock solution.

Preparation of Sample solution: The sample stock solution was made by precisely weighing twenty tablets and crushing them into a fine powder. A 100 mL volumetric flask was filled with a measured quantity of the powder, which

was equal to 100mg of sitagliptin phosphate and 10 mg of empagliflozin. To ensure full solubility, the powder was dissolved in a methanol: water combination and then sonicated. The stock solution (1Ml) was put into a volumetric flask (10mL) along with diluted with the diluent to the appropriate level to produce a solution having a 100µg/mL of concentration.

Method Development and Validation of HPLC:

The suggested analytical technique was verified based on the ICH requirements (Q2 R1) for parameters like ruggedness, specificity, linearity, and system applicability.

Forced Degradation Studies:

1mL of the filtered stock solution had been moved to a volumetric flask of 10 mL & diluted including diluent to 10 mL to conduct forced deterioration studies. For each stress condition, three sample solutions were ready at a concentration of 10 ppm for EMPA and 100 ppm for SITA. These solutions were subjected to the following stress conditions:

Acidic Degradation:

Acidic degradation was induced by mixing 1N HCl of 1 mL to the sample. After 24 hours, the mixture had beeen neutralized using 1mL of NaOH (1N). Finally, solution was made up of target concentration.

Alkaline Degradation:

Alkaline degradation was performed by adding 1 mL of NaOH (1N) to the sample. After 24 hours, with 1N HCl of 1 mL, the mixture was neutralized. Finally, the solution was made up of target concentration.

Oxidative Degradation:

Oxidative degradation was carried out by treating the sample with 1 mL of 30% (v/v) hydrogen peroxide (H_2O_2) for 24 hours. Finally, the solution was made up of target concentration.

Thermal Degradation:

To perform thermal degradation, API was positioned in a petri dish as well as baked at 60°C for two hours. To evaluate the sample's stability, a 10 µL solution had been mixed into the system.

Photolytic Degradation:

The sample has been exposed to UV light for two hours in a UV chamber in order to assess photolytic deterioration. Ten microliters of solution were added to the device in order to record the chromatograms and evluate the sample stability.

RESULTS AND DISCUSSION:

System suitability: Following the recommended protocol, a standard solution of empagliflozin and sitagliptin phosphate was made and six injections were made into the HPLC system. The suitability parameters of the system were assessed using the standard chromatograms and the percentage RSD of the tailing factor, retention duration, theoretical plates, as well as peak regions from 6 replicate injections. The results have been shown in Table 2, and all parameters were determined to be within the permissible range.

Table 2. System suitability for Empagliflozin and Sitagliptin phosphate

Parameters	Empagliflozin	Sitagliptin phosphate
Retension time	3.327	5.625
Peak area	894328	16390
Theoretical plates	7388	7574
Symmetry factor	1.187	1.292

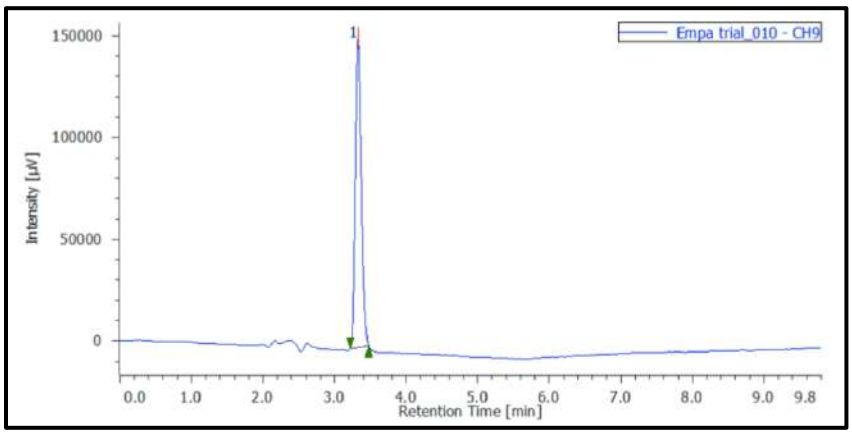

Figure 3:System suitability chromatogram of Empagliflozin

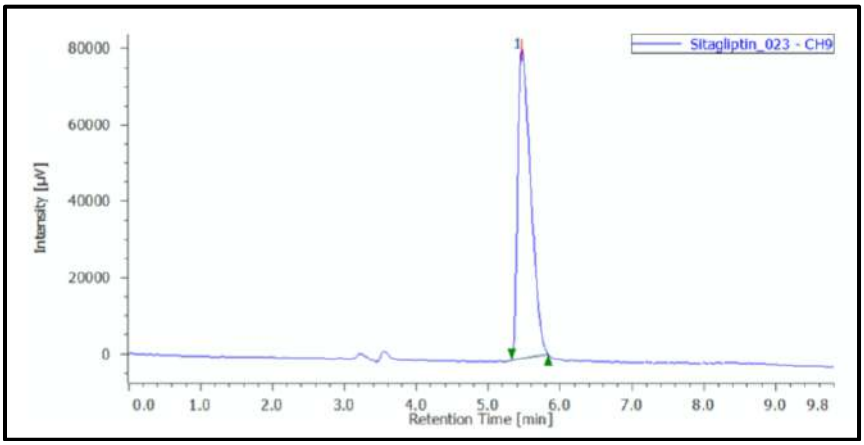

Figure 4: System suitability chromatogram of Sitagliptin phosphate

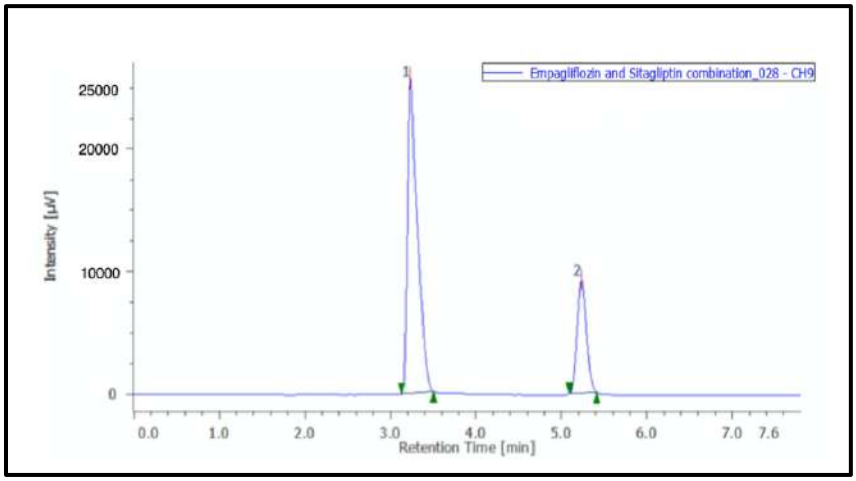

Figure 5: Optimized Chromatogram of Empagliflozin and Sitagliptin phosphate

Specificity: The analytical method must be capable of distinguishing and resolving all process impurities, including both known and unknown degradation impurities, observed during stress testing.

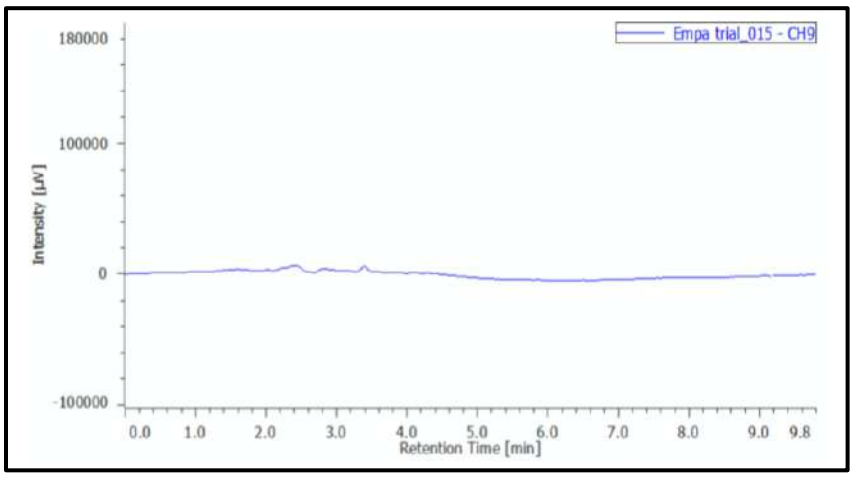

Figure 6: Specificity of Empagliflozin

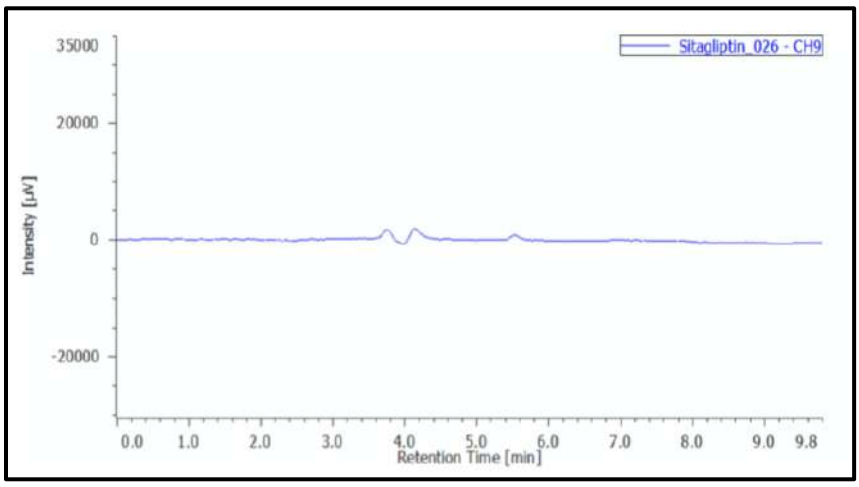

Figure 7: Specificity of Sitagliptin phosphate

Linearity: Standard solutions were prepared with concentrations of 10-60µg/mL for Empagliflozin and 100-600µg/mL for Sitagliptin phosphate. After injecting these solutions, a graph showing the relationship between concentration and peak area was created. The resulting straight line confirmed the linearity of the methodology. The linearity data is described in Table 3 for Empagliflozin and sitagliptin phosphate.

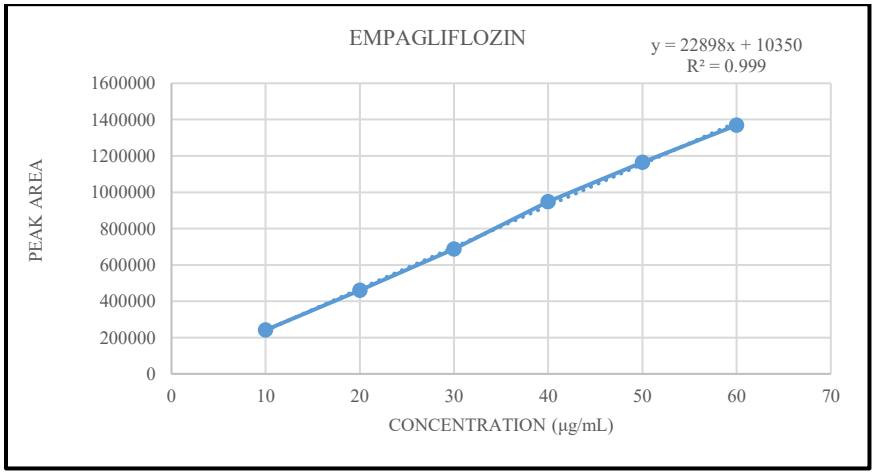

Figure 8: Calibration curve of Empagliflozin

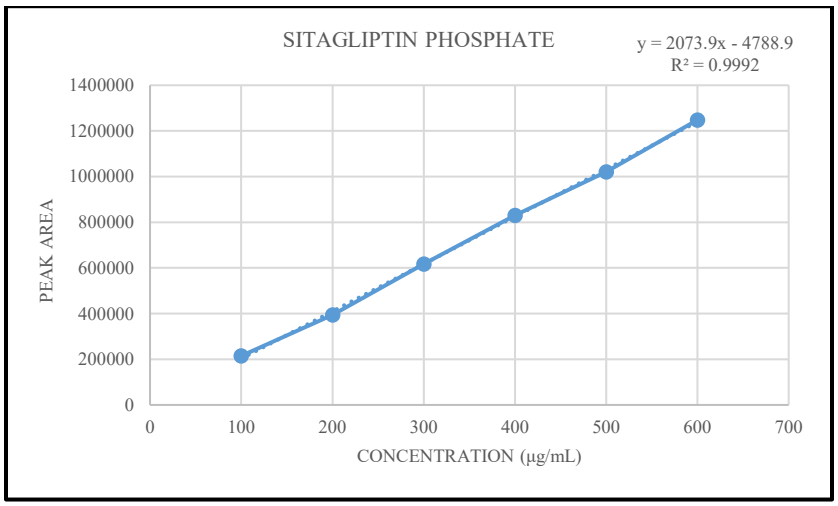

Figure 9: Calibration curve of Sitagliptin phosphate

Table 3. Linearity of Empagliflozin and Sitagliptin phosphate

Sr. No.	Empagliflozin		Sitagliptin phosphate	
	Concentration(µg/mL)	Peak Area	Concentration(µg/mL)	Peak Area
1.	10	241351	100	214831
2.	20	459555	200	394694
3.	30	688195	300	617200
4.	40	947555	400	830745
5.	50	1164849	500	1020966
6.	60	1369156	600	1248123

Accuracy: To evaluate recovery, known quantities of the drugs were added to the placebo at levels corresponding to 50percent, 100percent, and 150percent of the labeled claim of the marketed formulation. For every stage, three samples were made. After the solutions had been examined, the calibration curve was used to calculate the recovery percentage. Tables 4 and 5 display the accuracy findings.

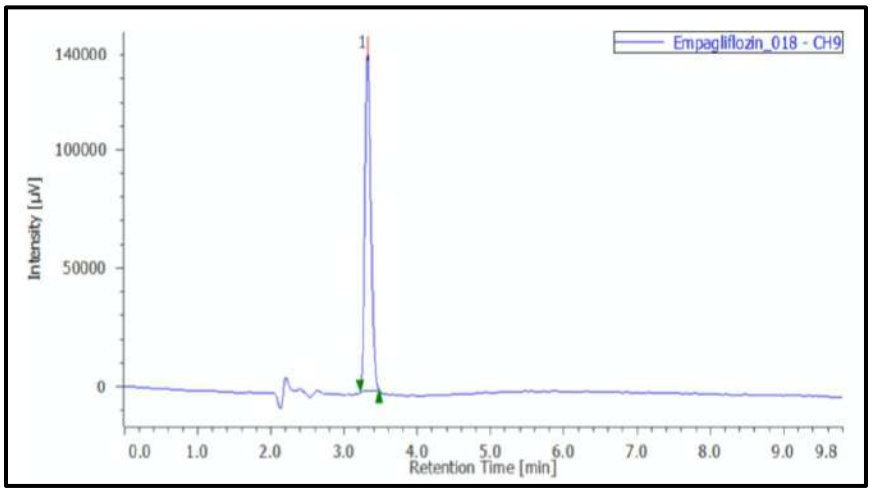

Figure 10: Chromatogram of Empagliflozin for accuracy (80%)

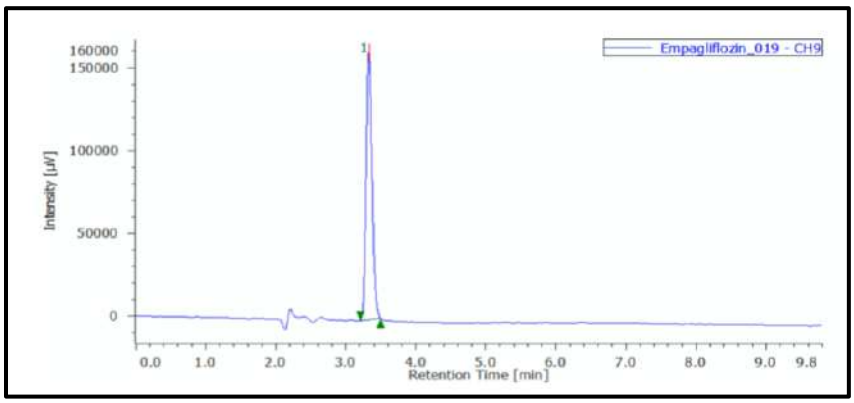

Figure 11: Chromatogram of Empagliflozin for accuracy (100%)

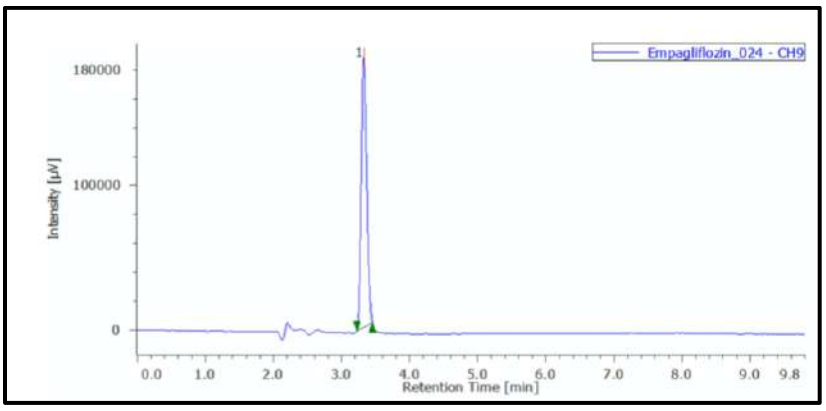

Figure 12: Chromatogram of Empagliflozin for accuracy (120%)

Table 4. Accuracy data of Empagliflozin

Level	Peak area	calculated concentration	% recovery	mean concentration	standard deviation	%RSD
0.8	832951	35.92457857	99.30%	35.75737328	0.236720267	0.662018055
	831496	35.8610359				
	822920	35.48650537				
1	927410	40.04978601	100.72%	40.29292223	0.294053238	0.729788811
	940461	40.61974845				
	931061	40.20923225				
1.2	1016492	43.94016945	101.54%	44.6851399	0.666465527	1.49147016
	1045907	45.22477946				
	1038252	44.89047078				

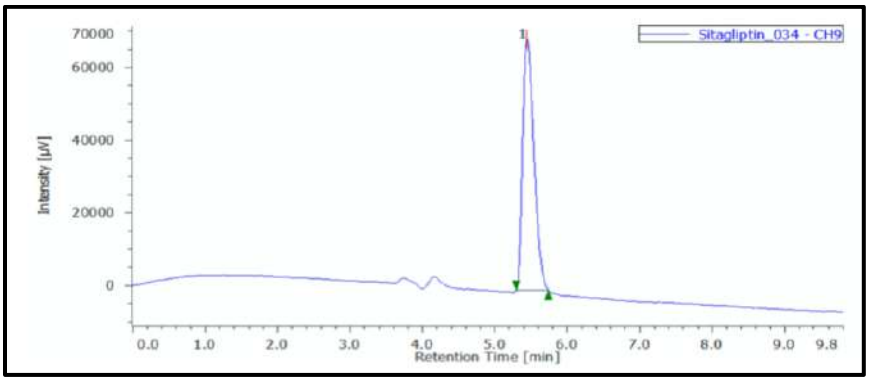

Figure 13: Chromatogram of Sitagliptin phosphate for accuracy (80%)

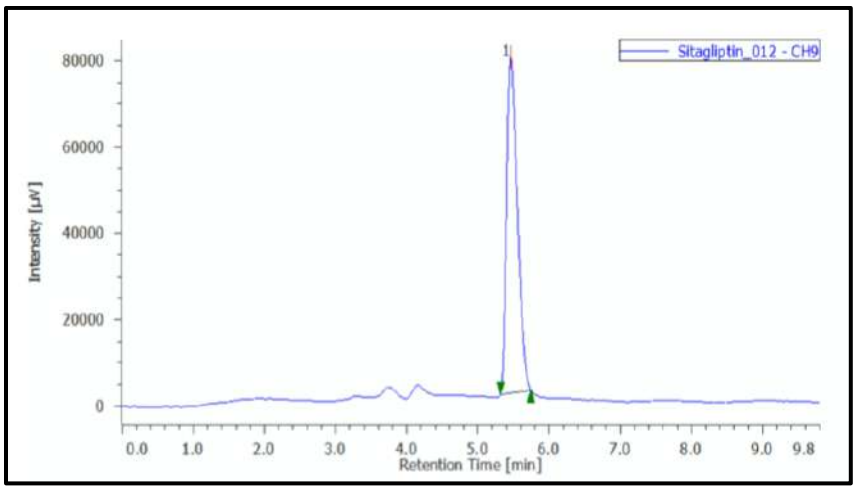

Figure 14: Chromatogram of Sitagliptin phosphate for accuracy (100%)

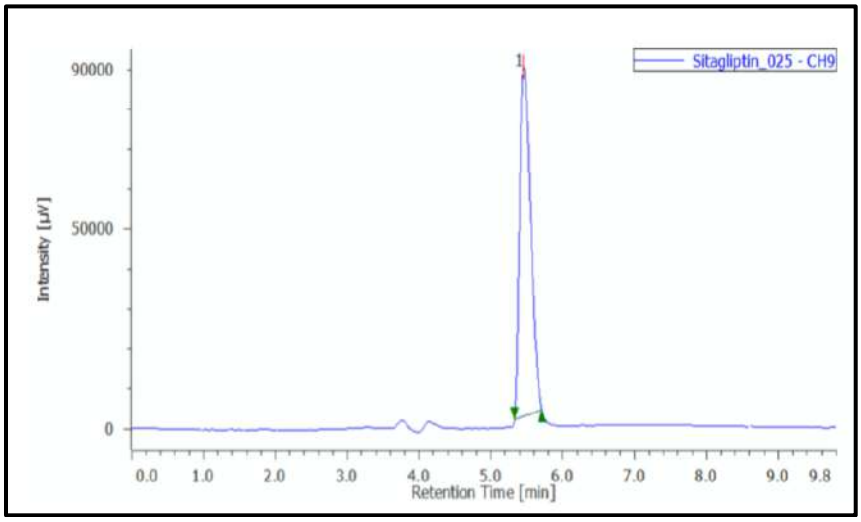

Figure 15: Chromatogram of Sitagliptin phosphate for accuracy (120%)

Table 5: Accuracy data of Sitagliptin phosphate

Level	Peak area	calculated concentration	% recovery	mean concentration	standard deviation	%RSD
0.8	745674	357.242442	99.43%	357.9727888	0.9159627	0.25587495
	749320	359.000482				
	746572	357.675442				
1	833028	399.363084	100.23%	400.9523603	1.67616579	0.41804612
	839956	402.70365				
	835988	400.790347				
1.2	918994	440.814456	100.26%	441.1754504	2.38520415	0.54064752
	915213	438.991321				
	925021	443.720575				

Precision: Intra-day precision was assessed by preparing samples from the same batch at three different concentrations, with 3 replicates for all concentrations. Inter-day precision was evaluated by analyzing the dosage form in triplicate over three consecutive days. Additionally, precision

was confirmed by injecting the standard solution 6 times. The outcomes were reported as %RSD.

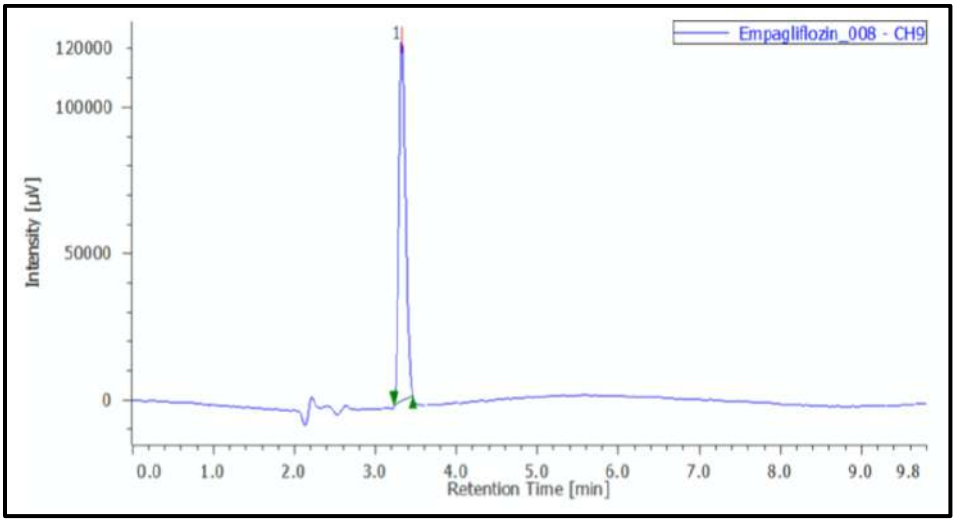

Figure 16: Chromatogram of Empagliflozin for intraday precision

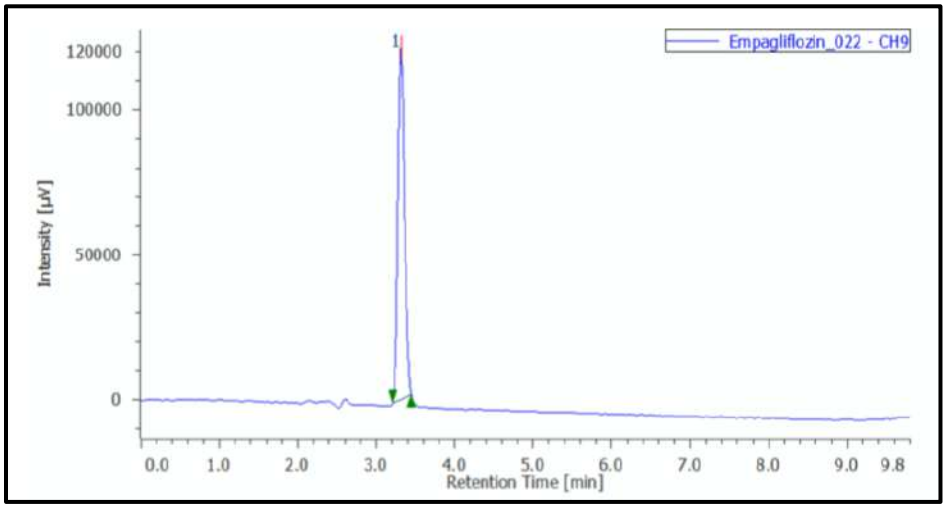

Figure 17: Chromatogram of Empagliflozin for interday precision

Table 6: Results of Intraday and Interday precision by RP-HPLC method for Empagliflozin

Sr. No.	Sample Name	Intraday	Interday
	Concentration 30 µg/mL	Area	
1	TEST_1	706872	691116
2	TEST_2	697375	693340
3	TEST_3	689391	672151
4	TEST_4	700765	703531
5	TEST_5	704219	704655
6	TEST_6	700835	709021
Mean		699909.50	695636
SD		6094.25	13417.00
%RSD		0.87	1.93

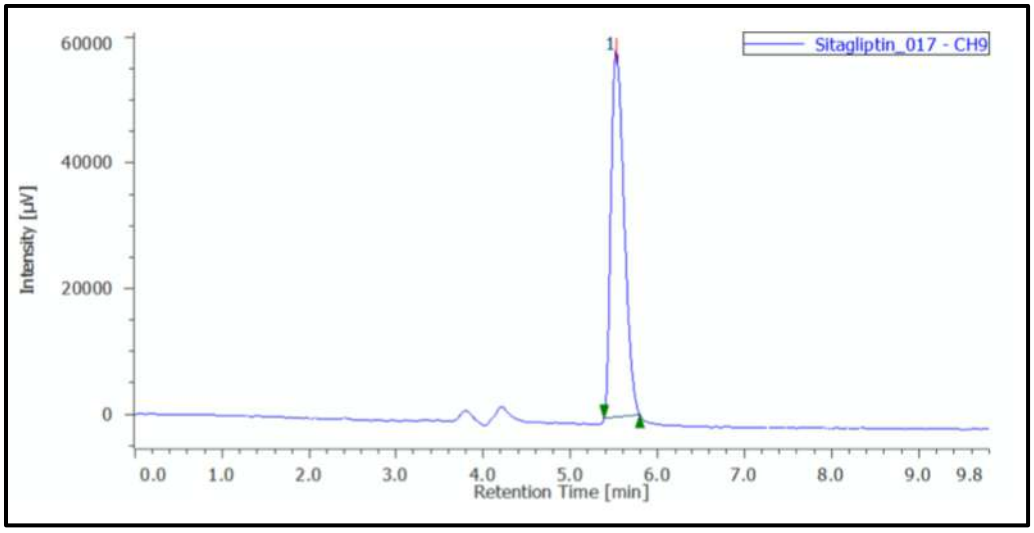

Figure 18: Chromatogram of Sitagliptin phosphate for intraday precision

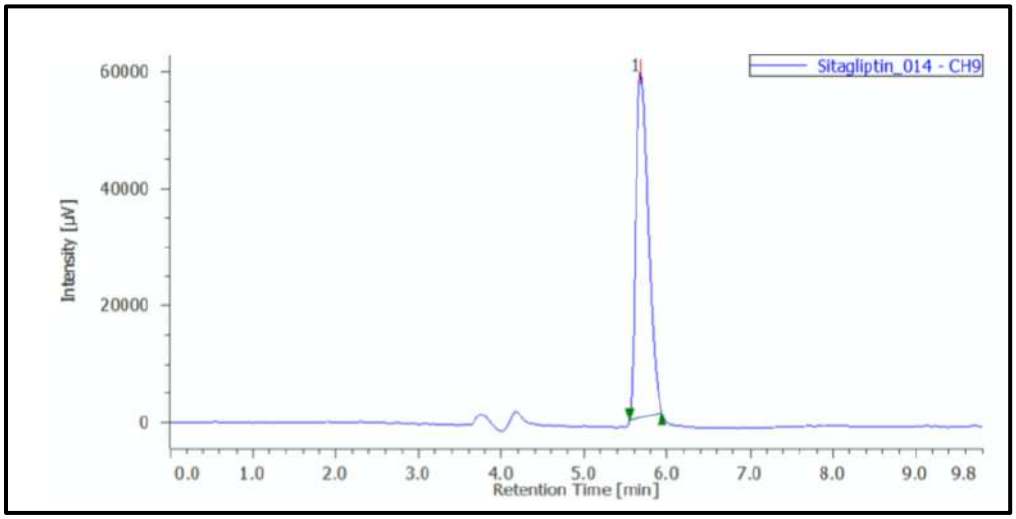

Figure 19: Chromatogram of Sitagliptin phosphate for interday precision

Table 7: Results of Intraday and Interday precision by RP-HPLC method for Sitagliptin phosphate

Sr. No.	Sample Name	Intraday	Interday
	Concentration 300 µg/mL	Area	
1	TEST_1	617945	616654
2	TEST_2	624804	604425
3	TEST_3	611992	613485
4	TEST_4	606876	621457
5	TEST_5	616273	621189
6	TEST_6	611850	627563
Mean		614956.67	617462.17
SD		6186.61	7978.89
%RSD		1.01	1.29

Limit of Detection (LOD) and Limit of Quantitation (LOQ):

The LOQ & LOD had been used to assess the method's sensitivity. These were computed by infusing a succession of diluted solutions with known concentrations, yielding signal-to-noise ratios of 10:1 for LOQ as well as 3:1 for LOD. In contrast to LOQ values of 15.23µg/mL and 136.68µg/mL, respectively, the LOD values for EMPA and SITA have been 5.02 µg per mL and 45.10 µg per mL. These results demonstrate that the method is highly sensitive.

Robustness:

Intentional modifications to the chromatographic conditions were used to evaluate the method's robustness. This involved varying parameters such as flow rate (± 2%), temperature (± 5°C), and wavelength (± 2nm). The flow rate had been adjusted between 0.8mL per min and 1.2mL per min, and the mobile phase's composition was altered by 5%. The method showed consistent performance under lower flow rate conditions. The %RSD values obtained during the study were below 2%, indicating that the method is robust and reliable.

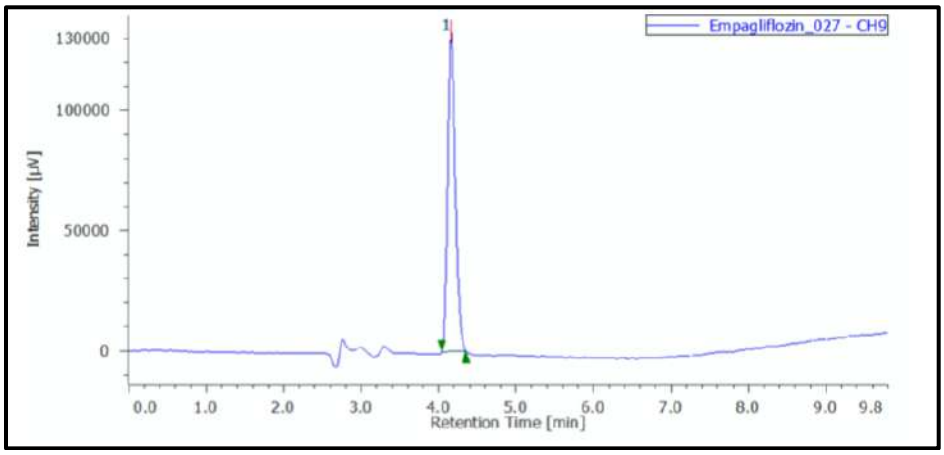

Figure 20: Chromatogram of Empagliflozin for robustness (0.8ml per min)

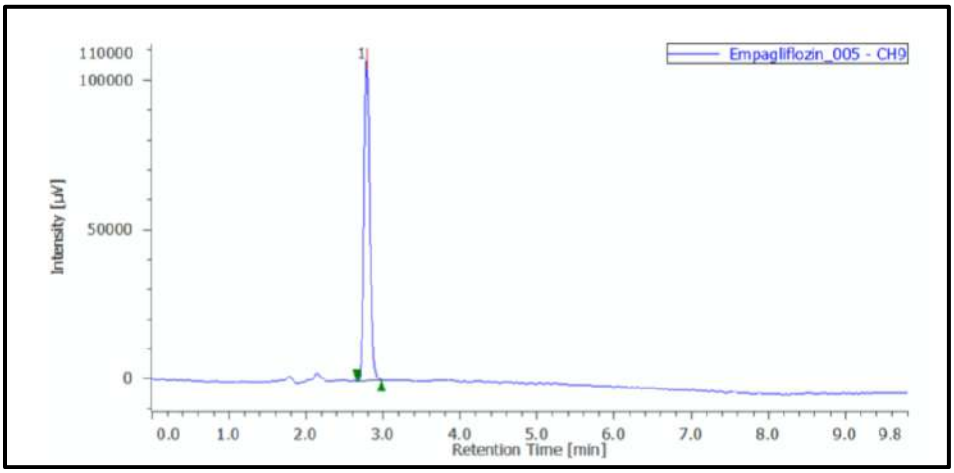

Figure 21: Chromatogram of Empagliflozin for robustness (1.2 ml/min)

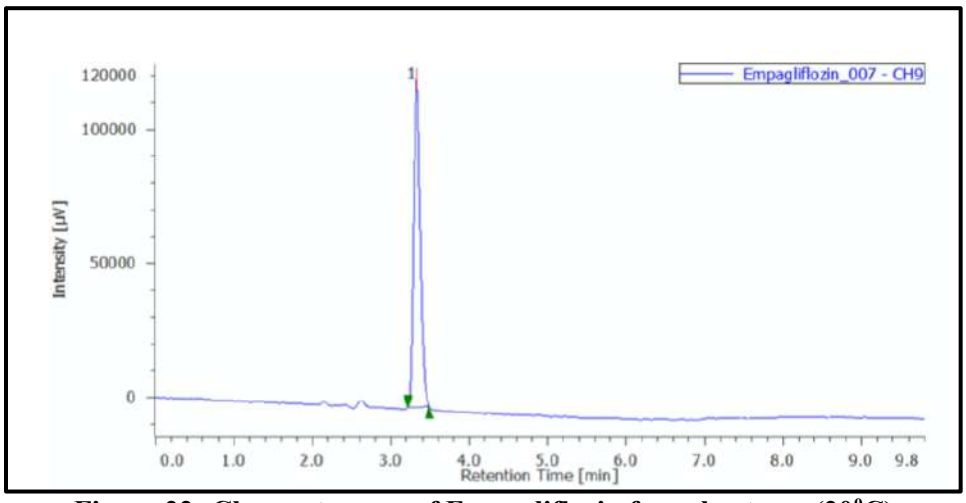

Figure 22: Chromatogram of Empagliflozin for robustness (20^{0}C)

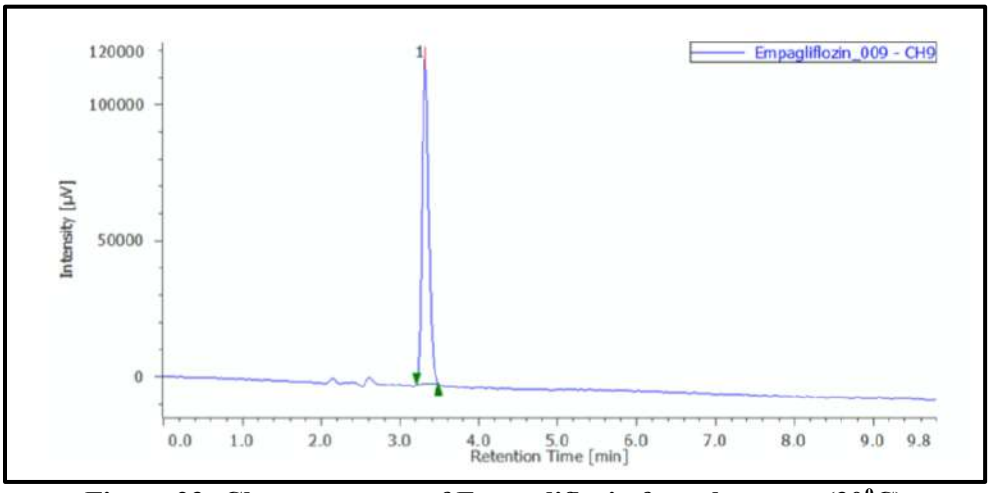

Figure 23: Chromatogram of Empagliflozin for robustness (30^{0}C)

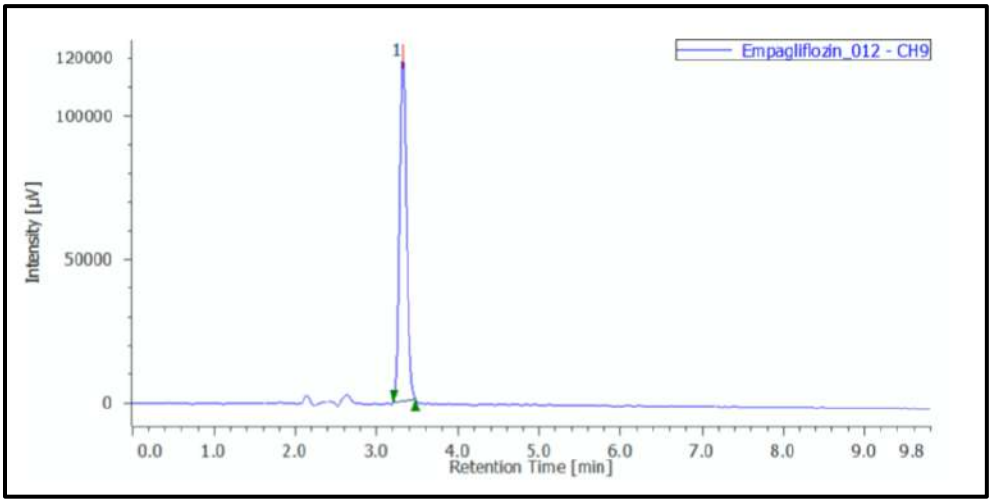

Figure 24: Chromatogram of Empagliflozin for robustness (222nm)

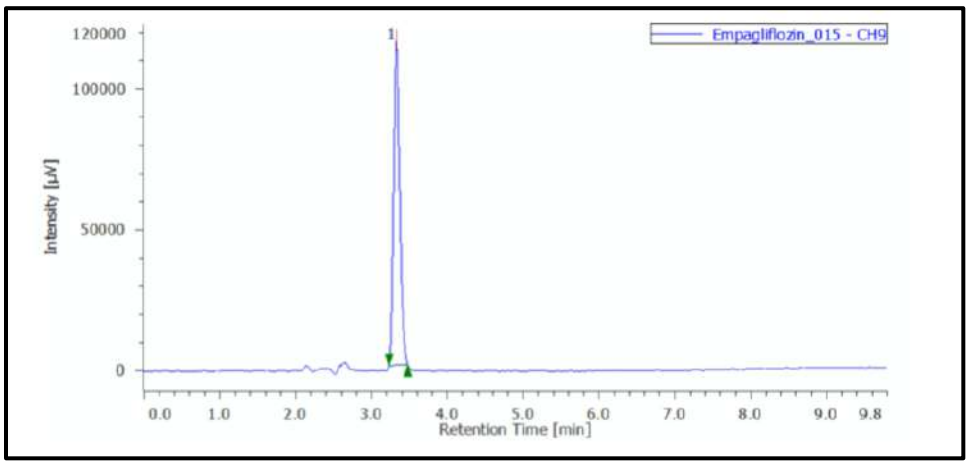

Figure 25: Chromatogram of Empagliflozin for robustness (226nm)

Table 8: Robustness results at different temperature, flow rate and wavelength for Empagliflozin

SR NO.			1	2	3	MEAN	SD	%RSD
FLOW RATE	1.2ml/min	AREA	572348	578570	560597	570505	9127.14	1.60
		RT	2.783	2.787	2.783	3	0.00	0.08
		NTP	6626	6711	6714	6684	49.96	0.75
	0.8ml/min	AREA	912164	893682	884795	896880	13962.00	1.56
		RT	4.16	4.15	4.147	4	0.01	0.16
		NTP	8599	8724	8936	8753	170.36	1.95
TEMP	20°C	AREA	713298	724268	705846	714471	9266.82	1.30
		RT	3.337	3.327	3.313	3	0.01	0.36
		NTP	7342	7326	7315	7328	13.58	0.19
	30°C	AREA	710366	723936	715779	716694	6831.08	0.95
		RT	3.317	3.317	3.313	3	0.00	0.07
		NTP	7276	7264	7254	7265	11.02	0.15
WAVELENTH	222nm	AREA	703083	708675	707382	706380	2927.56	0.41
		RT	3.32	3.32	3.31	3	0.01	0.17
		NTP	7483	7413	7267	7388	110.21	1.49
	226nm	AREA	664787	663413	672451	666884	4870.15	0.73
		RT	3.327	3.317	3.32	3	0.01	0.15
		NTP	7675	7567	7392	7545	142.82	1.89

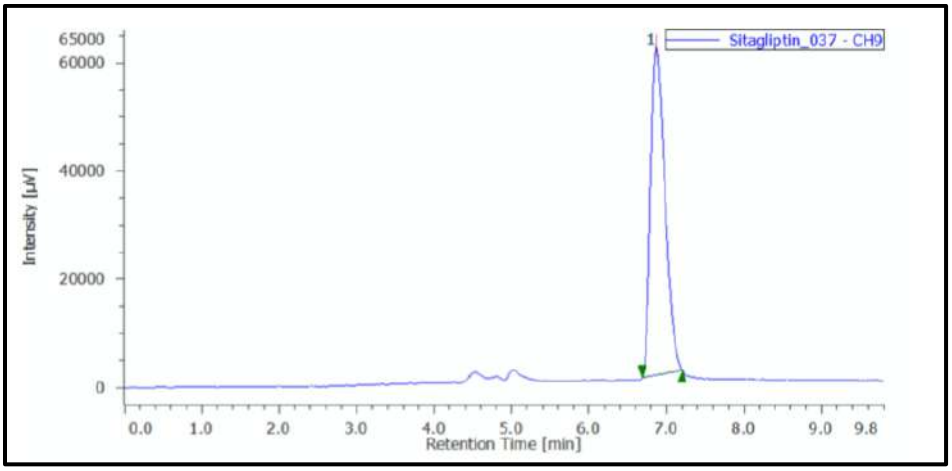

Figure 26: Chromatogram of Sitagliptin phosphate for robustness (0.8ml/min)

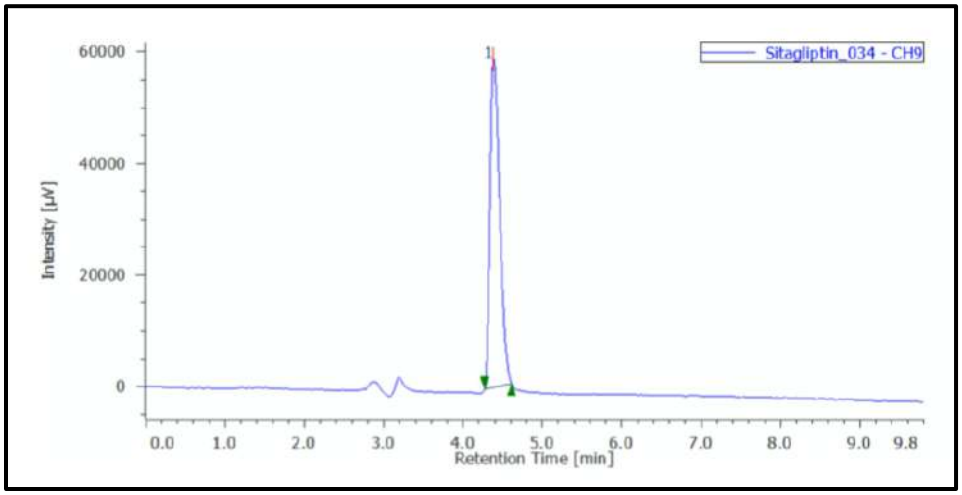

Figure 27: Chromatogram of Sitagliptin phosphate for robustness (1.2 ml/min)

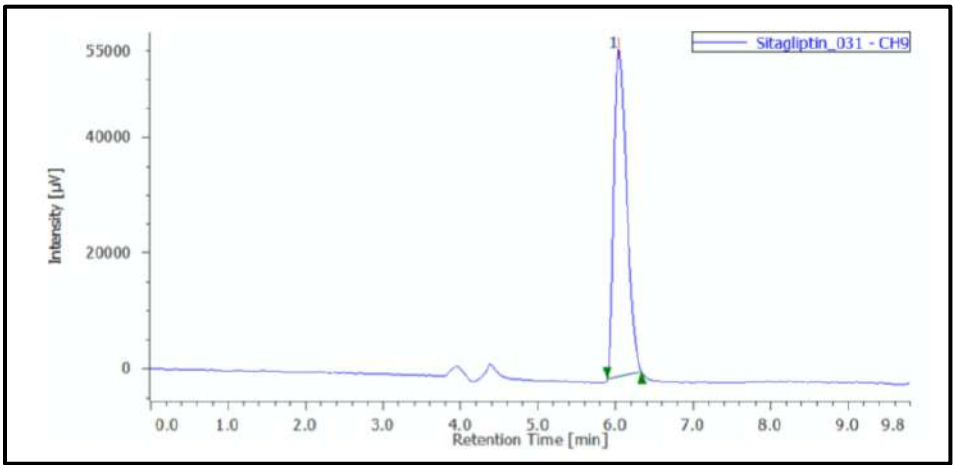

Figure 28: Chromatogram of Sitagliptin phosphate for robustness (20^{0}C)

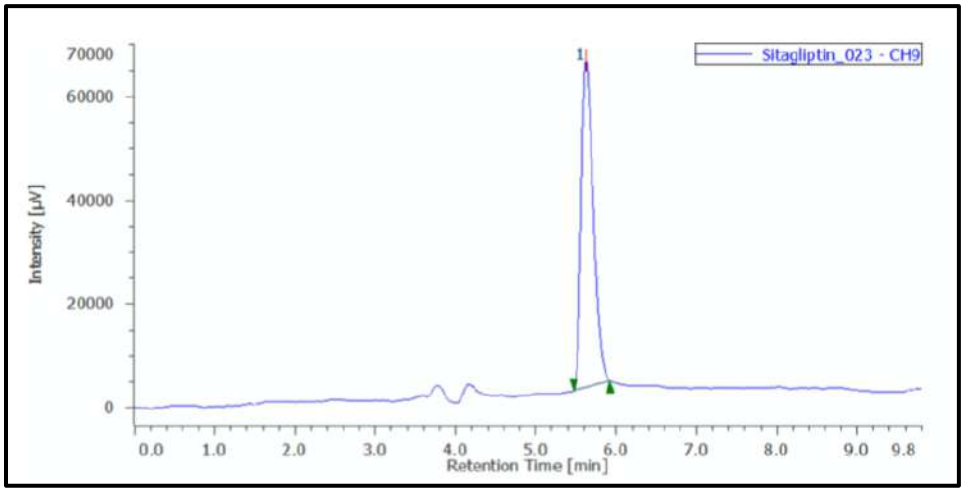

Figure 29: Chromatogram of Sitagliptin phosphate for robustness (30^{0}C)

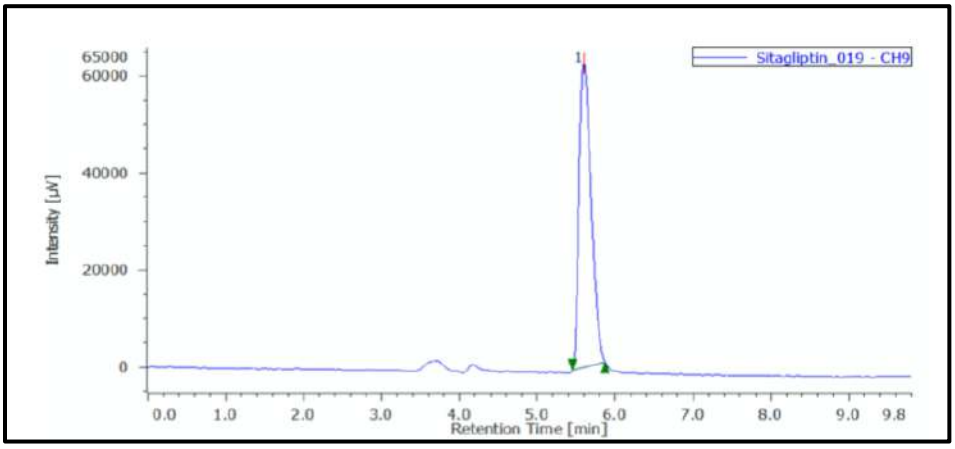

Figure 30: Chromatogram of Sitagliptin phosphate for robustness (265nm)

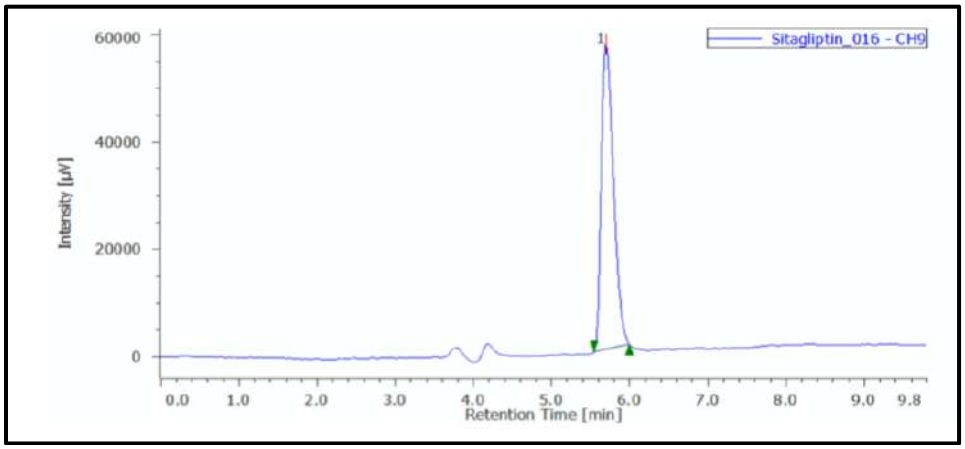

Figure 31: Chromatogram of Sitagliptin phosphate for robustness (269nm)

Table 9: Robustness outcomes at different temperatures, flow rates, as well as wavelength for Sitagliptin phosphate

SR NO.			1	2	3	MEAN	SD	%RSD
FLOW RATE	1.2ml/min	AREA	791405	771222	766150	776259	13359.73	1.72
		RT	6.863	6.803	6.787	7	0.04	0.59
		NTP	6074	6139	6130	6114	35.22	0.58
	0.8ml/min	AREA	497640	486223	482453	488772	7907.86	1.62
		RT	4.383	4.407	4.403	4	0.01	0.29
		NTP	5963	6010	6003	5992	25.36	0.42
TEMP	20°C	AREA	668324	653316	651324	657655	9293.44	1.41
		RT	5.973	6.04	6.027	6	0.04	0.59
		NTP	6025	5996	5988	6003	19.47	0.32
	30°C	AREA	651310	663556	658772	657879	6171.61	0.94
		RT	5.773	5.623	5.603	6	0.09	1.64
		NTP	6144	6354	6360	6286	123.01	1.96
WAVELENTH	265nm	AREA	675853	677577	669985	674472	3980.03	0.59
		RT	5.597	5.683	5.703	6	0.06	0.99
		NTP	5889	6027	6085	6000	100.68	1.68
	269nm	AREA	620252	612540	602649	611814	8823.95	1.44
		RT	5.693	5.68	5.687	6	0.01	0.11
		NTP	6020	6072	6158	6083	69.69	1.15

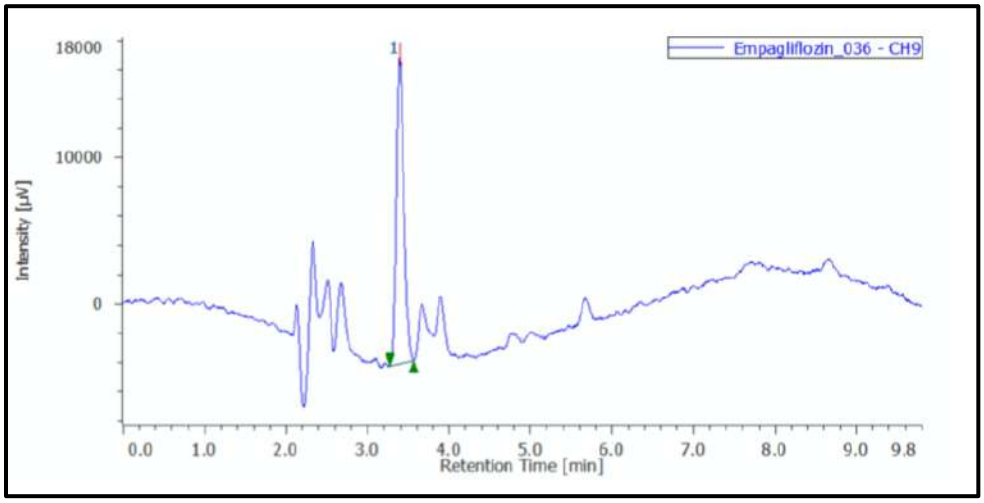

Figure 32: Chromatogram of Acid degradation of Empagliflozin

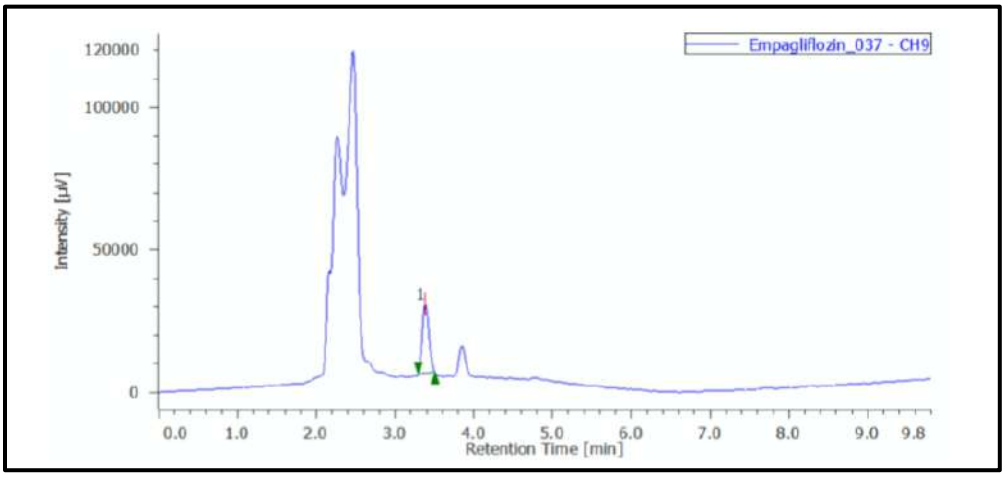

Figure 33: Chromatogram of Alkaline degradation of Empagliflozin

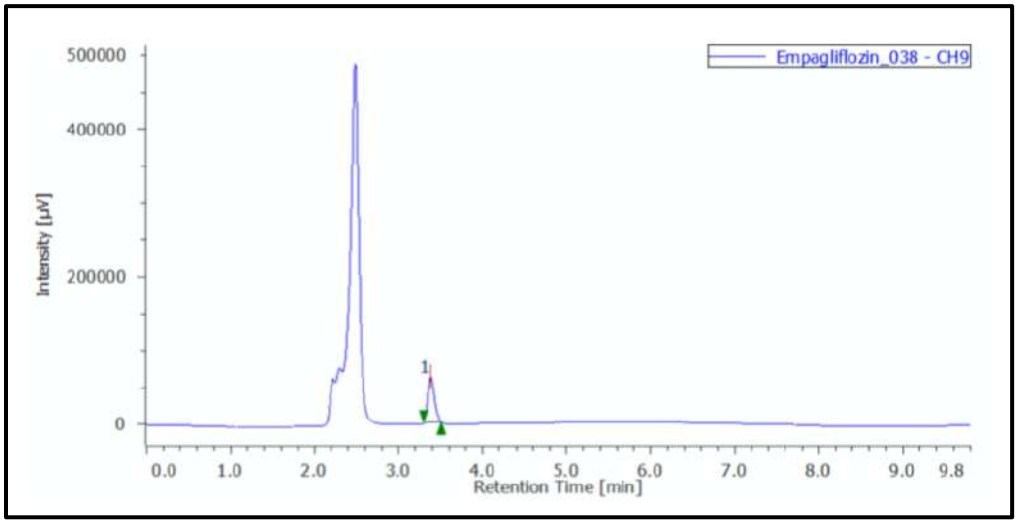

Figure 34: Oxidative degradation of empagliflozin chromatogram

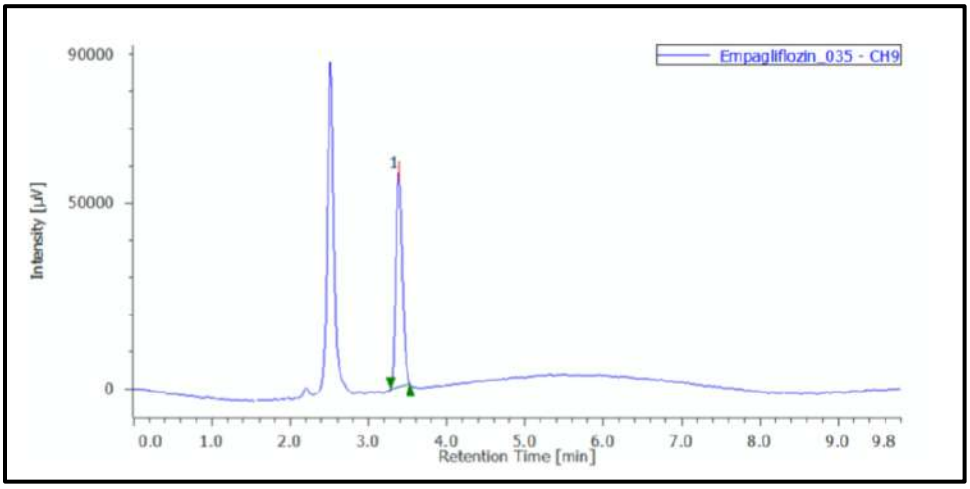

Figure 35: Chromatogram of Empagliflozin Photolytic Degradation

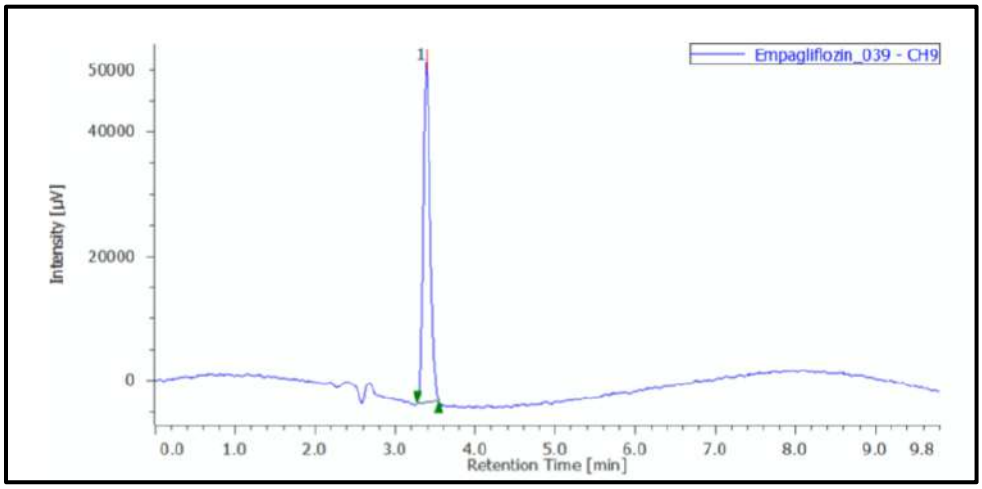

Figure 36: Chromatogram of Thermal degradation of Empagliflozin

Table 10: Degradation study of Empagliflozin

Sr. No.	Type	Condition	Time(hrs)	Conc	Area	% Degradation
1	Acid	1M HCL	24	10	135910	0.43
2	Alkaline	1M NaOH	24	10	140194	0.41
3	Oxidative	30% H_2O_2	24	10	349773	0.44
4	Thermal	60°C	2	10	331673	0.37
5	Photolytic	UV Chamber	2	10	340165	0.4

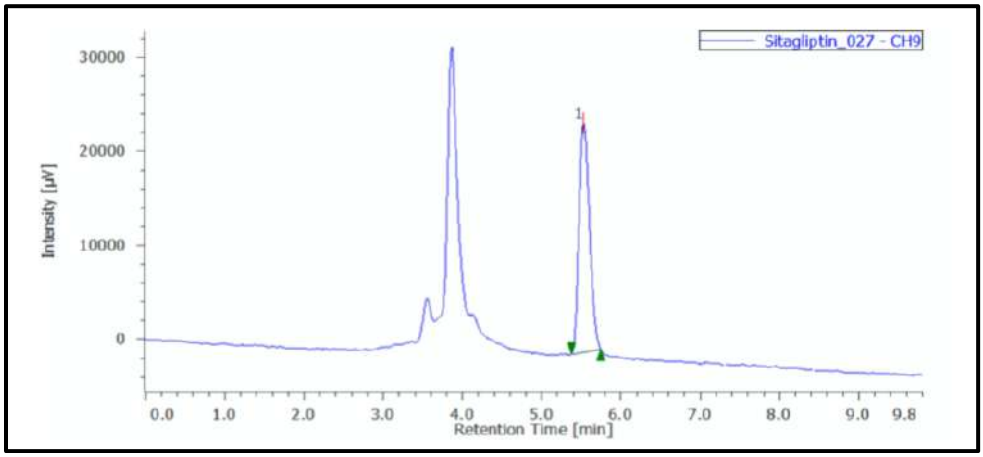

Figure 37: Chromatogram of Acid degradation of Sitagliptin phosphate

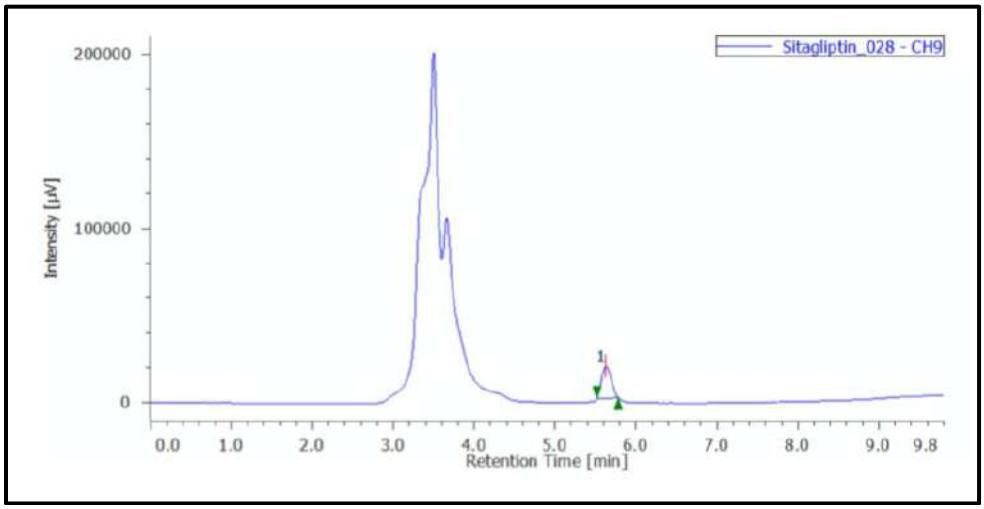

Figure 38: Chromatogram of Alkaline degradation of Sitagliptin phosphate

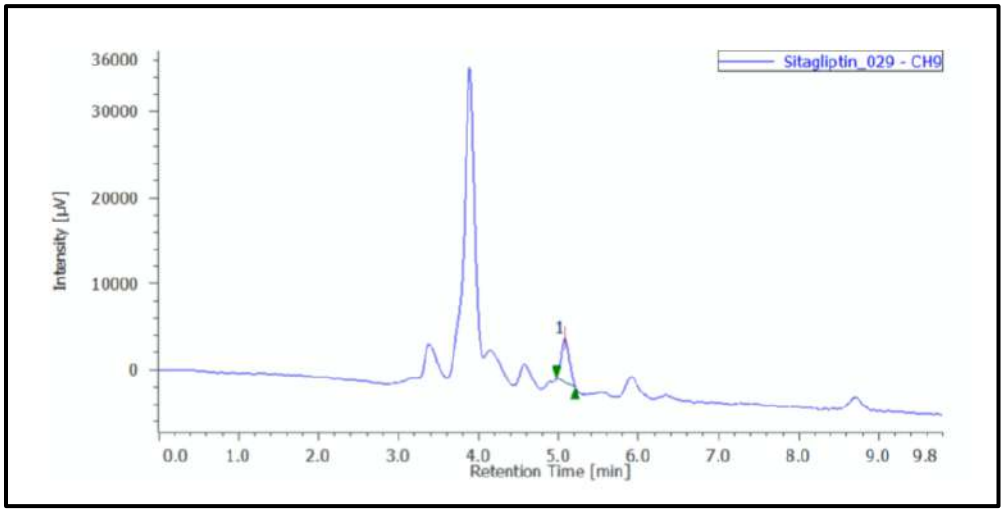

Figure 39: Chromatogram of Sitagliptin Phosphate Oxidative Degradation

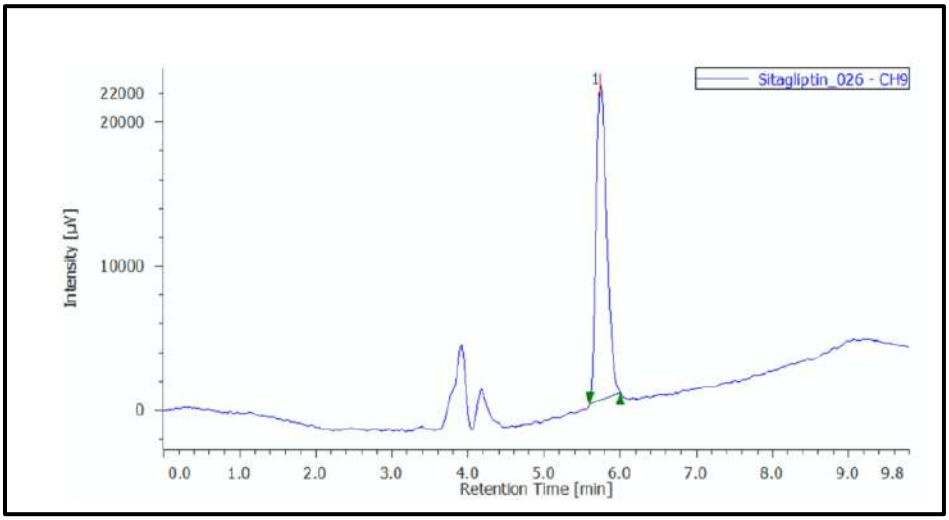

Figure 40: Chromatogram of Sitagliptin phosphate Photolytic degradation

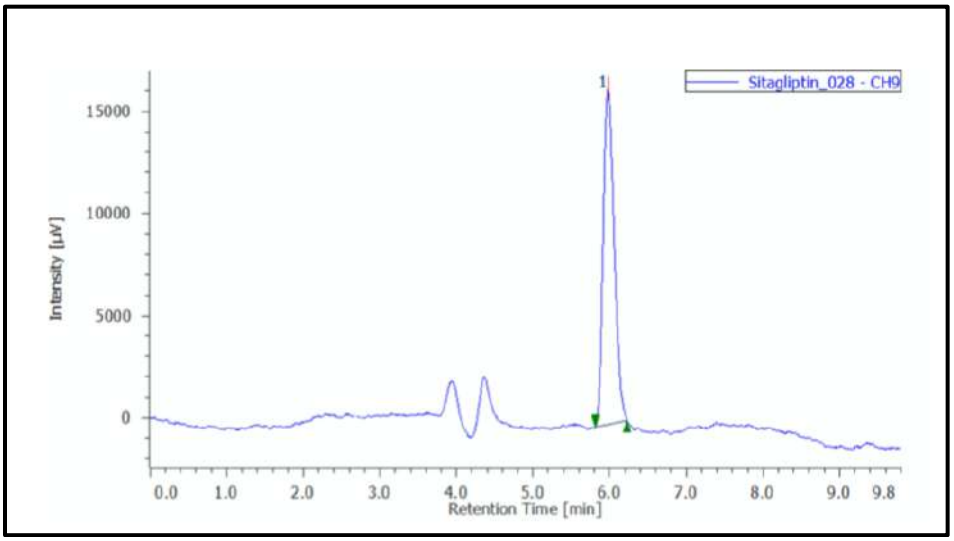

Figure 41: Chromatogram of Thermal degradation of Sitagliptin phosphate

Table 11: Degradation study of Sitagliptin phosphate

Sr. No.	Type	Condition	Time(hrs)	Conc	Area	% Degradation
1	Acid	1M HCL	24	10	224130	0.04
2	Alkaline	1M NaOH	24	10	151560	0.29
3	Oxidative	30% H_2O_2	24	10	36113	0.83
4	Thermal	60ºC	2	10	163247	0.24
5	Photolytic	UV Chamber	2	10	213282	0.007

CONCLUSION

To estimate empagliflozin and sitagliptin phosphate simultaneously, a stability-indicating RP-HPLC method had been created and verified. As stated by ICH criteria, the approach was determined to be robust, specific, accurate, and exact. To evaluate the durability of the medications, stress degradation tests were conducted in alkaline, acidic, thermal, oxidative, and photolytic environments. The method successfully separated drugs from their degradation products, confirming its suitability as a stability-indicating technique. This technique is dependable for stability testing and routine quality

control of empagliflozin and sitagliptin phosphate in pharmaceutical formulations. It is suitable for monitoring product quality and identifying degradation products throughout the drug's shelf life.

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