Ind. J. Pharm. Edu. Res., 2026; 60(3s):s1261-s1266.
https://www.ijper.org

Original Article

Establishment of a Sensitive and Stability-Indicating RP-HPLC Protocol for Co-Quantification of Empagliflozin and Sitagliptin

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ABSTRACT

Aim/Background: To develop a stability-indicating RP-HPLC method for simultaneous quantification of Empagliflozin (EMPA) and Sitagliptin (SITA) in a synthetic mixture for quality control and stability studies. **Materials and Methods:** Separation used a C_{18} column with phosphate buffer-methanol-acetonitrile (40:20:40,%v/v/v) at 1.0 mL/min, detected at 224 nm. Validation per ICH Q2(R1) assessed linearity, precision, accuracy, specificity, and robustness. Stress tests included acidic, alkaline, oxidative, thermal, and photolytic conditions. **Results:** EMPA (2.8 min) and SITA (3.7 min) showed linearity (R^2 >0.99) over 20-120 µg/mL (EMPA) and 200-1200 µg/mL (SITA), with %RSD < 2% and recovery 99-102%. Detection limits were 1.8 µg/mL (EMPA) and 4.7 µg/mL (SITA). Stress tests revealed moderate EMPA degradation (acidic/alkaline) and high SITA degradation (thermal/photolytic), with distinct degradation peaks. This precise, accurate RP-HPLC method is stability-indicating and suitable for routine quality control and stability analysis of EMPA-SITA mixtures per ICH guidelines. **Conclusion:** The developed RP-HPLC method is precise, accurate, and reliable for simultaneous estimation of EMPA and SITA. Its stability-indicating nature ensures suitability for routine quality control and stability studies of Synthetic Mixture.

Keywords: Empagliflozin, Sitagliptin, RP-HPLC, Stability-Indicating Method, Forced Degradation, Method Validation.

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Received: 12-01-2026;
Revised: 26-03-2026;
Accepted: 04-05-2026.

INTRODUCTION

Type 2 Diabetes Mellitus (T2DM) is a chronic metabolic disorder characterized by insulin resistance and relative insulin deficiency, leading to persistent hyperglycemia. Globally, the prevalence of T2DM is rising rapidly due to sedentary lifestyles, obesity, and dietary habits. Effective glycemic control remains a cornerstone in preventing long-term complications of the disease.[1-4]

Among the various antidiabetic agents, empagliflozin-a Sodium-Glucose Co-Transporter 2 (SGLT2) inhibitor-and sitagliptin-a Dipeptidyl Peptidase-4 (DPP-4) inhibitor-have gained popularity due to their complementary mechanisms. Empagliflozin promotes urinary glucose excretion, while sitagliptin enhances incretin levels to regulate insulin secretion and glucagon levels, providing synergistic effects in glycemic control. Combination therapy with these agents has shown improved clinical outcomes and patient compliance, particularly in patients inadequately controlled on monotherapy.[1-2]

The clinical benefit of this combination has also been supported by trials such as NCT01289990, which evaluated the safety and efficacy of empagliflozin as an add-on to sitagliptin (with or without metformin) in patients with T2DM.[3]

Numerous analytical methods have been developed for the individual estimation of empagliflozin or sitagliptin using HPLC, UPLC, and LC-MS/MS techniques.[4-6] However, only a limited number of studies have addressed their simultaneous estimation in combined pharmaceutical formulations. Existing methods often lack robustness or fail to assess stability-indicating parameters essential for quality control during formulation development and shelf-life assessment.[7,8]

The novelty of the present study lies in the development and validation of a simple, specific, and stability-indicating Reverse-Phase HPLC (RP-HPLC) method for the simultaneous determination of empagliflozin and sitagliptin in combined dosage forms. The method is fully validated as per ICH Q2(R1) guidelines for parameters such as linearity, accuracy, precision, specificity, robustness, and forced degradation, ensuring its applicability for routine quality control and stability studies.

DOI: 10.5530/ijper.20262811

MATERIALS AND METHODS

Chemicals and Reagents

A stability-indicating RP-HPLC method was developed and validated for the simultaneous quantification of empagliflozin and sitagliptin in combined formulations. The method effectively separates both drugs from degradation products and meets ICH guidelines for routine quality control.

Instrumentation and Chromatographic Conditions

Chromatographic analysis was conducted on a Shimadzu LC system equipped with a photodiode array detector and a C18 column. The separation utilized a mobile phase of phosphate buffer (pH 3.0), methanol, and acetonitrile in a 40:20:40 ratio, which was pre-filtered and degassed. The method operated at a 1.0 mL/min flow rate with detection at 224 nm and a 20 µL sample injection. The system demonstrated high efficiency, yielding distinct, symmetrical peaks for both compounds, and met established criteria for resolution, theoretical plates, and peak shape.

Preparation of Standard and Sample Solutions

EMPA and SITA (1000 µg/mL) were prepared in methanol and sonicated. Calibration standards were generated by diluting stocks to 20-120 µg/mL (EMPA) and 200-1200 µg/mL (SITA) with mobile phase, and analyzed in triplicate for linearity. Sample solutions were prepared by extracting a synthetic mixture equivalent to 25 mg EMPA and 100 mg SITA in methanol, filtered, and diluted to yield 50 µg/mL EMPA and 200 µg/mL SITA for assay.

Method validation Parameters

Linearity: Measures the proportional relationship between analyte concentration and detector response, with $R^2 > 0.99$ for EMPA (20-120 µg/mL) and SITA (200-1200 µg/mL).

Specificity: Ensures no interference from excipients or degradation products at EMPA (2.8 min) and SITA (3.7 min) retention times.

Accuracy: Reflects closeness to true value, with recoveries of 99.66-101.36% (EMPA) and 97.86-101.56% (SITA) at 50-150% spiking levels.

Precision: Indicates repeatability, with intra-day %RSD of 0.78% (EMPA) and 1.03% (SITA), and inter-day %RSD of 1.5% (EMPA) and 1.7% (SITA).

LOD/LOQ: Detects lowest measurable concentrations, with LOD of 1.8 µg/mL (EMPA) and 4.7 µg/mL (SITA), and LOQ of 5.6 µg/mL (EMPA) and 14.2 µg/mL (SITA).

Robustness: Assesses method reliability under minor variations, with %RSD < 2% for changes in flow rate (±0.1 mL/min), wavelength (±4 nm), and mobile phase ratio (±4%).

Forced Degradation Studies

To evaluate the stability-indicating capability of the method, forced degradation (stress testing) was carried out on EMPA and SITA - individually and in combination under various stress conditions as recommended by ICH Q1A (R2):

Acidic degradation

Individual and combined samples of EMPA and SITA were subjected to 0.1 N HCl and incubated at 50°C for 2 hr. After cooling, the solutions were neutralized with 0.1 N NaOH, diluted with mobile phase to approximately 60 µg/mL (EMPA) and 600 µg/mL (SITA), and analyzed by HPLC.

Alkaline degradation

Samples were treated with 0.1 N NaOH under identical conditions, then neutralized with 0.1 N HCl and prepared for chromatographic analysis at the same concentrations.

Oxidative degradation

The analytes were exposed to 6% hydrogen peroxide at 40°C for 2 hr, and excess peroxide was neutralized as needed with sodium bisulfite prior to dilution for HPLC evaluation.

Thermal degradation

Drug powders and their mixture were spread thinly and heated in a dry oven at 70°C for 2 hr. Cooled samples were dissolved in methanol, brought to volume with mobile phase, filtered, and injected for analysis.

Photolytic degradation

Solid samples were exposed to sunlight for 12 hr, approximating 1.2 million lux hours. These were subsequently dissolved and diluted for HPLC as described above.

RESULTS

Method validation

Linearity

Calibration curves for empagliflozin and sitagliptin exhibited strong linearity across the ranges of 20-120 µg/mL and 200-1200 µg/mL, respectively as presented in Table 1. The linear regression equations relating peak area to analyte concentration were $y = 8916x + 7553.5$ for empagliflozin and $y = 382.86x + 5304.1$ for sitagliptin, with correlation coefficients (R^2) of 0.9954 and 0.9979, indicating excellent linearity for both compounds, indicating excellent linearity of response across the tested range. There was no significant deviation from linearity at the upper or lower concentration levels, confirming the defined range is appropriate for quantification of low (20% of nominal) to high (120% of nominal) analyte levels.

Specificity

The specificity of the method was confirmed by the absence of interference from excipients or degradation products at the retention times of Empagliflozin and Sitagliptin. This demonstrates that the method accurately distinguishes and quantifies the analytes in the presence of potential sample matrix components, as shown in Figure 1.

Accuracy

Recovery studies yielded high accuracy. The percentage recoveries of EMPA were 99.66%, 100.45%, and 101.36% at the 50%, 100%, and 150% spiking levels, respectively. For SITA, the recoveries were 97.86%, 99.72%, and 101.56% at the corresponding levels. All recovery values are close to 100% and well within acceptable limits (98-102%), demonstrating that the method is accurate and that common excipients or degradation products do not interfere with quantification. Which as presented in Table 2.

Precision

The method showed excellent precision. In repeatability (intra-day precision), the %RSD of peak areas for six replicate injections of the standard mixture (60 µg/mL EMPA + 600 µg/mL SITA) was 0.78% for EMPA and 1.03% for SITA. Intermediate precision (day-to-day) also remained low, with overall %RSD values of 1.5% for EMPA and 1.7% for SITA across three different days. These low RSD values (2%) confirm that the method produces consistent results under the same operating conditions over short intervals and is reproducible on different days, meeting the criterion for precision.

LOD and LOQ

The method showed high sensitivity, with limits of detection of 1.8 µg/mL for empagliflozin and 4.7 µg/mL for sitagliptin. Limits of quantitation were 5.6 µg/mL and 14.2 µg/mL, respectively, enabling accurate measurement well below the lowest calibration standards.

Robustness

The robustness of the developed HPLC method was evaluated by deliberately varying critical chromatographic parameters, including flow rate (±0.1 mL/min), detection wavelength (±4 nm), and mobile phase composition (±4% for each component). These minor, deliberate changes did not result in significant variations in the mean peak areas or retention times for Empagliflozin and Sitagliptin, as indicated by low %RSD values (<2%). The results confirm that the method is robust and reliable for routine analysis, demonstrating minimal influence from typical operational variations the results are shown in Table 3.

Forced Degradation Study

The developed HPLC method was applied to EMPA and SITA samples subjected to various forced degradation conditions (Table 4). In all cases, the empagliflozin and sitagliptin peaks in the stressed samplesh retained their original retention times, and any additional peaks from degradation were baseline-separated from the drug peaks, demonstrating the method's specificity.

Table 4 summarizes the results of forced-degradation studies. Under acidic and alkaline conditions, EMPA showed moderate degradation (Acidic Condition 12.96% and Aalkaline conditions 15.11%) while SITA degraded slightly more (Acidic Condition 13.85% and Aalkaline conditions 15.27%), with the remaining percentages above 85% for both drugs. Under oxidative

Table 1: Linearity data of Empa and Sita.

Empagliflozin			
Sl. No.	**Conc µg/mL**	**Mean peak area±SD (*n*=6)**	**%RSD**
1.	20	181993±2158.49	1.19
2.	40	351819±5473.02	1.56
3.	60	531672±9453.7	1.78
4.	80	660472±11375.98	1.72
5.	100	894885±14500.64	1.62
6.	120	1078671±15311.81	1.42
Sitagliptin			
Sl. No.	**Conc µg/mL**	**Mean peak area±SD (*n*=6)**	**%RSD**
1.	200	70623±1339.98	1.90
2.	400	151663±2603.43	1.72
3.	600	228168±4017.31	1.76
4.	800	292877±4509.42	1.54
5.	1000	370438±6832.21	1.84

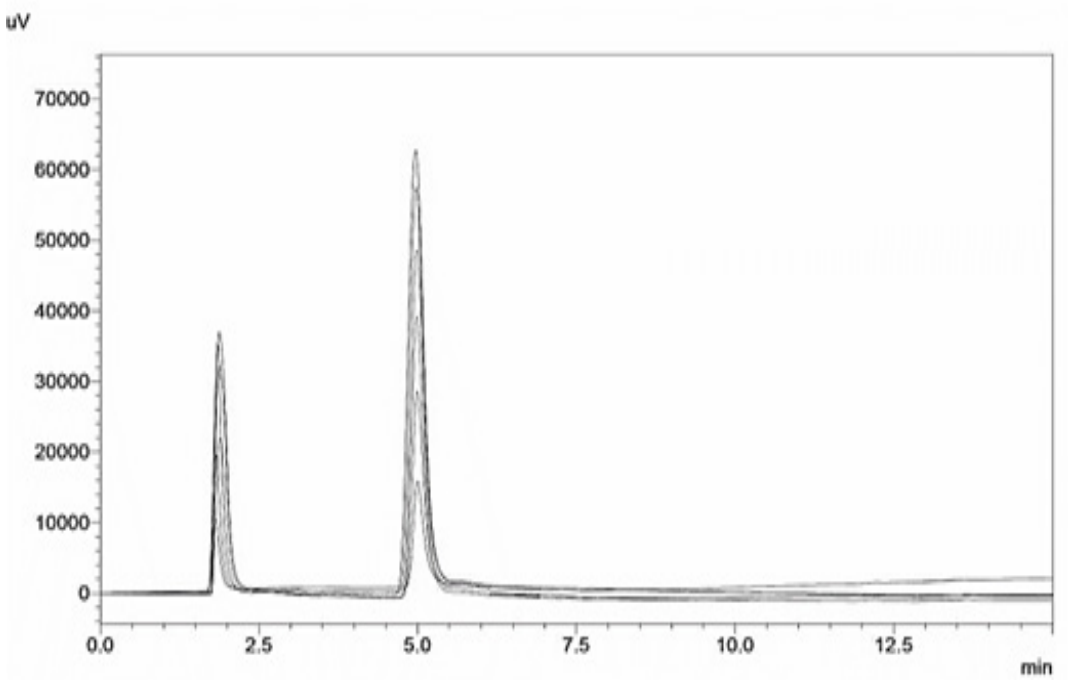

Figure 1: Overlay chromatograph of Empagliflozin and Sitagliptin.

Table 2: Accuracy data of Empagliflozin and Sitagliptin.

Level (%)	Target Conc. (µg/mL)	Spiked Conc. (µg/mL)	Total Conc. (µg/mL)	Peak Area	Conc. Found (µg/mL)	% Recovery
Empagliflozin						
0	40	0	40	351491.22	40.27	100.67
50	40	20	60	534711.39	60.82	101.36
100	40	40	80	703303.61	79.73	99.66
150	40	60	100	893945.37	101.11	101.11
Sitagliptin						
0	400	0	400	150232.31	406.25	101.56
50	400	200	600	227707.86	608.61	101.43
100	400	400	800	294436.08	782.90	97.86
150	400	600	1000	374679.52	992.49	99.25

conditions, degradation was lower (EMPA 8.72%, SITA 10.24%), and under thermal stress EMPA degraded by only 5.6% (retaining 94.4% intact), whereas SITA was extensively degraded (95.94% degraded). Photolytic stress caused 6.87% degradation of EMPA, but 94.13% of SITA was degraded. Importantly, all degradation products were baseline-resolved from the parent drug peaks.

DISCUSSION

The optimized RP-HPLC method demonstrated rapid and efficient analysis of both empagliflozin and sitagliptin with a short run time. The use of a C18 column with a phosphate-methanol-acetonitrile mobile phase provided sharp, symmetric peaks and high resolution, which is critical for routine quality control. System suitability parameters (peak asymmetry, theoretical plates) met acceptable criteria, confirming that the method's performance is reliable.

Linearity across the tested ranges showed excellent correlation coefficients (R^2 >0.995) for both drugs, indicating that the detector response is proportional to concentration. The obtained LOD and LOQ values are low enough to allow detection of trace levels, comparable to or better than previously reported method. Precision and accuracy results (RSD <2%, recoveries near 100%) meet the ICH acceptance criteria, confirming the method's reproducibility and lack of bias. These validation results are consistent with those reported in litrature review for similar assays of empagliflozin and sitagliptin, supporting the method's validity.[5,6]

Table 3: Robustness.

Sl. No.	Factor	Drug	Level	Mean area (*n*=3)	%RSD
1.	Change in the Flow Rate	Empagliflozin	0.9 mL/min	534855.62±4672.82	0.87
			1.1 mL/min	528629.43±7086.23	1.34
		Sitagliptin	0.9 mL/min	235230.31±2553.68	1.09
			1.1 mL/min	235025.86±2365.74	1.01
2.	Change in wavelength	Empagliflozin	220 nm	528433.68±5029.76	0.95
			228 nm	532679.8±4296.06	0.81
		Sitagliptin	220 nm	234552.48±3408.24	1.45
			228 nm	231737.41±3514.21	1.52
3.	Change in mobile phase ratio phase Buffer (pH 3.0 adjusted wit OPA and 0.1% TEA): Methanol: Acetonitrile in the volume ratio 30:35:35 v/v	Empagliflozin	38:22:40	536417.3±4539.8	0.85
			42:18:40	536417.3±4539.8	0.85
		Sitagliptin	38:22:40	237922.7±1609.03	0.68
			42:18:40	230009.73±2349.23	1.02

Table 4: Forced Degradation Results for Empagliflozin (EMPA) and Sitagliptin (SITA) (Values indicate the percentage of drug substance degraded under each condition.)

Stress Condition	EMPA Degraded (%)	EMPA Remaining (%)	SITA Degraded (%)	SITA Remaining (%)
Acidic (0.1 N HCl, 50°C, 2 hr)	12.96%	87.04%	13.85%	86.15%
Alkaline (0.1 N NaOH, 50°C, 2 hr)	15.11%	84.89%	15.27%	84.73%
Oxidative (6% H_2O_2, 40°C, 2 hr)	8.72%	91.28%	10.24%	89.76%
Thermal (70°C, 2 hr)	5.58%	94.42%	95.94%	4.06%
Photolytic (Sunlight, 12 hr)	6.70%	93.30%	94.13%	5.87%

Forced degradation studies revealed the stability-indicating power of the method. Empagliflozin was relatively stable to oxidative, thermal, and photolytic stress, with only moderate degradation in strong acid or base, whereas sitagliptin showed high susceptibility to thermal and photolytic conditions. This difference may be attributed to the chemical structures of the drugs: sitagliptin's primary amine is known to degrade under heat and light. The observed degradation behavior underscores the need for appropriate storage conditions, especially for sitagliptin-containing products. Importantly, all degradation products were well resolved from the parent drug peaks, allowing for accurate quantitation even in stressed samples. Such specificity is a hallmark of a stability-indicating method.[7,8]

Overall, the developed method is simple, fast, and highly selective. It successfully satisfies regulatory requirements for analytical method validation and can be applied for simultaneous estimation of empagliflozin and sitagliptin in combined formulations and stability samples. By ensuring baseline separation of degradation products, the assay provides confidence in the reliability of quality-control measurements for these antidiabetic drugs.

CONCLUSION

A sensitive and stability-indicating RP-HPLC method was established and validated for the simultaneous estimation of empagliflozin and sitagliptin. The method is simple, precise, accurate, and robust. It effectively separates the drugs from their degradation products and meets ICH guidelines, making it suitable for quality control and stability analysis of their Synthetic Mixture.

ABBREVIATIONS

DM: Diabetes mellitus; **T2DM:** Type 2 diabetes mellitus; **SGLT2:** Sodium-glucose cotransporter 2; **DPP-4:** Dipeptidyl peptidase IV; **RP-HPLC:** Reversed-phase high-performance liquid chromatography; **ICH:** International Conference on Harmonisation; **LOD:** Limit of detection; **LOQ:** Limit of quantitation; **RSD:** Relative standard deviation; **PDA:** Photodiode array; **SITA:** Sitagliptin; **EMPA:** Empagliflozin.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

SUMMARY

A new RP-HPLC method was successfully developed for simultaneous determination of empagliflozin and sitagliptin. The method provides rapid separation (≈5 min) with well-resolved peaks and a simple mobile phase composition. Validation results demonstrated excellent linearity, sensitivity, precision, and accuracy for both drugs. Stress testing confirmed the method's stability-indicating capability, as drug peaks were unaffected by common degradation products. These attributes make the method suitable for routine assay and stability studies of Synthetic mixture empagliflozin-sitagliptin formulations.

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Cite this article: Desai R, Dalwadi M, Upadhyay U. Establishment of a Sensitive and Stability-Indicating RP-HPLC Protocol for Co-Quantification of Empagliflozin and Sitagliptin. Indian J of Pharmaceutical Education and Research. 2026;60(3s):s1261-s1266.