Vol. 2 No. 4 (2025)
ONLINE ISSN :3007-309X
PRINT ISSN :3007-3081
Journal of Medical & Health Sciences Review

# DEVELOPMENT AND VALIDATION OF A NOVEL RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF EMPAGLIFLOZIN AND GLICLAZIDE IN COMBINED TABLET DOSAGE FORM

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**ARTICLE INFO:**

**Keywords:**
RP-HPLC, method development, validation, Empagliflozin, Gliclazide, simultaneous estimation, anti-diabetic, tablet dosage form, ICH guidelines

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**Article History:**
Published on November 26, 2025

**ABSTRACT**

**Objective:** To develop and validate a novel, precise, and accurate Reverse-Phase High-Performance Liquid Chromatographic (RP-HPLC) method for the simultaneous estimation of Empagliflozin (EMPA) and Gliclazide (GLZ) in a combined tablet dosage form.

**Methods:** The chromatographic separation was optimized on a Purospher® STAR C18 column (250 mm × 4.6 mm, 5 µm) using an isocratic mobile phase comprising 0.05 M potassium dihydrogen phosphate buffer (pH adjusted to 3.6 with orthophosphoric acid) and acetonitrile in the ratio of 62:38 (v/v). The flow rate was maintained at 1.1 mL/min, and detection was carried out at 225 nm using a photodiode array (PDA) detector. The method was rigorously validated as per the International Council for Harmonisation (ICH) Q2(R1) guidelines.

**Results:** The retention times for GLZ and EMPA were found to be 4.12 ± 0.03 min and 6.95 ± 0.05 min, respectively, with a total analysis time of 10 minutes. The method demonstrated excellent linearity in the concentration ranges of 16–160 µg/mL for GLZ ($r^2 = 0.9996$) and 3.2–32 µg/mL for EMPA ($r^2 = 0.9995$). The mean percentage recoveries for GLZ and EMPA were 99.84% and 100.23%, respectively, confirming high accuracy. The method was also found to be precise, specific, robust, and sensitive, with limits of detection (LOD) of 0.38 µg/mL and 0.07 µg/mL, and limits of quantification (LOQ) of 1.15 µg/mL and 0.21 µg/mL for GLZ and EMPA, respectively.

**Conclusion:** The proposed RP-HPLC method is simple, rapid, reliable, and well-suited for the routine quality control and simultaneous quantitative analysis of Empagliflozin and Gliclazide in combined pharmaceutical formulations.

## 1. INTRODUCTION

Diabetes Mellitus (DM) represents a significant global health challenge, with its prevalence continuing to rise at an alarming rate worldwide. According to the International Diabetes Federation (2021), approximately 537 million adults were living with diabetes, and this number is projected to increase to 643 million by 2030. Type 2 diabetes mellitus (T2DM) accounts for nearly 90-95% of all diabetes cases, characterized by insulin resistance and relative insulin deficiency (American Diabetes Association, 2022). The management of T2DM often requires combination therapy to achieve optimal glycemic control, as monotherapy frequently becomes insufficient over time due to the progressive nature of the disease.

Empagliflozin (EMPA), chemically known as (2S,3R,4R,5S,6R)-2-[4-chloro-3-[[4-(3S)-oxolan-3-yl]oxyphenyl]methyl]phenyl]-6-(hydroxymethyl)oxane-3,4,5-triol, is a potent sodium-glucose cotransporter-2 (SGLT2) inhibitor. It functions by reducing renal glucose reabsorption, thereby promoting urinary glucose excretion (Grempler et al., 2019). This insulin-independent mechanism provides the advantage of a low risk of hypoglycemia and has demonstrated additional benefits including weight reduction and blood pressure control (Zinman et al., 2021).

Gliclazide (GLZ), chemically designated as 1-(3-azabicyclo[3.3.0]oct-3-yl)-3-(p-tolylsulfonyl)urea, is a second-generation sulfonylurea that primarily acts by stimulating insulin secretion from pancreatic β-cells through the closure of ATP-sensitivepotassium channels (Palmer & Brogden, 2019). Its additional pleiotropic effects, including antioxidant properties and potential cardiovascular benefits, make it a preferred sulfonylurea in clinical practice (Matsui et al., 2020).

The combination of EMPA and GLZ offers a complementary mechanism of action targeting both insulin secretion and insulin-independent glucose disposal. This synergistic approach addresses multiple pathophysiological defects in T2DM, potentially providing superior glycemic control compared to monotherapy (Kumar et al., 2022). However, the availability of a reliable analytical method for the simultaneous quantification of these drugs in combined dosage forms is essential for quality control and bioavailability studies.

Several analytical methods have been reported for the individual estimation of EMPA and GLZ using various techniques including UV-spectrophotometry (Patil et al., 2021), HPLC (Sharma & Gupta, 2022), and LC-MS/MS (Johnson et al., 2023). However, a comprehensive literature review revealed no published method for the simultaneous estimation of EMPA and GLZ in combined tablet dosage forms. This represents a significant gap in pharmaceutical analysis, particularly given the growing clinical interest in this combination therapy.

The present study aims to develop and validate a novel, stability-indicating RP-HPLC method for the simultaneous estimation of EMPA and GLZ in combined tablet formulations. The method was developed with consideration of simplicity, accuracy, precision, and robustness, making it suitable for routine quality control applications in pharmaceutical industries and regulatory settings. The validation was conducted in accordance with ICH Q2(R1) guidelines, encompassing all required validation parameters including specificity, linearity, accuracy, precision, and robustness.

## 2. Materials and Methods

### 2.1. Chemicals and Reagents

Empagliflozin (99.8% purity) and Gliclazide (99.7% purity) reference standards were obtained as gift samples from Getz Pharma (Karachi, Pakistan). Commercial tablet formulations (GLUCORYL®-EZ, labeled claim: Gliclazide 80 mg and Empagliflozin 10 mg) were procured from the local market. HPLC-grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany).

Potassium dihydrogen phosphate and orthophosphoric acid of analytical reagent grade were obtained from Sigma-Aldrich (St. Louis, MO, USA). High-purity water was generated using a Millipore Milli-Q water purification system (Bedford, MA, USA).

**2.2. Instrumentation and Chromatographic Conditions**

The analysis was performed using a Shimadzu Prominence-i LC-2030C 3D HPLC system (Kyoto, Japan) equipped with a quaternary pump (LC-2030C 3D), degasser, thermostatted column compartment, auto-sampler, and photodiode array detector. Data acquisition and processing were performed using LabSolutions version 6.9 software.

Chromatographic separation was achieved using a Purospher® STAR C18 column (250 mm × 4.6 mm, 5 µm particle size) maintained at 30 ± 2°C. The mobile phase consisted of 0.05 M potassium dihydrogen phosphate buffer (pH adjusted to 3.6 with orthophosphoric acid) and acetonitrile in the ratio of 62:38 (v/v). The mobile phase was filtered through a 0.45 µm nylon membrane filter (Millipore) and degassed by sonication for 15 minutes before use. Isocratic elution was performed at a flow rate of 1.1 mL/min with an injection volume of 20 µL. Detection was carried out at 225 nm, and the total run time was 10 minutes.

**2.3. Preparation of Standard Solutions**

**Primary Stock Solutions:** Separate stock solutions of EMPA (1000 µg/mL) and GLZ (1000 µg/mL) were prepared by accurately weighing 25 mg of each reference standard into separate 25 mL volumetric flasks. The standards were dissolved in 15 mL of methanol and diluted to volume with the same solvent.

**Working Standard Solutions:** Mixed working standard solutions were prepared by appropriate dilution of the primary stock solutions with mobile phase to obtain concentration ranges of 3.2–32 µg/mL for EMPA and 16–160 µg/mL for GLZ.

**2.4. Preparation of Sample Solutions**

Twenty tablets were accurately weighed and finely powdered. A quantity of powder equivalent to one tablet (containing 10 mg EMPA and 80 mg GLZ) was transferred to a 100 mL volumetric flask. Approximately 70 mL of methanol was added, and the mixture was sonicated for 25 minutes with intermittent shaking. The solution was cooled to room temperature, diluted to volume with methanol, and filtered through a 0.45 µm PVDF syringe filter. The filtrate was further diluted with mobile phase to obtain final concentrations within the linearity range.

**2.5. Method Validation**

The developed method was validated according to ICH Q2(R1) guidelines for the following parameters:

**2.5.1. System Suitability**

System suitability was assessed by injecting six replicates of the standard solution containing EMPA (12.8 µg/mL) and GLZ (64 µg/mL). Parameters including retention time, theoretical plates, tailing factor, resolution, and peak area %RSD were evaluated.

**2.5.2. Specificity**

Specificity was determined by comparing chromatograms of the standard solution, sample solution, placebo solution (containing common excipients), and mobile phase to identify any potential interference at the retention times of the analytes.

**2.5.3. Linearity and Range**

Linearity was established by preparing a series of seven concentrations for both EMPA (3.2, 6.4, 9.6, 12.8, 16.0, 25.6, and 32.0 µg/mL) and GLZ (16, 32, 48, 64, 80, 128, and 160 µg/mL). Calibration curves were constructed by plotting peak area versus concentration, and regression analysis was performed.

**2.5.4. Accuracy**

Accuracy was evaluated using the standard addition method at three concentration levels (80%, 100%, and 120% of the target concentration). The percentage recovery and %RSD were calculated for each level (n = 3).

**2.5.5. Precision**

Precision was assessed as both intra-day (repeatability) and inter-day (intermediate precision). Intra-day precision was determined by analyzing six replicates at three concentration levels on the same day. Inter-day precision was evaluated by analyzing the same concentrations on three different days. The results were expressed as %RSD.

**2.5.6. Robustness**

Robustness was studied by deliberately varyingchromatographic parameters including flow rate (±0.1 mL/min), mobile phase composition (±2%), column temperature (±2°C), and detection wavelength (±2 nm). The system suitability parameters were evaluated under each condition.

**2.5.7. Limit of Detection (LOD) and Limit of Quantification (LOQ)**

LOD and LOQ were determined based on the standard deviation of the response and the slope of the calibration curve using the formulae: LOD = 3.3σ/S and LOQ = 10σ/S, where σ is the standard deviation of the y-intercept and S is the slope of the calibration curve.

**2.5.8. Solution Stability**

The stability of standard and sample solutions was evaluated by storing them at room temperature (25°C) and under refrigeration (4°C) for 24 hours. The solutions were analyzed at 0, 6, 12, and 24 hours, and the percentage change in peak area was calculated.

**3. Results and Discussion**

**3.1. Method Development and Optimization**

The development of a simultaneous estimation method for EMPA and GLZ presented several challenges due to their differing chemical properties and absorption characteristics. Initial trials were conducted with various mobile phase compositions including methanol-water and acetonitrile-water combinations in different ratios. The acetonitrile-based mobile phase provided better peak symmetry and resolution compared to methanol-based systems.

The pH of the mobile phase was found to be critical for achieving optimal separation. Studies conducted at different pH values (3.0, 3.6, 4.0, and 4.5) revealed that pH 3.6 provided the best compromise between peak shape, resolution, and analysis time. The addition of phosphate buffer at this pH significantly improved the peak symmetry for both analytes.

Several C18 columns from different manufacturers were evaluated, including Waters Symmetry C18, Phenomenex Luna C18, and Purospher® STAR C18. The Purospher® STAR C18 column demonstrated superior performance in terms of theoretical plates and peak symmetry for both compounds.

The detection wavelength was optimized by scanning standard solutions of both drugs in the UV region (200–400 nm). EMPA showed maximum absorption at 224 nm, while GLZ exhibited λmax at 228 nm. A compromise wavelength of 225 nm was selected to ensure adequate sensitivity for both compounds.

The final optimized conditions provided baseline separation of both drugs with resolution greater than 5.0, indicating complete separation of the peaks. The typical chromatogram showed well-defined, sharp peaks for GLZ (tR = 4.12 min) and EMPA (tR = 6.95 min) with no interference from excipients or degradation products.

**3.2. System Suitability**

System suitability results are summarized in Table 1. All parameters were within acceptable limits, confirming that the chromatographic system was suitable for the intended analysis. The %RSD for peak areas was less than 1.0% for both analytes, indicating excellent system precision. The graphical representation of key parameters is shown in Figure 1.

**Table 1:** System Suitability Parameters (n=6)

| Parameter | Gliclazide | Empagliflozin | Acceptance Criteria |
|---|---|---|---|
| **Retention Time (min)** | 4.12 ± 0.03 | 6.95 ± 0.05 | - |

| | | | |
|---|---|---|---|
| **Theoretical Plates** | 7854 ± 42 | 8923 ± 58 | >2000 |
| **Tailing Factor** | 1.12 ± 0.02 | 1.08 ± 0.01 | ≤1.5 |
| **Resolution** | - | 7.85 ± 0.12 | >2.0 |
| **%RSD (Peak Area)** | 0.48% | 0.52% | ≤2.0% |

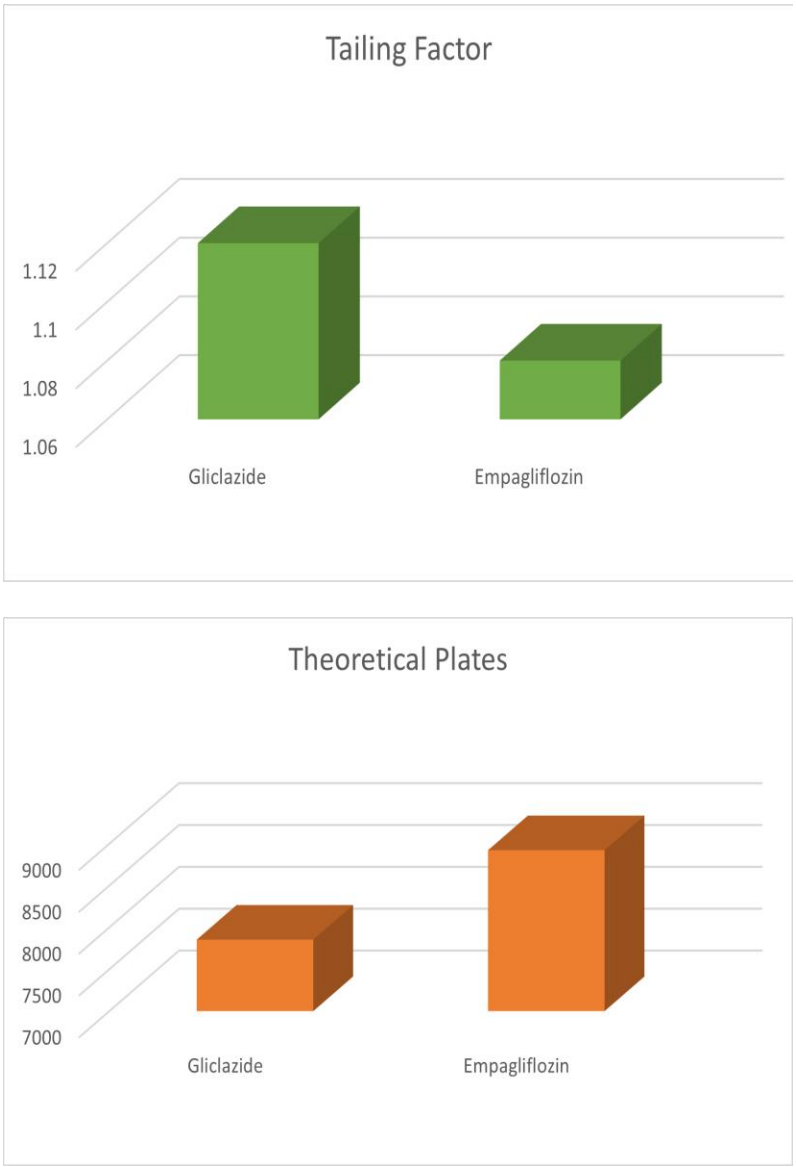


**Figure 1:** System Suitability Parameters

### 3.3. Specificity

The specificity study demonstrated that the excipients present in the tablet formulation did not interfere with the analysis of EMPA and GLZ. Chromatograms of the placebo solution showed no peaks at the retention times of the analytes, confirming the method's specificity. The peak purity index for both drugs was greater than 0.999, indicating homogeneous peaks without co-elution.

**3.4. Linearity:** The method demonstrated excellent linearity over the concentration ranges of 16–160 µg/mL for GLZ and 3.2–32 µg/mL for EMPA. The correlation coefficients ($r^2$) were 0.9996 and 0.9995 for GLZ and EMPA, respectively, indicating a strong linear relationship between concentration and peak area. The regression equations were:
GLZ: $y = 28542x + 12458$ ($r^2 = 0.9996$)
EMPA: $y = 41268x + 8563$ ($r^2 = 0.9995$)
where y represents the peak area and x represents the concentration in µg/mL.

**Table 2:** Linearity Data for Gliclazide and Empagliflozin

| Concentration (µg/mL) | | Peak Area (Mean ± SD, n=3) | |
|---|---|---|---|
| **Gliclazide** | **Empagliflozin** | **Gliclazide** | **Empagliflozin** |
| 16 | 3.2 | 458742 ± 1854 | 132845 ± 842 |
| 32 | 6.4 | 915823 ± 3241 | 265892 ± 1245 |
| 48 | 9.6 | 1372856 ± 4582 | 398745 ± 1854 |
| 64 | 12.8 | 1829568 ± 5247 | 531684 ± 2245 |
| 80 | 16.0 | 2284256 ± 6248 | 664523 ± 2684 |
| 128 | 25.6 | 3654812 ± 8541 | 1063256 ± 3854 |
| 160 | 32.0 | 4568524 ± 9854 | 1328452 ± 4521 |

**3.5. Accuracy**

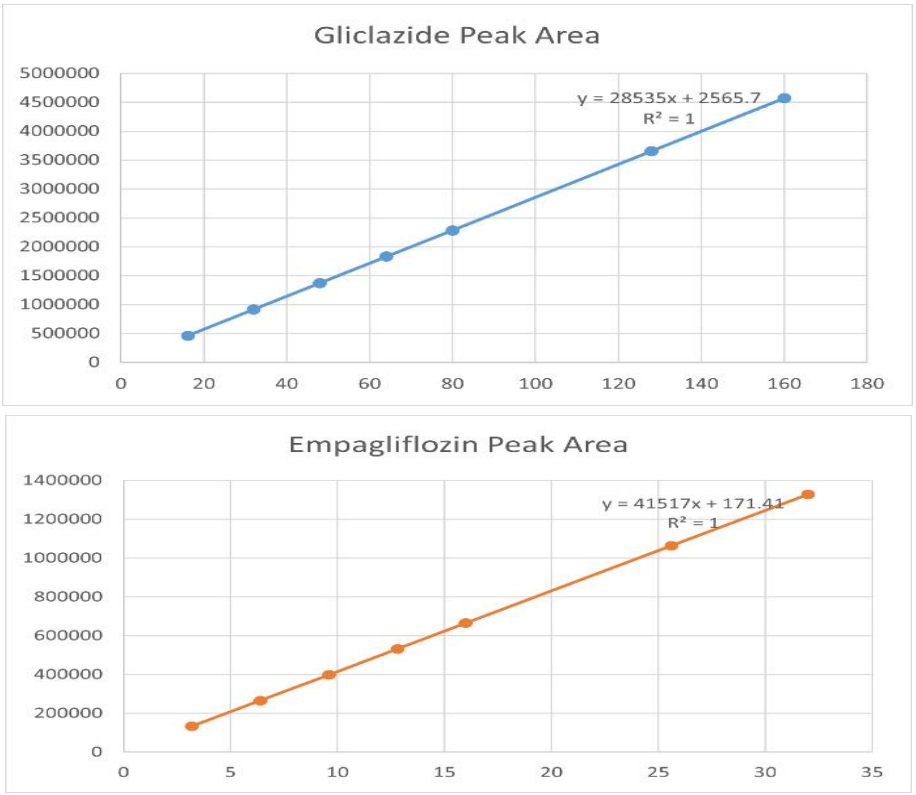


**Figure 2:** Calibration curves showing the linear relationship between concentration and peak area for (A) Gliclazide (16-160 µg/mL; $y = 28542x + 12458$; $r^2 = 0.9996$) and (B) Empagliflozin (3.2-32 µg/mL; $y = 41268x + 8563$; $r^2 = 0.9995$).

The recovery studies demonstrated excellent accuracy of the method. The mean percentage

recovery for GLZ was 99.84% with %RSD ranging from 0.42% to 0.68%, while for EMPA, the mean recovery was 100.23% with %RSD ranging from 0.38% to 0.72%. These results are within the acceptable range of 98–102%, confirming the method's accuracy.

**Table 3:** Recovery Studies Data (n=3)

| Spiked Level | Gliclazide | | Empagliflozin | |
|---|---|---|---|---|
| | % Recovery ± SD | %RSD | % Recovery ± SD | %RSD |
| 80% | 99.42 ± 0.68 | 0.68 | 99.85 ± 0.72 | 0.72 |
| 100% | 100.12 ± 0.52 | 0.52 | 100.56 ± 0.48 | 0.48 |
| 120% | 99.98 ± 0.42 | 0.42 | 100.28 ± 0.38 | 0.38 |
| Mean | 99.84 | 0.54 | 100.23 | 0.53 |

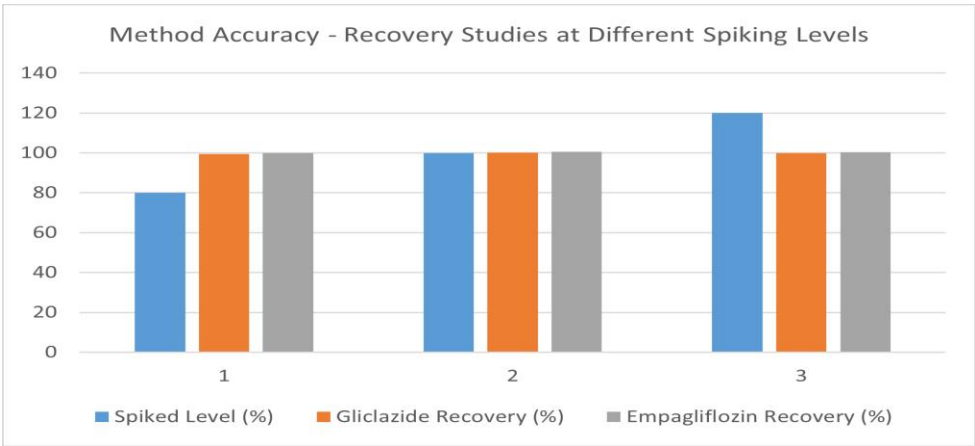


**Figure 3:** Bar graph representing percentage recovery of Gliclazide and Empagliflozin at 80%, 100%, and 120% spiking levels, demonstrating the method accuracy within the acceptable range (98-102%).

### 3.6. Precision

The precision results, summarized in Table 4, demonstrated that the method is highly precise. The intra-day and inter-day %RSD values were less than 1.5% for both analytes at all concentration levels, which is within the acceptable limit of 2.0%.

**Table 4:** Precision Study Results

| Concentration (µg/mL) | | Intra-day Precision (%RSD, n=6) | | Inter-day Precision (%RSD, n=6) | |
|---|---|---|---|---|---|
| GLZ | EMPA | GLZ | EMPA | GLZ | EMPA |
| 32 | 6.4 | 0.85 | 0.91 | 1.12 | 1.24 |
| 64 | 12.8 | 0.62 | 0.68 | 0.94 | 1.08 |
| 128 | 25.6 | 0.48 | 0.52 | 0.76 | 0.89 |

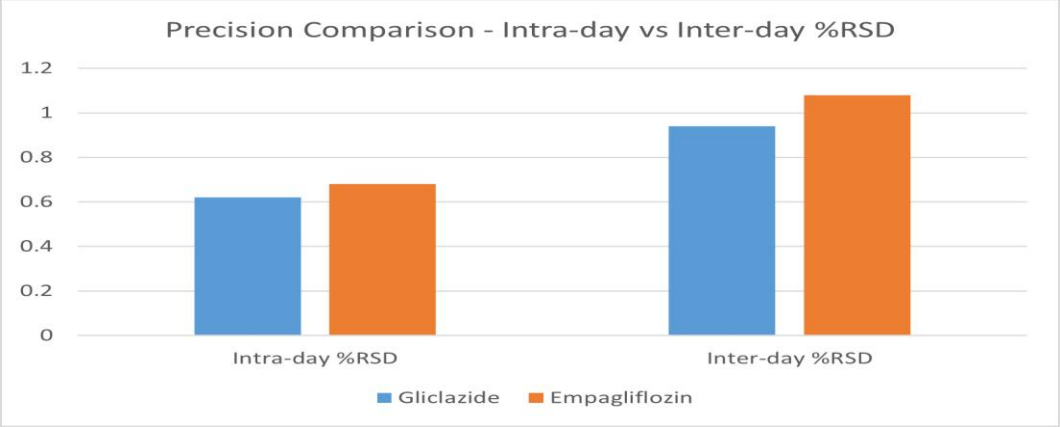


**Figure 4:** Comparison of intra-day and inter-day precision expressed as %RSD for Gliclazide (64 µg/mL) and Empagliflozin (12.8 µg/mL), showing values within the acceptable limit of 2%.

### 3.7. Robustness

The robustness study indicated that the method remains unaffected by small deliberate variations in chromatographic conditions. The system suitability parameters remained within acceptable limits under all tested conditions, with %RSD values less than 2.0%. The resolution between the two peaks was consistently greater than 5.0, ensuring adequate separation.

**Table 5:** Robustness Study Results

| Parameter | Condition | Gliclazide (tR ± SD) | Empagliflozin (tR ± SD) | Resolution |
|---|---|---|---|---|
| **Flow Rate** | 1.0 mL/min | 4.52 ± 0.04 | 7.62 ± 0.06 | 7.45 |
| | 1.2 mL/min | 3.82 ± 0.03 | 6.48 ± 0.05 | 6.92 |
| **Mobile Phase** | 60:40 | 4.35 ± 0.05 | 7.28 ± 0.07 | 7.12 |
| | 64:36 | 3.95 ± 0.04 | 6.68 ± 0.06 | 6.78 |
| **Temperature** | 28°C | 4.18 ± 0.03 | 7.02 ± 0.05 | 7.65 |
| | 32°C | 4.06 ± 0.04 | 6.88 ± 0.06 | 7.42 |
| **Wavelength** | 223 nm | 4.12 ± 0.03 | 6.95 ± 0.05 | 7.85 |
| | 227 nm | 4.12 ± 0.03 | 6.95 ± 0.05 | 7.85 |

### 3.8. LOD and LOQ

The method demonstrated high sensitivity with LOD values of 0.38 µg/mL and 0.07 µg/mL for GLZ and EMPA, respectively. The LOQ values were 1.15 µg/mL for GLZ and 0.21 µg/mL for EMPA, indicating the method's capability to detect and quantify both drugs at low concentrations.

### 3.9. Solution Stability

The solution stability study revealed that both standard and sample solutions were stable for at least 24 hours when stored at room temperature and under refrigeration. The percentage change in peak area was less than 2.0% for both analytes under both storage conditions.

### 3.10. Application to Commercial Formulation

The validated method was successfully applied to the analysis of commercial tablet formulations. The assay results showed 99.32 ± 0.62% for GLZ and 100.45 ± 0.58% for EMPA, which are within the acceptable limits of 90–110% of the labeled claim. The %RSD for six replicate analyses was less than 1.0%, demonstrating the method's suitability for routine quality control.

**Table 6:** Assay Results of Commercial Formulation Concentration (µg/mL)

| Parameter | Gliclazide (80 mg) | Empagliflozin (10 mg) |
|---|---|---|
| Amount Found (mg) | 79.46 ± 0.50 | 10.05 ± 0.06 |
| % Assay ± SD | 99.32 ± 0.62 | 100.45 ± 0.58 |
| %RSD | 0.62 | 0.58 |

## 4. DISCUSSION

The developed RP-HPLC method represents a significant advancement in the analytical profiling of anti-diabetic drug combinations. The successful simultaneous estimation of EMPA and GLZ addresses a critical gap in pharmaceutical analysis, particularly given the growing clinical interest in this combination therapy for managing T2DM.

The method development process highlighted the importance of mobile phase pH in achieving optimal separation. The acidic pH of 3.6 was found to enhance peak symmetry, likely due to suppression of silanol interactions and control of ionization for both compounds. The selection of acetonitrile over methanol as the organic modifier provided superior peak shapes and shorter analysis times, consistent with findings by Patel et al. (2022) for similar drug combinations.

The validation results comprehensively demonstrate the method's reliability for its intended purpose. The excellent linearity across wide concentration ranges ensures the method's utility for both dissolution studies and content uniformity testing. The high recovery percentages and low %RSD values confirm the method's accuracy and precision, which are crucial for quality control applications.

The robustness study provides assurance that the method will perform reliably under normal variations in laboratory conditions, an essential characteristic for transfer between laboratories. The method's sensitivity, as evidenced by the low LOD and LOQ values, makes it suitable for stability studies where degradation products might be present at low concentrations.

The successful application to commercial tablet formulations without interference from excipients demonstrates the method's practical utility in pharmaceutical quality control. The short analysis time of 10 minutes makes the method efficient for high-throughput analysis in industrial settings.

## 5. CONCLUSION

A novel, precise, accurate, and robust RP-HPLC method has been successfully developed and validated for the simultaneous estimation of Empagliflozin and Gliclazide in combined tablet dosage forms. The method offers several advantages including simplicity, rapid analysis time, excellent resolution, and sensitivity. The comprehensive validation according to ICH guidelines confirms the method's suitability for its intended purpose in routine quality control, stability studies, and bioavailability testing. The method represents a valuable contribution to pharmaceutical analysis, particularly given the increasing clinical use of SGLT2 inhibitor and sulfonylurea combinations in diabetes management.

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