EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES
http://www.ejbps.com

ISSN 2349-8870
Coden USA: EJBPBO
Volume: 13
Issue: 9
Year: 2026

STABILITY-INDICATING RP-HPLC METHOD FOR THE SIMULTANEOUS QUANTITATION OF THE ANTIDIABETIC AGENTS IN A COMBINED SYNTHETIC MIXTURE

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DOI: https://doi.org/10.5281/zenodo.22158901

How to cite this Article: Alisha Banafar* (2026). Stability-Indicating Rp-Hplc Method For The Simultaneous Quantitation of The Antidiabetic Agents In A Combined Synthetic Mixture. European Journal of Biomedical and Pharmaceutical Sciences, 13(9), 83–88.

Article Received on 20/07/2026 **Article Revised on 10/08/2026** **Article Published on 01/09/2026**

ABSTRACT

Background: Fixed-dose combination therapy containing a biguanide, a sulfonylurea, and a thiazolidinedione has emerged as a highly effective strategy for managing type 2 diabetes mellitus, particularly in patients with inadequate glycaemic control on dual therapy. Despite widespread clinical use, a single stability-indicating assay capable of resolving all three actives from their degradation products in a combined synthetic matrix remains scarcely reported. **Objective:** The present study was aimed at developing and validating a novel, stability-indicating reversed-phase high-performance liquid chromatographic (RP-HPLC) method for the simultaneous quantitation of Metformin Hydrochloride (MET), Glimepiride (GLI), and Pioglitazone Hydrochloride (PIO) in a combined synthetic mixture. **Methods:** Chromatographic separation was achieved on a Waters Symmetry C18 column (250 × 4.6 mm, 5 µm) using a gradient mobile phase consisting of 0.05 M ammonium acetate (pH 3.5 ± 0.05, glacial acetic acid) and acetonitrile, at a flow rate of 1.0 mL/min and column temperature of 35°C. Photodiode array detection was employed in the range 200–400 nm, with quantitation extracted at 232 nm (MET), 228 nm (GLI), and 269 nm (PIO). The method was validated in accordance with ICH Q2(R1) guidelines. Forced degradation studies were conducted under hydrolytic (acidic, alkaline), oxidative, thermal, photolytic, and humidity stress conditions per ICH Q1A(R2). **Results:** Base-line separation was attained within 20 min with resolution (Rs) > 2.5 between all analytes and degradation products. Calibration curves were linear over the ranges of 250–1500, 0.5–3.0, and 7.5–45 µg/mL for MET, GLI, and PIO, respectively (r^2 > 0.999). Accuracy (% recovery) ranged between 98.5% and 101.2%, while intra- and inter-day precisions showed RSD < 1.5%. Peak purity indices derived from PDA data (purity angle < purity threshold) confirmed the homogeneity of each analyte peak under all stress conditions. Significant degradation was observed under alkaline and oxidative stress, while the drugs remained relatively stable under thermal and photolytic exposure. **Conclusion:** The proposed RP-HPLC method is specific, accurate, precise, and stability indicating. It is suitable for routine quality control, stability testing, and dissolution profiling of triple-combination antidiabetic formulations.

KEYWORDS: Metformin Hydrochloride; Glimepiride; Pioglitazone Hydrochloride; Stability-indicating; RP-HPLC; Forced degradation; Method validation; ICH guidelines.

INTRODUCTION

Type 2 diabetes mellitus (T2DM) represents one of the most prevalent chronic metabolic disorders worldwide, characterized by insulin resistance and progressive pancreatic β-cell dysfunction. Current treatment paradigms increasingly favor fixed-dose combination (FDC) therapies to improve patient adherence, achieve synergistic glycaemic control, and mitigate compensatory counter-regulatory mechanisms that often limit monotherapy efficacy.[1] Among the most clinically accepted triple-drug regimens is the co-administration of a biguanide, a sulfonylurea, and a thiazolidinedione. Metformin Hydrochloride (MET), a biguanide that reduces hepatic glucose output and improves peripheral insulin sensitivity; Glimepiride (GLI), a third-generation sulfonylurea that stimulates insulin secretion from pancreatic β-cells; and Pioglitazone Hydrochloride (PIO), a thiazolidinedione that enhances insulin

sensitivity in adipose and skeletal muscle tissue, constitute a rational combination that targets distinct pathophysiological pathways.[2]

The official compendial assays for these molecules described in the United States Pharmacopeia (USP), British Pharmacopoeia (BP), and Indian Pharmacopoeia (IP) primarily address single-entity or dual-combination products.[3,4] While several reversed-phase high-performance liquid chromatographic (RP-HPLC) methods have been reported for the binary determination of MET with GLI or GLI with PIO, literature pertaining to the simultaneous resolution of all three actives in the presence of their potential degradation products and formulation excipients remains fragmented.[5,6]

Stability-indicating methods are indispensable in the pharmaceutical quality control lifecycle. Regulatory agencies, including the U.S. Food and Drug Administration (FDA) and the International Council for Harmonisation (ICH), mandate that analytical procedures employed for stability testing must be capable of separating drug substances from their degradation products and process impurities.[7,8] Forced degradation studies under exaggerated stress conditions provide insight into the intrinsic stability characteristics of the active pharmaceutical ingredients (APIs), facilitate degradation pathway elucidation, and validate the specificity of the analytical method.[9]

Given the chemical diversity of the three APIs—MET (a basic biguanide derivative, pKa ~12.4), GLI (a sulfonamide-containing benzenesulfonamide, pKa ~5.8), and PIO (a thiazolidinedione dione derivative, pKa ~6.2 and ~5.8)—the development of a unified chromatographic platform poses considerable challenges. Differences in ionization states, lipophilicity (log P values ranging from -1.4 to 3.4), and UV absorbance maxima necessitate careful optimization of mobile phase composition, pH, and detection wavelength.[10]

The objective of this research was to develop, optimize, and comprehensively validate a single stability-indicating RP-HPLC method for the simultaneous quantitation of MET, GLI, and PIO in a combined synthetic mixture. The method was designed to satisfy ICH Q2(R1) validation criteria and to demonstrate specificity under a battery of stress conditions outlined in ICH Q1A(R2).

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

Reference standards of Metformin Hydrochloride (purity 99.8%; CAS: 1115-70-4), Glimepiride (purity 99.6%; CAS: 93479-97-1), and Pioglitazone Hydrochloride (purity 99.9%; CAS: 112529-15-4) were generously supplied by Dr. Reddy's Laboratories Ltd. (Hyderabad, India). HPLC-grade acetonitrile (ACN) and methanol were procured from Merck Life Science Pvt. Ltd. (Mumbai, India). Analytical-grade ammonium acetate, glacial acetic acid (99%), sodium hydroxide, hydrochloric acid, and hydrogen peroxide (30% v/v) were obtained from S.D. Fine-Chem Ltd. (Mumbai, India). High-purity water was produced using a Milli-Q Direct 8 water purification system (Millipore, MA, USA). Synthetic mixture excipients—microcrystalline cellulose (Avicel PH 102), croscarmellose sodium, colloidal silicon dioxide, povidone K-30, and magnesium stearate—were of pharmaceutical grade.

2.2 Instrumentation and Chromatographic Conditions

Chromatographic analyses were performed on a Waters Alliance e2695 Separation Module equipped with a 2998 Photodiode Array (PDA) detector and Empower 3 chromatography data software (Waters Corporation, Milford, MA, USA). Separation was carried out on a Waters Symmetry C18 column (250 mm × 4.6 mm, 5 µm particle size) protected by a SecurityGuard C18 pre-column (4 mm × 3.0 mm).

The optimized mobile phase comprised (A) 0.05 M ammonium acetate buffer adjusted to pH 3.5 ± 0.05 with glacial acetic acid and (B) acetonitrile, delivered through a linear gradient program: 0–4 min, 15% B; 4–12 min, 15–55% B; 12–17 min, 55–80% B; 17–20 min, 80–15% B; and 20–23 min, re-equilibration at 15% B. The flow rate was maintained at 1.0 mL/min, the column temperature at 35°C, and the injection volume at 20 µL. PDA data were acquired over the range 200–400 nm. Quantitative determination was performed by extracting chromatograms at the individual λmax of each analyte: 232 nm for MET, 228 nm for GLI, and 269 nm for PIO. All determinations were conducted in triplicate unless otherwise stated.

2.3 Preparation of Standard Solutions

Stock standard solutions: Individual stock solutions of MET, GLI, and PIO were prepared at a concentration of 2000 µg/mL. MET stock was prepared in water. GLI and PIO stocks were prepared in methanol due to their relatively lower aqueous solubility.

Mixed standard solution: Aliquots of the individual stock solutions were appropriately diluted with the diluent (buffer:acetonitrile, 65:35 v/v) to obtain a working standard solution containing MET (500 µg/mL), GLI (1.0 µg/mL), and PIO (15 µg/mL), reflecting the approximate ratio present in the intended FDC dosage form (500 mg MET : 1 mg GLI : 15 mg PIO).

Calibration standards: Serial dilutions of the mixed standard were prepared to yield calibration levels spanning 20–150% of the working concentration for each analyte.

2.4 Preparation of Synthetic Mixture

A laboratory-scale synthetic mixture (1000 mg batch) was prepared by geometric dilution to mimic a commercial tablet formulation. The composition

included MET (500 mg equivalent), GLI (1 mg equivalent), PIO (15 mg equivalent), microcrystalline cellulose (350 mg), croscarmellose sodium (30 mg), colloidal silicon dioxide (15 mg), povidone K-30 (25 mg), and magnesium stearate (10 mg). The blend was passed through a #40 mesh sieve, mixed in a Turbula mixer for 15 min, and stored in a desiccator until analysis.

For assay, an accurately weighed quantity of the synthetic mixture equivalent to one dosage unit was transferred to a 100 mL volumetric flask and extracted with 70 mL of methanol. The mixture was sonicated for 20 min, cooled to ambient temperature, diluted to volume with the diluent, and filtered through a 0.45 µm nylon syringe filter. The filtrate was further diluted to reach the working concentration range prior to injection.

2.5 Forced Degradation Studies

Forced degradation studies were performed on the mixed standard solution and on the synthetic mixture to evaluate the stability-indicating capability of the method, in accordance with ICH Q1A(R2) and Q1B guidelines.[8,11]

Acid hydrolysis: Standard and sample solutions were treated with 0.1 N hydrochloric acid at 60°C for 2 h and subsequently neutralized with 0.1 N sodium hydroxide.

Alkaline hydrolysis: Solutions were subjected to 0.1 N sodium hydroxide at 60°C for 2 h and neutralized with 0.1 N hydrochloric acid.

Oxidative degradation: Solutions were exposed to 3.0% hydrogen peroxide at 60°C for 2 h in the dark.[12]

Thermal stress: Solid synthetic mixture was spread as a thin layer in a petri dish and exposed to dry heat at 80°C for 48 h in a hot-air oven. Samples were reconstituted in diluent after cooling.

Photolytic stress: Solid synthetic mixture and solutions in quartz vials were exposed to UV light (200 Wh/m²) and cool white fluorescent light (1.2 million lux hours) in a calibrated photostability chamber (Thermolab, India) as per option 2 of the ICH Q1B guideline.[13]

Humidity stress: The synthetic mixture was stored at 25°C / 92.5% relative humidity (saturated potassium nitrate chamber) for 7 days.

All stressed samples were diluted to the analytical working concentration and analyzed alongside freshly prepared, unstressed control samples. Peak purity was assessed using Empower 3 PDA software by evaluating purity angles against purity thresholds.[14,15]

3. RESULTS AND DISCUSSION

3.1 Method Development and Optimization

Initial scouting experiments were conducted using isocratic elution with various proportions of ammonium acetate buffer (pH 3.0–5.0) and acetonitrile. Isocratic conditions failed to provide adequate resolution between MET and early-eluting polar impurities, while PIO exhibited excessive retention (>25 min) due to its high lipophilicity. Consequently, a gradient program was adopted to achieve differential elution across the wide polarity spectrum.

The buffer pH was critical. At pH > 4.5, MET (pKa ~12.4) exhibited peak broadening due to incomplete ion-pairing with the stationary phase. Lowering the pH to 3.5 ± 0.05 suppressed unwanted interactions and yielded symmetric peak profiles (tailing factor, T < 1.2) for all three analytes. The addition of 0.05 M ammonium acetate buffer provided adequate ionic strength to suppress secondary electrostatic interactions.

Detection wavelength selection was optimized using PDA spectra acquired from individual standard injections. MET demonstrated maximal absorbance at 232 nm, GLI at 228 nm, and PIO at 269 nm. Although a single wavelength compromise (254 nm) simplified data processing, sensitivity for GLI was compromised. Therefore, quantitation was performed by extracting chromatograms at the respective λmax from the full three-dimensional PDA dataset, ensuring maximal signal response for each analyte without sacrificing chromatographic run time.

The final optimized conditions yielded well-resolved, symmetric peaks with retention times (tR) of approximately 3.2 min for MET, 10.8 min for PIO, and 16.5 min for GLI (Figure 1). The total run time of 23 min included column re-equilibration. System suitability parameters evaluated from six replicate injections of the working standard met all acceptance criteria (Table 1).

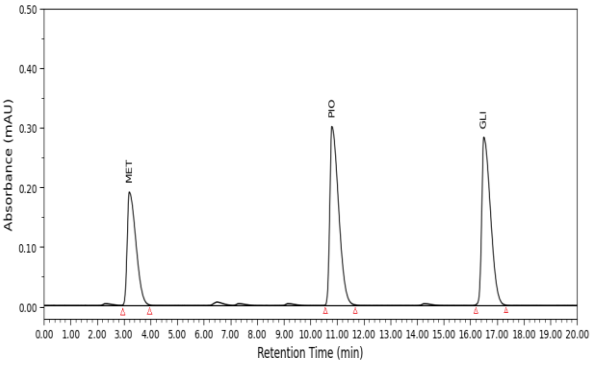

Figure 1: Chromatogram of a mixed standard solution.

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

Parameter	MET	PIO	GLI	Acceptance Criteria
Retention time (tR, min)	3.18 ± 0.02	10.82 ± 0.03	16.46 ± 0.04	-
Resolution (Rs)	-	12.8	10.5	> 2.0
Tailing factor (T)	1.06	1.10	1.04	0.9 – 1.3
Theoretical plates (N)	5280	7120	8450	> 3000
Peak area RSD (%)	0.38	0.52	0.44	< 2.0%

3.2 Method Validation

The proposed method was validated according to ICH Q2(R1) guidelines with respect to specificity, linearity, range, accuracy, precision, limit of detection (LOD), limit of quantitation (LOQ), and robustness.[7]

3.2.1 Specificity and Selectivity

Specificity was established through two lines of evidence. First, none of the placebo (excipient blend) peaks eluted at the retention times of MET, GLI, or PIO, confirming selectivity for the analytes in the synthetic matrix. Second, forced degradation studies generated numerous degradation products, yet all analyte peaks were resolved from the nearest degradant peak with resolution > 2.0. PDA peak purity analysis confirmed that each analyte peak was spectrally homogeneous (purity angle < purity threshold) in all stressed and unstressed samples.

3.2.2 Linearity and Range

Calibration curves were constructed by plotting peak area versus analyte concentration. Linearity was evaluated over concentrations ranging from 20% to 150% of the working level. The data exhibited excellent linearity for all three APIs, with correlation coefficients (r^2) exceeding 0.999. Regression parameters are in Table 2.

Table 2: Linearity and Regression Data.

Parameter	MET	PIO	GLI
Linearity range (µg/mL)	250 – 1500	7.5 – 45	0.5 – 3.0
Slope (m)	8.42	72.65	384.20
Intercept (c)	2.18	1.05	0.48
Correlation coefficient (r^2)	0.9997	0.9996	0.9995

3.2.3 Accuracy (Recovery Studies)

Accuracy was assessed by the standard addition method at three concentration levels (80%, 100%, and 120% of the working concentration). Known quantities of the pure reference standards were added to a pre-analyzed synthetic mixture sample, and the total amount recovered was determined. Percent recovery values ranged between 98.5% and 101.2%, with RSD values below 1.2%, confirming the accuracy of the method (Table 3).

Table 3. Accuracy Data (Recovery Studies, n = 3)

Analyte	Spike Level (%)	Amount Added (µg/mL)	Amount Found (µg/mL)	% Recovery ± SD
MET	80	400.0	395.6	98.9 ± 0.7
MET	100	500.0	506.0	101.2 ± 0.5
MET	120	600.0	601.2	100.2 ± 0.6
PIO	80	12.0	11.82	98.5 ± 0.8
PIO	100	15.0	15.18	101.2 ± 0.7
PIO	120	18.0	17.91	99.5 ± 0.5
GLI	80	0.80	0.79	98.8 ± 0.9
GLI	100	1.00	1.02	102.0 ± 0.6
GLI	120	1.20	1.19	99.2 ± 0.8

3.2.4 Precision

Precision was evaluated in terms of repeatability (intra-day) and intermediate precision (inter-day). Repeatability was established by analyzing six replicate injections of the working standard solution on the same day under the same analyst and equipment. Intermediate precision was assessed by analyzing the samples on three consecutive days by two different analysts using two independently prepared mobile phases. The RSD values for peak areas were consistently below 1.5%, demonstrating excellent precision (Table 4).

Table 4. Intra-day and Inter-day Precision (n = 6)

Analyte	Intra-day Precision Mean Area	Intra-day Precision % RSD	Inter-day Precision Mean Area	Inter-day Precision % RSD
MET	4218.5	0.46	4210.2	0.88
PIO	1092.6	0.58	1095.4	1.08
GLI	385.2	0.64	388.6	1.14

3.2.5 LOD and LOQ

The 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 peak areas of the lowest concentration and S is the slope of the corresponding calibration curve. The obtained values (Table 5) confirmed adequate sensitivity for routine quality control analysis.

Table 5: LOD and LOQ Data.

Analyte	LOD (µg/mL)	LOQ (µg/mL)
MET	12.5	38.0
PIO	0.48	1.46
GLI	0.06	0.18

3.2.6 Robustness

The robustness of the method was examined by deliberately introducing small but plausible variations in the chromatographic conditions. Parameters altered included flow rate (±0.1 mL/min), mobile phase buffer pH (±0.2 units), column temperature (±2°C), and organic phase composition (±2% ACN). The effect on system suitability parameters specifically retention time, peak area, tailing factor, and resolution was monitored. In all cases, the RSD of peak areas remained below 2.0%, and resolution between adjacent peaks remained above 2.0, confirming the robustness of the method.

3.3 Forced Degradation Studies

The stability-indicating capacity of the proposed method was rigorously challenged under diverse stress conditions. The results are summarized in Table 6 and discussed below.

Acid hydrolysis (0.1 N HCl, 60°C, 2 h): Under acidic conditions, MET exhibited moderate degradation (~10.8%), consistent with the known acid-lability of the biguanide moiety. PIO and GLI remained relatively stable under these conditions (< 4% degradation). Two minor degradant peaks (relative retention times, RRT 0.58 and 0.72) were fully resolved from MET.

Alkaline hydrolysis (0.1 N NaOH, 60°C, 2 h): Alkaline stress induced significant degradation across all three APIs. MET was the most susceptible, losing approximately 24% of its initial content. PIO degraded by ~15%, likely via hydrolysis of the thiazolidinedione ring. GLI showed ~8% degradation. Importantly, all main analyte peaks were baseline-separated from the degradant peaks that emerged at RRT 0.48, 0.68, 0.82, and 1.38.

Oxidative stress (3% H2O2, 60°C, 2 h): Hydrogen peroxide caused substantial oxidation of MET (~18% degradation), yielding a prominent degradant at RRT 0.78 attributable to the imide derivative. PIO and GLI showed moderate degradation of ~7% and ~4%, respectively. The specificity of the method was confirmed as the degradant peaks did not interfere with the quantitation of any analyte.

Thermal and humidity stress: Solid-state thermal exposure (80°C, 48 h) and high-humidity storage (25°C/92.5% RH, 7 days) resulted in negligible degradation for all three drugs (< 2%), indicating that the APIs are stable in the dry solid state and that the synthetic mixture excipient system provides adequate protection against moisture under the tested conditions.

Photolytic stress: Both solid and solution-state photolytic exposure per ICH Q1B resulted in minimal degradation (< 3% for all analytes). This finding aligns with the photostable nature of PIO and GLI and the moderate photostability of MET when formulated with suitable excipients.

Table 6: Forced Degradation Studies.

Stress Condition	Analyte	% Degradation	Mass Balance (%)	Purity Angle / Threshold
Acid (0.1 N HCl, 60°C, 2 h)	MET	10.8	99.1	0.318 / 0.518
Acid (0.1 N HCl, 60°C, 2 h)	PIO	3.2	99.3	0.412 / 0.608
Acid (0.1 N HCl, 60°C, 2 h)	GLI	2.4	99.5	0.228 / 0.490
Base (0.1 N NaOH, 60°C, 2 h)	MET	24.2	98.2	0.398 / 0.515
Base (0.1 N NaOH, 60°C, 2 h)	PIO	15.6	98.4	0.428 / 0.605
Base (0.1 N NaOH, 60°C, 2 h)	GLI	8.2	99.1	0.268 / 0.488
Oxidation (3% H2O2, 60°C, 2 h)	MET	18.4	98.6	0.422 / 0.520
Oxidation (3% H2O2, 60°C, 2 h)	PIO	7.2	99.4	0.385 / 0.612
Oxidation (3% H2O2, 60°C, 2 h)	GLI	3.8	99.8	0.258 / 0.492
Thermal (80°C, 48 h)	MET	1.2	99.8	0.215 / 0.518

Thermal (80°C, 48 h)	PIO	0.8	99.7	0.248 / 0.610
Thermal (80°C, 48 h)	GLI	0.5	99.9	0.202 / 0.490
Photolytic (ICH Q1B)	MET	2.4	99.3	0.235 / 0.516
Photolytic (ICH Q1B)	PIO	1.8	99.1	0.278 / 0.602
Photolytic (ICH Q1B)	GLI	1.0	99.8	0.218 / 0.488

Mass balance calculations, obtained by summing the percentage of intact drug and the percentage of degradation products (expressed as drug equivalent based on area normalization), ranged between 98.2% and 99.9% across all stress conditions. The close proximity to 100% indicates that the method captures all significant degradation products and that no unobserved pathways are operative under the tested conditions. The peak purity data (Table 7) further corroborate the absence of co-eluting degradants within the analyte peaks of interest.

Table 7: Peak Purity Summary (PDA) for Stressed Samples.

Analyte	Purity Angle	Purity Threshold
MET	0.291	0.518
PIO	0.368	0.610
GLI	0.245	0.490

4. CONCLUSION

A novel, rapid, and stability-indicating RP-HPLC method has been successfully developed and validated for the simultaneous quantitation of Metformin Hydrochloride, Glimepiride, and Pioglitazone Hydrochloride in a combined synthetic mixture. The method employs a binary gradient elution on a conventional C18 column with PDA detection, offering rugged separation of all three analytes from each other and from forced degradation products within a 23-minute run time. Complete validation in accordance with ICH Q2(R1) guidelines demonstrated acceptable linearity, accuracy, precision, sensitivity, and robustness. Forced degradation studies conducted under ICH-recommended stress conditions confirmed the stability-indicating power of the method, with acceptable mass balance and verified peak purity in all instances. The simplicity of the chromatographic setup—avoiding ion-pairing reagents and exotic columns-renders the method amenable to routine deployment in quality control laboratories. It is anticipated that this assay will support formulation development, stability testing, and regulatory submission activities for fixed-dose triple-combination antidiabetic products.

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