COMPARATIVE STUDIES BETWEEN REVERSE PHASE AND MIX MODE CHROMATOGRAPHY TOWARDS SIMULTANEOUS ESTIMATION OF SELECTED ANTIDIABETIC DRUGS

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ABSTRACT:
This study compares reverse-phase chromatography (RPC) and mixed-mode chromatography (MMC) for the simultaneous estimation of lobeglitazone, pioglitazone, and metformin in human plasma. Analytical challenges associated with the diverse physicochemical properties of these drugs-specifically the extreme hydrophilicity of metformin and moderate polarity of pioglitazone and lobeglitazone-were addressed using protein precipitation and solid-phase extraction techniques. RPC exhibited efficient separation for pioglitazone and lobeglitazone with satisfactory recoveries and resolution. However, metformin showed low retention and poor recovery due to its high polarity. MMC, particularly with HILIC-1 stationary phases, demonstrated improved reproducibility and recoveries for moderately polar drugs and better matrix interference management. Despite these advantages, metformin recovery remained suboptimal across all techniques. These findings highlight MMC's superiority for analyzing drug combinations with varying polarities, providing a robust framework for future analytical developments in therapeutic drug monitoring and pharmacokinetic studies.

KEYWORDS:
Reverse-phase chromatography, Mixed-mode chromatography, Lobeglitazone, Pioglitazone, Metformin, Simultaneous estimation, Drug recovery etc.

INTRODUCTION:

Chromatographic techniques are indispensable tools in pharmaceutical analysis, particularly for the simultaneous estimation of active pharmaceutical ingredients (APIs) in complex formulations. These techniques are essential for quality control, ensuring the safety and efficacy of combination drug therapies. With the growing prevalence of chronic diseases like hypertension and diabetes, the pharmaceutical industry has increasingly focused on developing fixed-dose combination therapies. Such combinations aim to improve patient compliance, simplify treatment regimens, and achieve synergistic therapeutic effects. However, analyzing these formulations presents significant challenges due to the diverse physicochemical properties of the individual drugs. [1]

Reverse-phase high-performance liquid chromatography (RP-HPLC) and mixed-mode chromatography (MMC) are two prominent chromatographic approaches widely used in pharmaceutical research and quality control. RP-HPLC utilizes a hydrophobic stationary phase and is well-suited for the separation of non-polar to moderately polar compounds. Its reliability, robustness, and widespread adoption make it a standard method for pharmaceutical analysis. On the other hand, [2] MMC integrates multiple interaction mechanisms, including hydrophobic, ionic, and polar interactions, within a single chromatographic system. This multi-dimensional approach allows for the separation of compounds with varying polarity and charge, making it particularly advantageous for complex mixtures.In this study, we aim to compare RP-HPLC and MMC for the simultaneous estimation of selected antidiabetic and antihypertensive drugs, focusing on metformin hydrochloride, pioglitazone hydrochloride, and lobeglitazone sulfate. These drugs represent a class of medications commonly used in managing type 2 diabetes, often co-prescribed with antihypertensives due to the high comorbidity between diabetes and hypertension. [3] [4]

Metformin

Metformin remains a cornerstone in the management of type 2 diabetes mellitus (T2DM) due to its robust efficacy in improving glycemic control through multiple complementary mechanisms. It primarily reduces hepatic glucose production by inhibiting gluconeogenesis and glycogenolysis. Additionally, it enhances insulin-mediated glucose uptake in skeletal muscles and adipose tissues, and decreases glucose absorption in the gastrointestinal tract. These actions collectively help in maintaining euglycemia without causing significant risks of hypoglycemia, a hallmark advantage of metformin therapy [5].

The pharmacological effects of metformin are mediated via the activation of AMP-activated protein kinase (AMPK), a central regulator of cellular energy homeostasis. By stimulating AMPK, metformin promotes fatty acid oxidation, inhibits lipogenesis, and improves insulin sensitivity. Another critical mediator is LKB1, a tumor suppressor protein and an upstream kinase of AMPK, which plays a pivotal role in regulating glucose metabolism [6].

Beyond glycemic control, recent studies have highlighted metformin’s pleiotropic benefits. It has shown potential in modulating the gut microbiota, particularly by increasing the population of beneficial bacteria such as Akkermansia muciniphila, which is linked to improved metabolic health. Furthermore, metformin exhibits anti-inflammatory effects by reducing pro-

inflammatory cytokines and oxidative stress markers. These additional benefits suggest that metformin may contribute to overall metabolic regulation and cardiovascular protection, making it a vital component of T2DM management. Chemically, metformin hydrochloride is a simple biguanide compound with the molecular formula $C_4H_{11}N_5$ and a molar mass of 129.16 g/mol. It is highly polar, as indicated by a log P value of -0.92, and has a pKa of 12.4, making it predominantly ionized at physiological pH. These properties pose challenges in chromatographic analysis, particularly with hydrophobic stationary phases commonly used in reverse-phase high-performance liquid chromatography (RP-HPLC) [7].

To address these challenges, analytical methods often incorporate ion-pairing agents or employ mixed-mode chromatography (MMC) to ensure adequate retention and resolution of metformin. Over the years, the development of precise and accurate HPLC methods has been pivotal in quantifying metformin in pharmaceutical formulations, biological matrices, and fixed-dose combinations. These advancements have significantly improved the reliability of therapeutic drug monitoring and enhanced patient safety, particularly in managing drug interactions and ensuring consistent therapeutic outcomes.

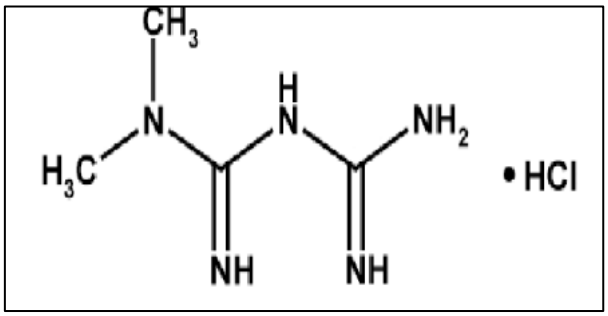

Figure 1: Structure of Metformin HCl

Pioglitazone

Pioglitazone hydrochloride is a thiazolidinedione (TZD) antidiabetic agent that plays a pivotal role in the management of type 2 diabetes mellitus (T2DM) by improving insulin sensitivity and reducing insulin resistance. It is particularly beneficial for T2DM patients with significant metabolic abnormalities, such as insulin resistance and dyslipidemia. By targeting the root causes of T2DM, pioglitazone provides comprehensive metabolic improvements and supports long-term disease management. [8].

The primary mechanism of action of pioglitazone involves activating the peroxisome proliferator-activated receptor-gamma (PPAR-γ), a nuclear receptor that is predominantly

expressed in adipose tissue, skeletal muscle, and the liver. Activation of PPAR-γ promotes the transcription of insulin-responsive genes involved in glucose and lipid metabolism. Pioglitazone enhances glucose uptake by increasing the expression of glucose transporter-4 (GLUT4) in adipose tissue and skeletal muscle, thereby facilitating insulin-mediated glucose transport and improving glycemic control. Additionally, it improves lipid profiles by reducing circulating free fatty acids and promoting their storage in adipose tissue, which mitigates lipotoxicity in non-adipose tissues such as the liver and muscle. This effect enhances insulin sensitivity. Pioglitazone also suppresses pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6), while increasing adiponectin levels, contributing to its cardiovascular and anti-inflammatory benefits. Furthermore, it decreases hepatic glucose production, thereby improving overall glucose homeostasis. [8].

Beyond its glycemic benefits, pioglitazone provides additional clinical advantages, especially for patients with metabolic syndrome. Its favorable effects on lipid metabolism include reducing triglyceride levels, improving high-density lipoprotein (HDL) cholesterol, and decreasing small, dense low-density lipoprotein (LDL) particles, which are highly atherogenic. Clinical trials, such as the PROactive (PROspective pioglitAzone Clinical Trial In macroVascular Events) study, have demonstrated that pioglitazone may reduce the progression of atherosclerosis and lower the risk of major adverse cardiovascular events in patients with T2DM and established cardiovascular disease. These benefits highlight pioglitazone's potential as a multifaceted therapy for T2DM. [10].

Chemically, pioglitazone hydrochloride has the molecular formula $C_{19}H_{20}N_2O_3S{\cdot}HCl$ and a molar mass of 392.90 g/mol. It is moderately lipophilic, with a log P value of 2.3, and has a pKa of 5.9, reflecting its weakly acidic nature. These properties make it amenable to analysis using reverse-phase high-performance liquid chromatography (RP-HPLC), particularly under neutral to slightly acidic mobile phase conditions. Pioglitazone's physicochemical properties and its co-administration in fixed-dose combinations with other antidiabetic agents necessitate precise analytical methods. RP-HPLC and other advanced chromatographic techniques enable reliable quantification and quality assurance, ensuring the efficacy and safety of pioglitazone-based therapies. [11].

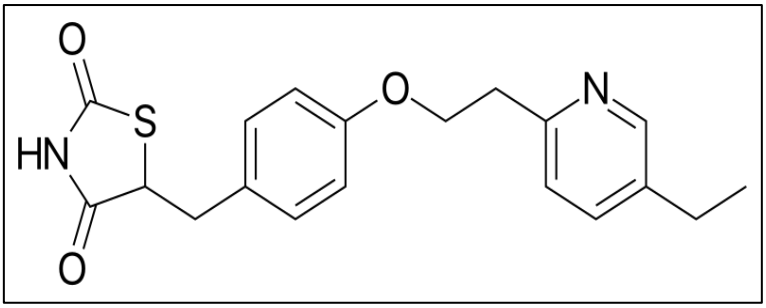

Figure 2: Structure of Pioglitazone

Lobeglitazone

Lobeglitazone is a potent thiazolidinedione (TZD) antidiabetic agent developed for managing type 2 diabetes mellitus (T2DM). It improves glycemic control by enhancing insulin sensitivity and reducing insulin resistance through selective activation of peroxisome proliferator-activated receptor-gamma (PPAR-γ). This activation promotes the expression of insulin-responsive genes, increasing glucose uptake in skeletal muscle and adipose tissue via GLUT4 and improving lipid metabolism by lowering triglycerides and raising HDL cholesterol levels. [12].

In addition to its metabolic effects, lobeglitazone exhibits anti-inflammatory properties by reducing pro-inflammatory cytokines like TNF-α and increasing adiponectin, which also contributes to its cardiovascular benefits. It decreases hepatic gluconeogenesis, further aiding in glucose regulation. Compared to earlier TZDs, lobeglitazone offers enhanced efficacy and a better safety profile, with a reduced risk of adverse effects such as bone fractures and bladder cancer. [13].

Chemically, lobeglitazone mesylate ($C_{24}H_{24}N_4O_5S \cdot CH_4O_3S$) has a molar mass of 588.71 g/mol, moderate lipophilicity (log P ~2.5), and a pKa of 6.2. Its properties are well-suited for reverse-phase high-performance liquid chromatography (RP-HPLC) analysis, ensuring precise quantification in pharmaceutical formulations. Lobeglitazone is often used alone or in combination therapies, offering a comprehensive approach to T2DM management with significant glycemic and lipid-modulating benefits. [14]. [15].

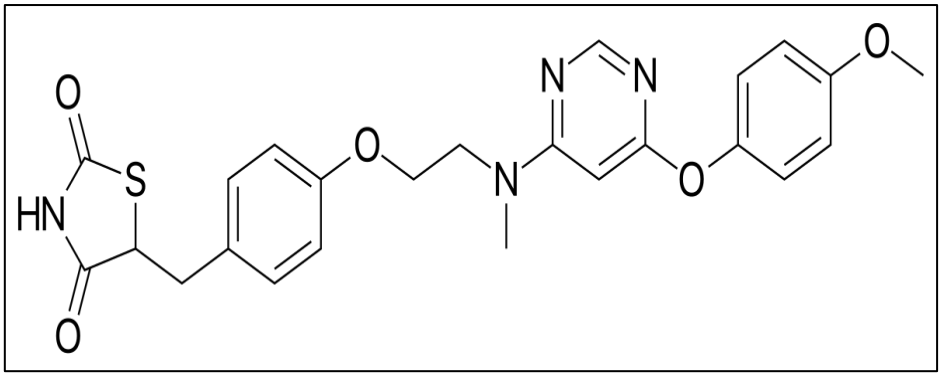

Figure 3: Structure of Lobeglitazone

The simultaneous estimation of these drugs in pharmaceutical formulations is essential for routine quality control and regulatory compliance. However, the combination of highly polar (metformin) and moderately lipophilic (pioglitazone and lobeglitazone) drugs poses unique analytical challenges. RP-HPLC, while robust for moderately polar compounds, may struggle with the retention of highly polar molecules like metformin. Conversely, MMC offers the advantage of combining hydrophobic and ionic interactions, potentially providing superior separation for such diverse compounds.

This study investigates the comparative performance of RP-HPLC and MMC, focusing on critical analytical parameters such as retention behavior, resolution, sensitivity, and selectivity. The findings will provide insights into the optimal chromatographic approach for the simultaneous estimation of metformin, pioglitazone, and lobeglitazone, contributing to advances in pharmaceutical analysis and improved methodologies for fixed-dose combination formulations.

MATERIALS AND METHODS:

MATERIALS:

The reference standards for lobeglitazone, pioglitazone, and metformin were procured from Yarrow Pharma Ltd. Ammonium acetate was obtained from Merck Ltd. (Mumbai, India), while HPLC-grade acetonitrile, methanol, and water were also sourced from Merck (Mumbai, India). Nylon membrane filters with pore sizes of 0.20 µm and 0.45 µm were purchased from UltraChrom Innovatives Pvt. Ltd. (India). All additional chemicals and reagents used in the study were of HPLC-grade to ensure accuracy and reliability in the analysis.

Selection of solvent and wavelength

Lobeglitazone and pioglitazone were found to be soluble in acetonitrile and methanol but only partially soluble in water. In contrast, metformin dissolved readily in water and methanol but was partially soluble in acetonitrile. Due to these solubility differences, individual standard

stock solutions of lobeglitazone, pioglitazone, and metformin were prepared using a solvent mixture comprising acetonitrile, methanol, and water in the ratio of 60:30:10 (% v/v). All three drugs exhibited maximum UV absorbance (λmax) around 230 nm, which was selected as the detection wavelength for simultaneous HPLC analysis of lobeglitazone, pioglitazone, and metformin. [16]

Preparation of standard solution

Precisely, 7 mg of lobeglitazone, pioglitazone, and metformin standards were individually weighed and dissolved in 7 mL of a solvent mixture comprising acetonitrile, methanol, and water (6:3:1, v/v) to prepare 1000 ppm (1000 µg/mL) stock solutions. Each solution was sonicated for 10–15 minutes to ensure complete dissolution. Serial dilutions were subsequently prepared as required for conducting validation studies, including assessments of repeatability, precision, and robustness. [17]

Chromatographic conditions

A 20 µL aliquot of freshly prepared stock solutions of lobeglitazone, pioglitazone, and metformin was injected into a Zodiac-100 C18 column (5 µm; 150 x 4.6 mm ID). The separation was performed using a gradient mobile phase comprising solvent A (15 mM ammonium acetate) and solvent B (methanol-acetonitrile, 20:80 v/v) at a flow rate of 1.0 mL/min over 18 minutes. The gradient program was set as 10% B (0–3 min), 75% B (3–12 min), and maintained at 75% B (12–18 min). The analysis was conducted at room temperature, with detection at 230 nm. [18]

System suitability studies

A freshly prepared homologous stock solution (100 ppm) of lobeglitazone, pioglitazone, and metformin was injected six times to assess precision through the relative standard deviation (RSD), ensuring values below 2%. Additionally, system suitability parameters such as retention time, capacity factor (k'), theoretical plates (N), tailing factor/peak asymmetry (As), and separation factor (α) were evaluated.[19]

Precision (intraday and Interday) studies of the proposed method

Stock solutions of lobeglitazone (100 ppm), pioglitazone (100 ppm), and metformin (50 ppm) were analyzed in triplicate within the same day (intraday) and over three consecutive days (interday). Mean, standard deviation, and relative standard deviation (RSD) were calculated, ensuring RSD values below 2% in compliance with ICH guidelines.[20]

Robustness for the chromatographic method

The robustness of the method was evaluated by varying the flow rate (1.0 ± 0.1 mL/min), organic modifier in solvent B (±2% across the gradient), and detection wavelength (230 ± 2 nm). The effects on retention time (tR), capacity factor (k'), resolution (Rs), and theoretical plates (N) were assessed.

Sample preparation for Linearity/Calibration studies

Individual stock solutions (1000 ppm) of lobeglitazone, pioglitazone, and metformin were prepared. A homogenous mixture was then formulated with metformin (70 ppm), lobeglitazone (80 ppm), and pioglitazone (80 ppm). Serial dilutions ranging from 2.18–70 ppm for metformin and 2.5–80 ppm for lobeglitazone and pioglitazone were prepared, ultrasonicated, and analyzed under the chromatographic conditions in Section 5.5. A calibration curve was plotted by correlating peak area with five known concentrations to determine the regression equation, regression coefficient (R^2), limit of quantification (LOQ), and limit of detection (LOD).[21]

Sample preparation for drug accuracy studies of metformin and pioglitazone

Five Pioglit-MF-15 tablets (500 mg metformin and 15 mg pioglitazone, manufactured by Glenmark Pharmaceuticals Ltd.) were weighed, and the average weight was calculated. The tablets were then crushed into a fine powder using a mortar and pestle. A 7 mg portion of the powder was accurately weighed, dissolved in 7 mL of acetonitrile-methanol-water (6:3:1, v/v), ultrasonicated for 5-10 minutes, and filtered through a 0.45 µ nylon filter. Serial dilutions were made to obtain final concentrations of 100 ppm for metformin and 10 ppm for pioglitazone, considered 100%. The solution was sonicated and analyzed according to the chromatographic conditions in Section 5.5. The drug recovery for metformin and pioglitazone was evaluated at 80%, 100%, and 120% concentrations and compared to the reference standard. [22]

Sample preparation for drug accuracy studies of lobeglitazone

Five Lobula 0.5 tablets (0.5 mg lobeglitazone, manufactured by Glenmark Pharmaceuticals Ltd.) were weighed, and the average weight was calculated. The tablets were crushed into a fine powder. An accurately weighed 7 mg of the powder was dissolved in 7 mL of acetonitrile-methanol-water (6:3:1, v/v), ultrasonicated for 5-10 minutes, and filtered through a 0.45 µ nylon filter. Serial dilutions were made to achieve a final concentration of 100 ppm lobeglitazone, considered as 100%. The solution was sonicated and analyzed following the chromatographic conditions outlined in Section 5.5. The drug recovery of lobeglitazone at 80%, 100%, and 120% concentrations was calculated based on peak area. [23]

RESULTS AND DISCUSSIONS:

UV spectroscopy of selected drugs (pioglitazone, metformin and lobeglitazone)

The UV absorption spectra of Lobeglitazone**,** Metformin**,** and Pioglitazone show distinct characteristics useful for their simultaneous estimation in pharmaceutical formulations. Lobeglitazone exhibits a broad absorption peak between 200-300 nm, while Metformin and Pioglitazone have sharp peaks around 230 nm. Metformin's spectrum displays a pronounced peak at 230 nm with a gradual decline, while Pioglitazone shows a similar pattern, making their separation challenging. However, Lobeglitazone's unique profile helps differentiate it from the other two drugs. These spectra enable effective quantification and analysis, especially with chromatography, ensuring accurate measurement of each drug in mixed formulations.

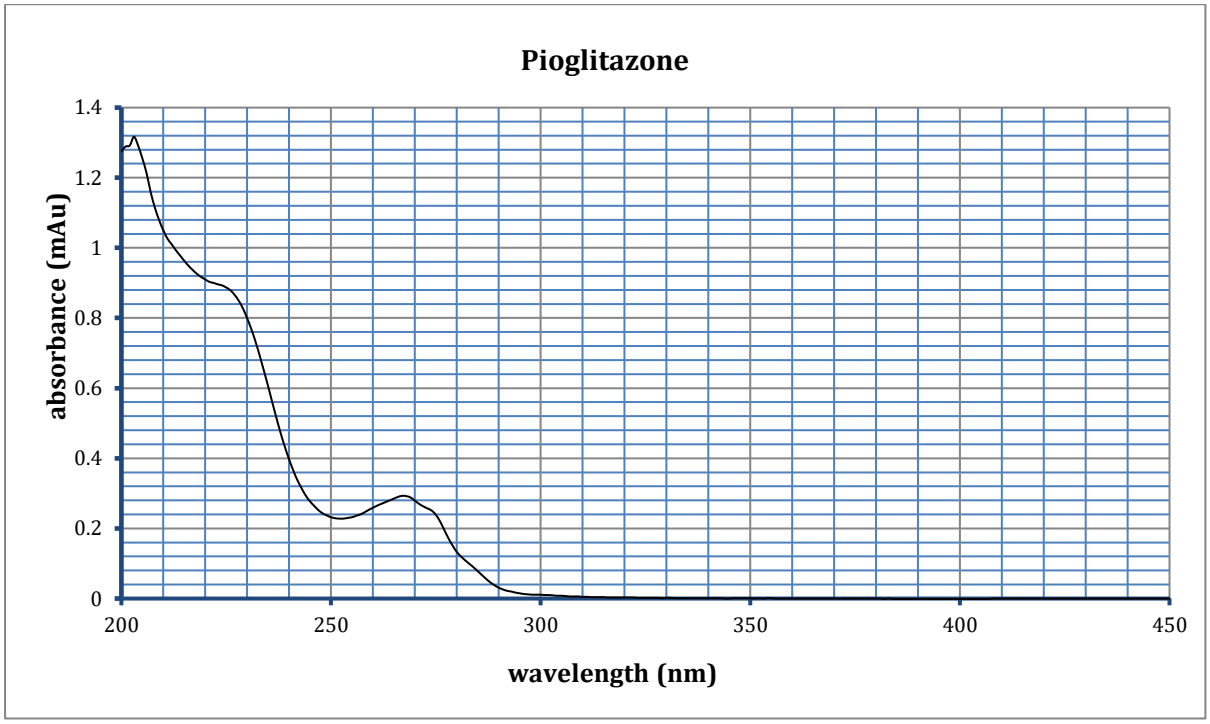

Figure 4: UV spectra of pioglitazone standard

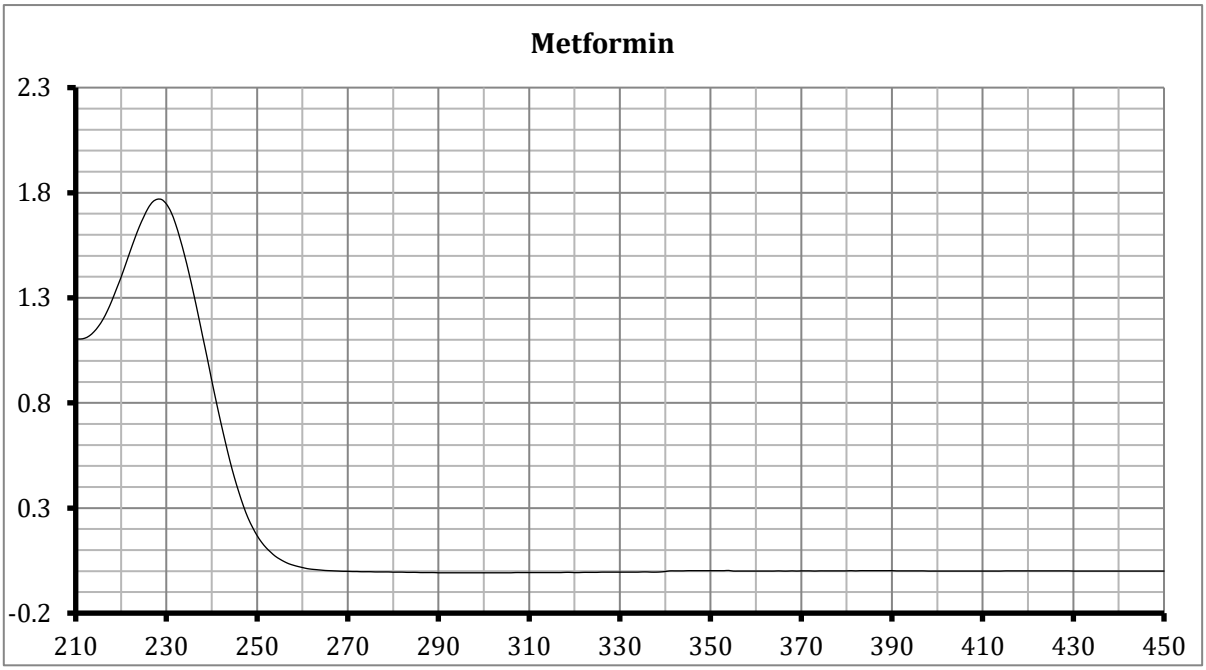

Figure 5: UV spectra of metformin standard

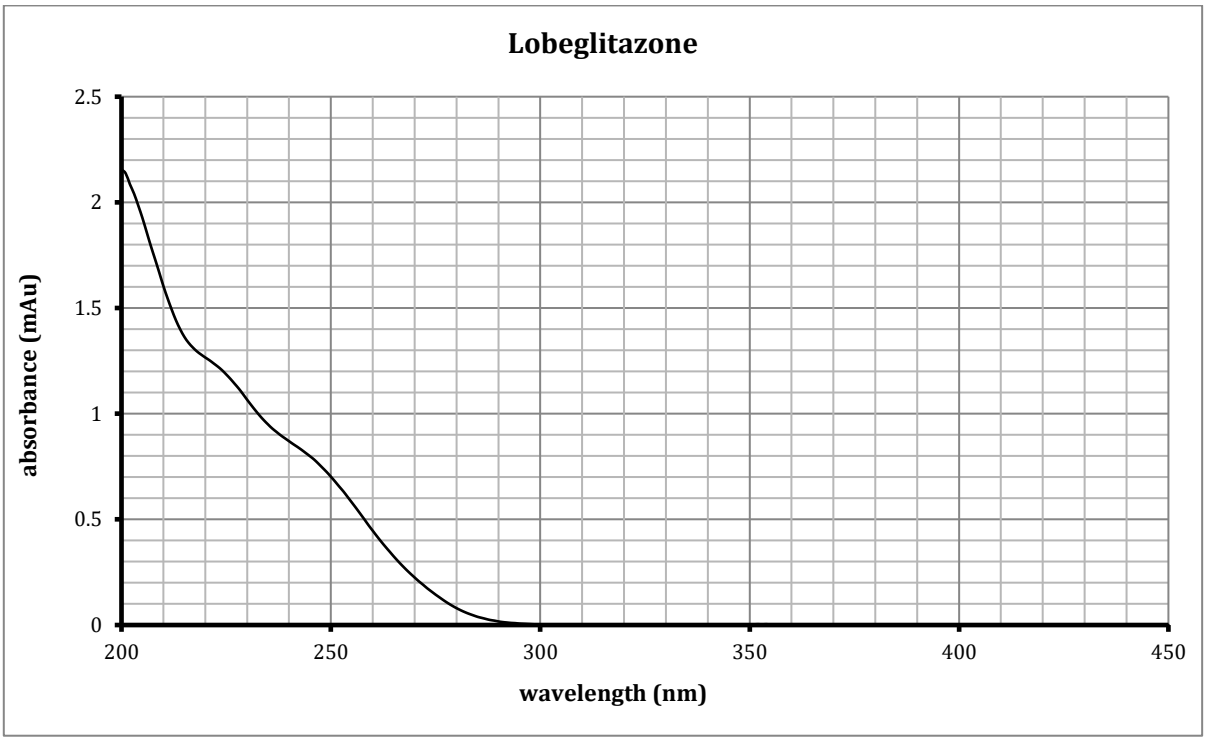

Figure 6: UV spectra of lobeglitazone standard

Significance of simultaneous estimation of metformin, pioglitazone and lobeglitazone

The simultaneous estimation of metformin, pioglitazone, and lobeglitazone in pharmaceutical formulations and human plasma has not been previously reported in the literature. This research uniquely compares two analytical techniques: C18-based reverse-phase chromatography and diol-based HILIC chromatography, an area not explored before. Previous studies often used

orthophosphoric acid and KH2PO4 buffers, which are incompatible with LC-MS/MS due to their non-volatile nature. Additionally, most studies fail to report retention factors (k') for highly polar drugs like metformin, a key parameter according to ICH guidelines. Moreover, quantification methods for these drugs have not adhered to ICH standards, highlighting the need for more precise and standardized analytical approaches.

HPLC analysis

The HPLC analysis for the simultaneous estimation of metformin (MTF), pioglitazone (PGZ), and lobeglitazone (LBG) was successfully carried out using reverse-phase chromatography with gradient elution. The method achieved excellent separation of all analytes, adhering to ICH guidelines, ensuring its suitability for further validation and recovery studies. Metformin had a retention time of 3.823 minutes, pioglitazone at 10.552 minutes, and lobeglitazone at 11.973 minutes, with resolution values indicating clear and distinct peaks. The tailing factors for all analytes were below 2, demonstrating peak symmetry, and the theoretical plates were sufficiently high, especially for lobeglitazone (28,815.79), confirming column efficiency. The area percentages for the drugs showed metformin contributing the largest proportion (53.714%), followed by pioglitazone (25.899%) and lobeglitazone (18.5406%). The separation factor (k') values confirmed appropriate retention for all analytes. Using a Zodiac C18 column with a mobile phase comprising ammonium acetate, methanol, and acetonitrile under a gradient elution program, the method achieved high resolution at a flow rate of 1 mL/min. Detection at a wavelength of 230 nm ensured accurate quantification of all drugs. This optimized method provides reliable separation and quantification, making it suitable for analyzing marketed formulations and plasma samples.

Table 1: Estimation of MTF, PBZ and LBG

Peak	Ret. Time	Area	Height	Area %	T.Plate	Resolution	Tailing F.	Separation
1	2.175	618425	63605	1.8462	959.363	--	1.044	0
MTF	3.823	17992602	1201991	53.714	2087.285	5.13	1.49	0
PGZ	10.552	8675469	812685	25.8992	21666.13	20.755	1.26	5.083
LBG	11.973	6210551	595708	18.5406	28815.79	4.996	1.14	1.17

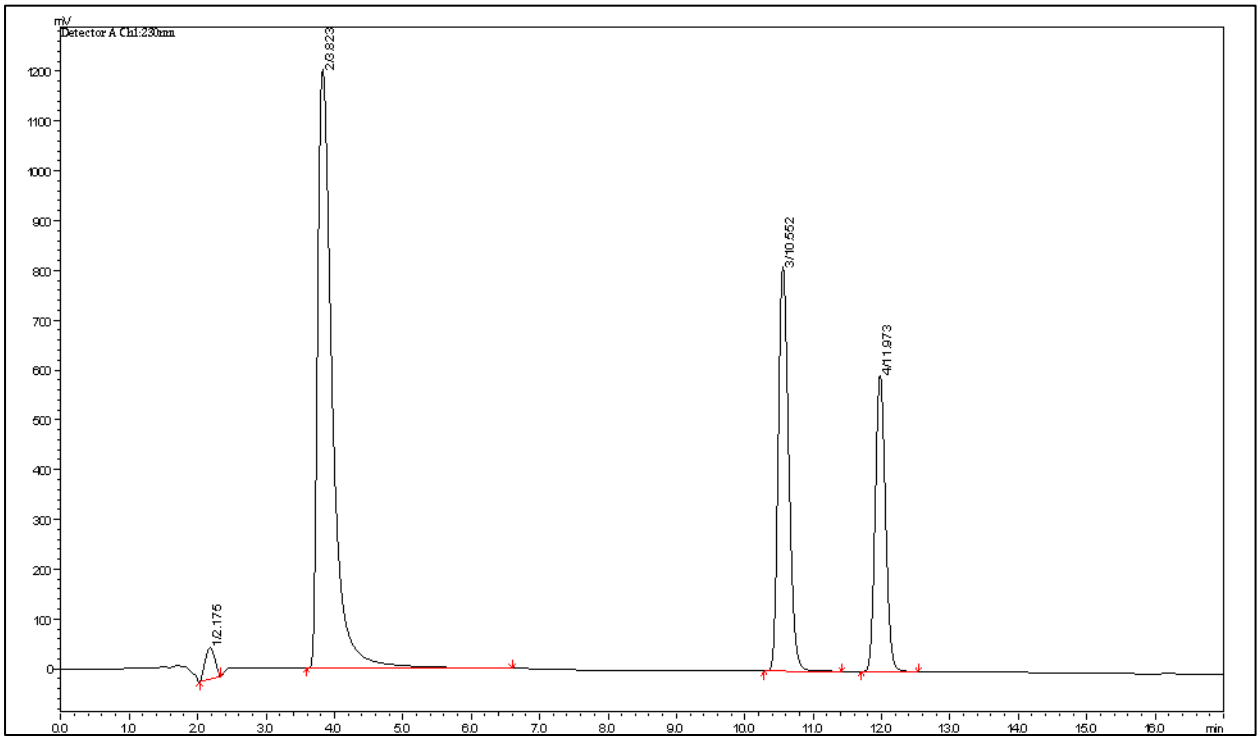

Figure 7: simultaneous quantification of MTF, LBG and PIG by RP-HPLC

Method validation

The method was validated according to ICH guidelines.

Repeatability study of MTF, LBG and PGZ

The repeatability study for metformin (MTF), pioglitazone (PGZ), and lobeglitazone (LBG) demonstrated excellent precision, with RSD values of 1.64%, 0.31%, and 1.17%, respectively, all within the ICH guideline limit of <2%. Retention times were 3.841, 10.443, and 11.862 minutes for MTF, PGZ, and LBG, respectively, with well-separated and symmetric peaks. MTF showed the highest area percentage (49.58%), followed by PGZ (27.82%) and LBG (19.99%). High theoretical plate numbers and acceptable tailing factors confirmed method reliability, making it suitable for simultaneous estimation.

Table 2: Repeatability study of metformin, pioglitazone and lobeglitazone

Peak	Drug Name	Ret. Time (min)	Mean Peak Area	Area %	T. Plate	Resolution	Tailing F.	RSD (%)
1	-	1.694	224801	0.7114	281.609	--	1.915	-
2	-	2.175	599738	1.898	921.39	1.392	0.99	-

3	Metformin	3.841	16878628	49.5819	2142.06	5.171	1.55	1.64
4	Pioglitazone	10.443	8680720	27.8183	21272.18	20.488	1.258	0.31
5	Lobeglitazone	11.862	6268691	19.9904	29996.44	5.067	1.193	1.17

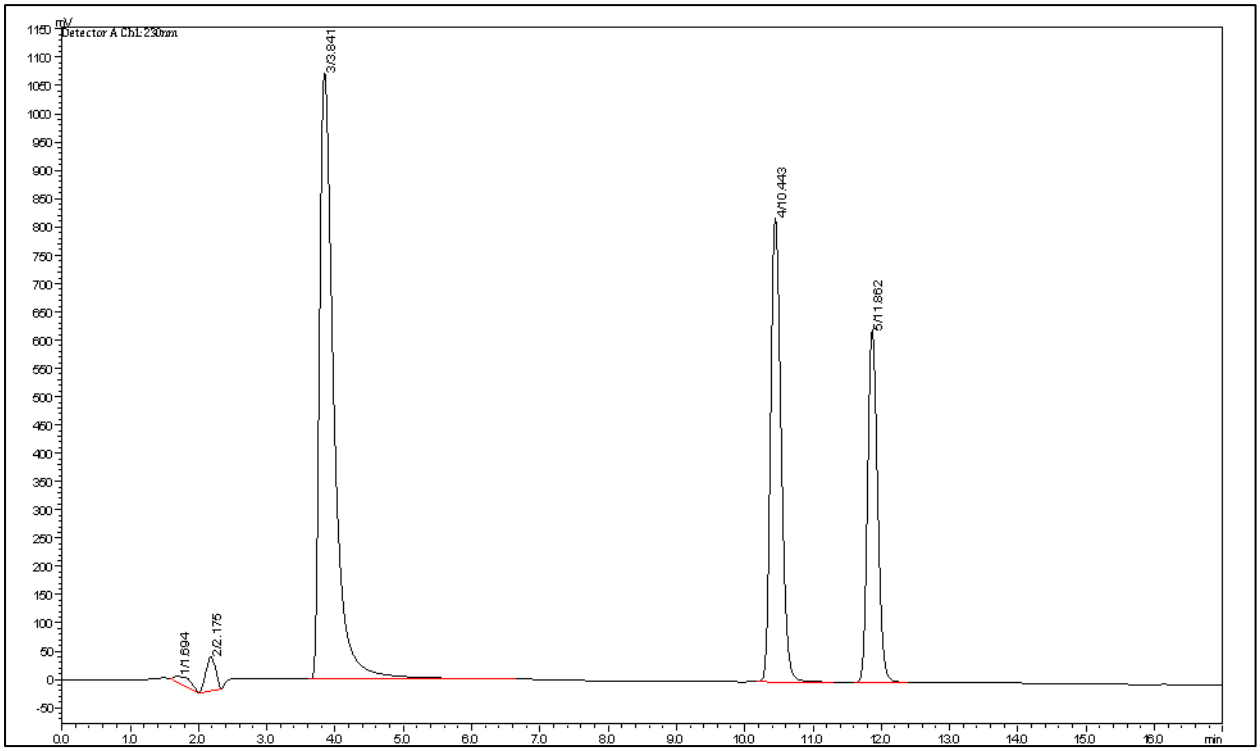

Figure 8: Repeatability study of MTF, PGZ and LBG

System suitability studies

The system suitability test confirmed that parameters such as theoretical plates (N), capacity factor (k'), resolution (R), separation factor (α), tailing factor (T), mean ± SD, and RSD% were within the acceptable range for six successive injections. The method for simultaneous quantification of metformin (MTF), pioglitazone (PGZ), and lobeglitazone (LBG) was validated per ICH guidelines, covering repeatability, linearity, precision, robustness, and recovery studies. Tailing factors for MTF, PGZ, and LBG were 1.55, 1.25, and 1.19, respectively, meeting the <1.8 criteria. Resolution values for PGZ and LBG were 20.48 and 5.06, exceeding the minimum requirement of 2. Theoretical plates for MTF, PGZ, and LBG

were 2142, 21272, and 29996, all above the threshold of 2000. These results confirm the method's reliability and reproducibility for simultaneous quantification.

Table 3: System Suitability Parameters for Metformin, Pioglitazone, and Lobeglitazone

Parameter	Metformin	Pioglitazone	Lobeglitazone	Acceptance Criteria
Theoretical Plates (N)	15022	60200	60200	≥ 2000
Capacity Factor (K')	2.33	4.84	4.84	≥ 0.5
Resolution (R)	---	23.47	23.47	≥ 1.5
Separation Factor (α)	4.07	2.29	2.29	> K'
Tailing Factor (T)	1.35	1.30	1.30	≤ 1.5
Retention Time (tR) (min)	6.60	11.57	11.57	> K'
Detection Wavelength (nm)	240	240	240	> 200
Repeatability (% RSD)	1.51	1.23	1.23	≤ 2%
Intra-Day Precision (% RSD)	0.43–1.07	0.42–1.65	0.42–1.65	≤ 2%
Inter-Day Precision (% RSD)	1.17–1.78	0.72–1.20	0.72–1.20	≤ 2%
Linearity Range (µg/mL)	3.9–62.5	3.9–62.5	3.9–62.5	NA
Regression Equation	y = 40707x + 54832	y = 128263x + 236005	y = 128263x + 236005	NA
SE of Intercept (Se)	0.9993	0.9995	0.9995	NA

SD of Intercept (Sa)	20462.15	55636.16	55636.16	NA
Correlation Coefficient (r^2)	0.9993	0.9995	0.9995	NA
LOQ (µg/mL)	11.24	4.34	4.34	NA
LOD (µg/mL)	3.37	1.30	1.30	NA

Precision studies

The precision of the developed HPLC method was evaluated by calculating the relative standard deviation (RSD) for repetitive measurements of metformin, pioglitazone, and lobeglitazone, both intraday and interday. As indicated in Table 4, the %RSD for all three drugs was consistently below 2%, demonstrating the method's high precision and minimal variation. The correlation between peak area and drug concentration further reinforces the method's reliability for simultaneous quantification. Specifically, intraday RSD for metformin ranged from 0.42% to 1.45%, for pioglitazone from 0.37% to 0.64%, and for lobeglitazone from 0.55% to 1.08%. Interday RSD for metformin was 0.87%, for pioglitazone ranged from 0.48% to 0.76%, and for lobeglitazone ranged from 0.69% to 1.20%. These results indicate robust precision and reproducibility, further confirmed by consistent retention times and resolution values.

Table 4: Intraday and Interday precision study of MTF, PGZ and LBG

Drug Name	Conc. (ppm)	Day	Average Area	Std. Deviation	%RSD	Retention Time (min)	Resolution
Metformin (MTF)	50 ppm	Intraday	16900992	241832.99	1.45	2.184	-
			16417420	241832.99	1.45	3.863	-
			16667463	241832.99	1.45	10.441	-
		Interday	16131231	139770.04	0.87	2.184	-

Pioglitazone (PGZ)	100 ppm	Intraday	8691863	32179.56	0.37	3.864	5.362
			8648211	32179.56	0.37	10.404	-
		Interday	8704045	65964.73	0.76	3.864	5.362
Lobeglitazone (LBG)	100 ppm	Intraday	6155075	34607.29	0.55	3.864	-
			6289302	34607.29	0.55	10.404	-
		Interday	6323256	45085.95	0.69	3.864	-

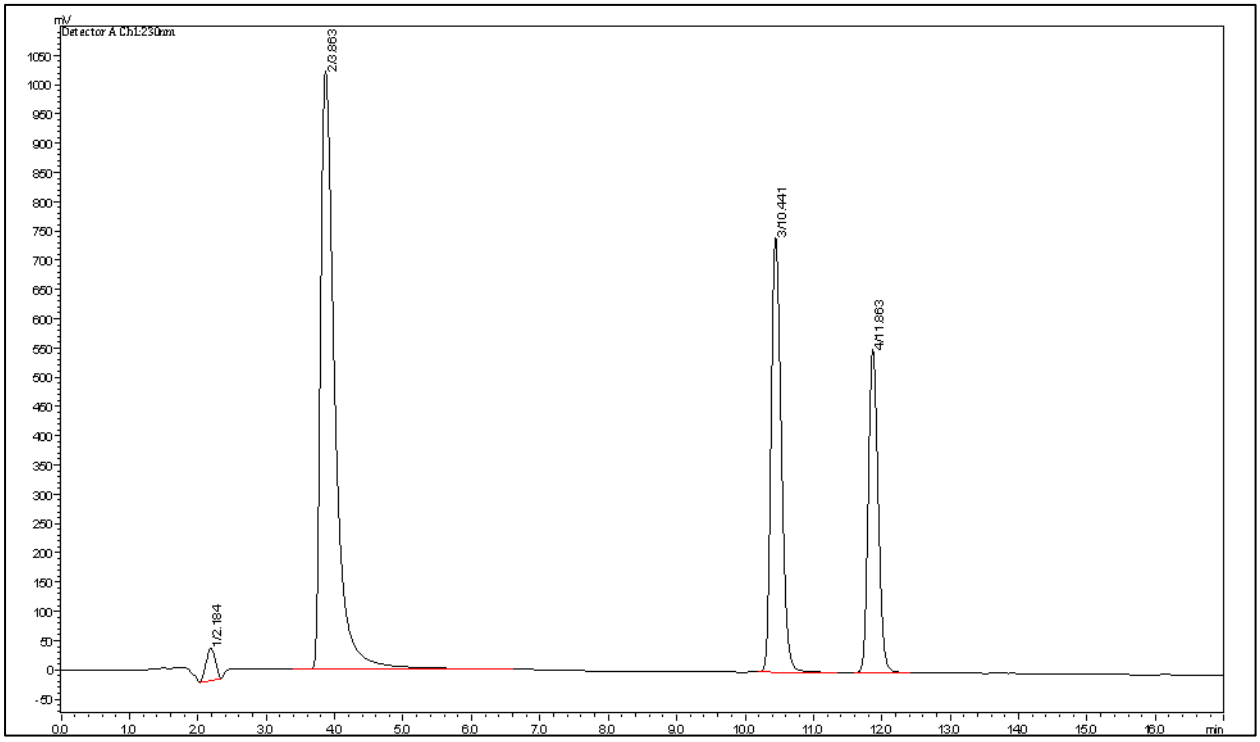

Figure 9: Intraday precision study of MTF, PGZ and LBG

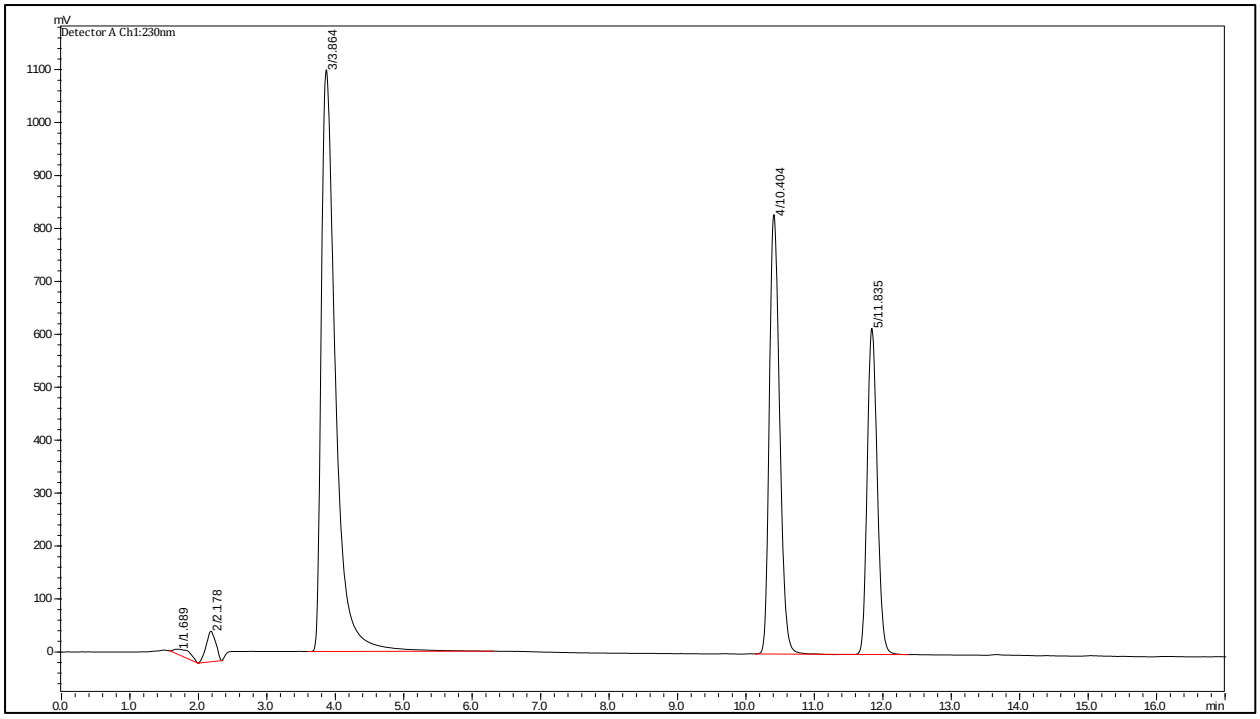

Figure 10: Interday precision study of MTF, PGZ and LBG

Robustness Study

Robustness of HPLC Method represents its ability to remain unaffected by small but deliberate changes in separation parameters to ascertain its reliability during routine analysis. In this method, robustness was established by making deliberate changes in flow rate (1.0±0.1 ml/mins), organic modifier as solvent B (±2%, v/v), and wavelength (230±2 nm). Robustness studies was determined by small variation in separation parameters like effect of flow rate, organic modifier composition, temperature, pH, wavelength, injection volume on selected separation variables including capacity factor (k'), resolution (Rs), tailing factor (*Tf*), separation factor, theoretical plates (N) and sometimes considered the peak area.

The table 5 presents the robustness study data for Metformin (MTF), Pioglitazone (PGZ), and Lobeglitazone (LBG), assessing the impact of flow rate, solvent B concentration, and wavelength adjustments. For flow rate changes, retention times increased for all drugs when the flow rate was reduced by 0.1 mL/min, but the percentage of drug found and RSD values remained stable. Solvent B adjustments (+2% and -2%) caused slight increases in retention time without affecting the amount of drug or RSD significantly. Similarly, wavelength shifts (+2 nm and -2 nm) led to minimal changes in retention time and consistent drug recovery and RSD values. Overall, these results demonstrate the robustness of the method for all three drugs under varied conditions.

Table 5: Robustness of the developed method for metformin, Pioglitazone and Lobeglitazone

Factor	MTF (Metformin)	PGZ (Pioglitazone)	LBG (Lobeglitazone)
Effect of Flow Rate (1.1 ml/min)			
Retention Time (min)	3.523	9.866	11.28
% Amount Found	49.93%	28.06%	20.36%
% RSD	0.42% to 1.45%	0.37% to 0.64%	0.55% to 1.08%
Effect of Flow Rate (0.9 ml/min)			
Retention Time (min)	3.854	9.686	11.195
% Amount Found	49.33%	28.18%	20.20%
% RSD	0.42% to 1.45%	0.37% to 0.64%	0.55% to 1.08%
Effect of Solvent C (27%)			
Retention Time (min)	3.854	9.686	11.195
% Amount Found	49.33%	28.18%	20.20%
% RSD	0.42% to 1.45%	0.37% to 0.64%	0.55% to 1.08%
Effect of Wavelength (232 nm)			
Retention Time (min)	3.878	10.39	11.813
% Amount Found	51.74%	26.44%	19.43%
% RSD	0.42% to 1.45%	0.37% to 0.64%	0.55% to 1.08%
Effect of Wavelength (228 nm)			
Retention Time (min)	3.877	10.39	11.813
% Amount Found	47.59%	29.65%	20.92%
% RSD	0.42% to 1.45%	0.37% to 0.64%	0.55% to 1.08%

Linearity studies

The linearity of the developed HPLC method was evaluated for metformin, pioglitazone, and lobeglitazone across concentrations of 100, 50, 25, 12.5, 6.25, and 3.12 ppm. The peak areas showed strong proportionality to concentrations, with regression coefficients (R^2) of 0.999 for

all drugs, indicating excellent linearity. The limits of detection (LOD) and quantification (LOQ) were calculated based on the regression slope and standard deviation, with values below 5 µg/mL for all analytes, highlighting the method's sensitivity. These findings confirm the method's reliability for detecting and quantifying low drug concentrations in pharmaceutical and biological samples.

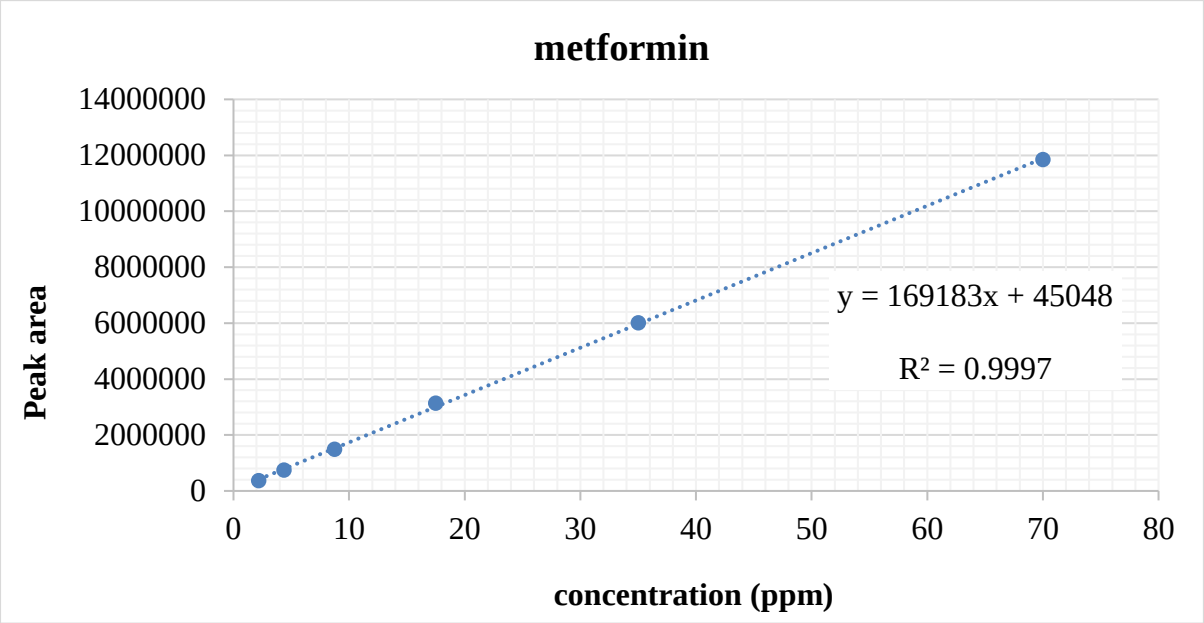

Figure 11: Calibration curve of metformin

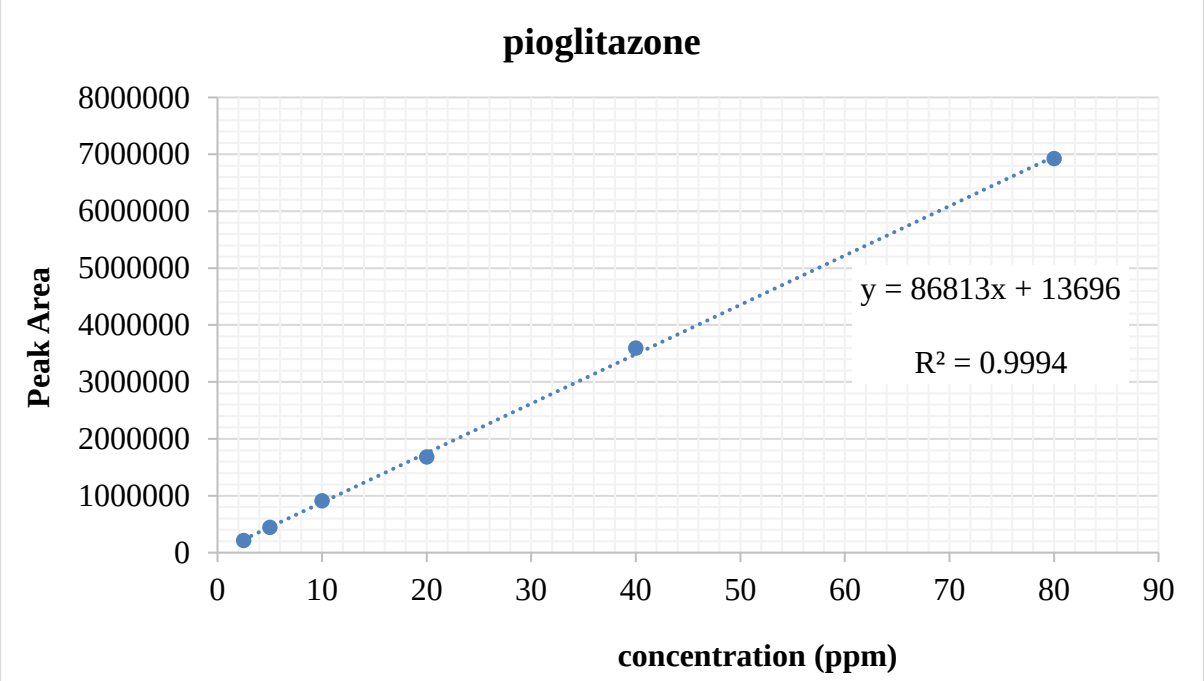

Figure 12: Calibration curve of pioglitazone

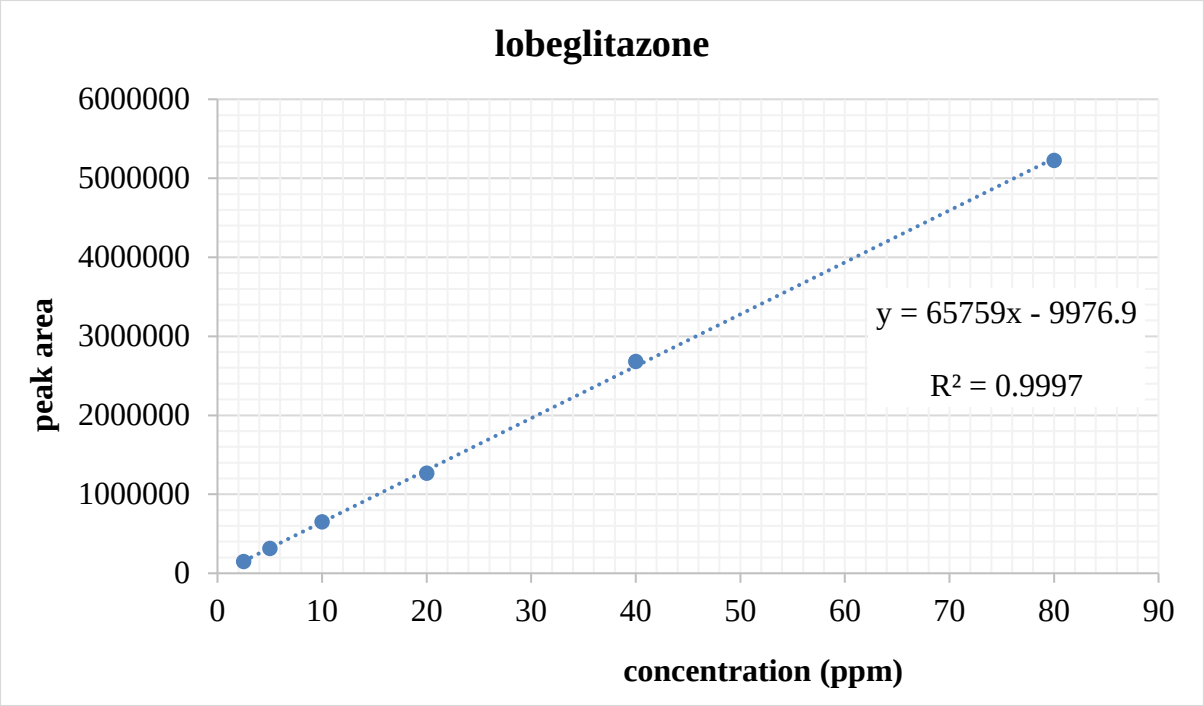

Figure 13: Calibration curve of lobeglitazone

Accuracy studies of marketed formulation

The percentage drug recovery was evaluated at three concentrations (80%, 100%, and 120%), injected thrice, to estimate saxagliptin and dapagliflozin in marketed formulations. Results indicated recovery rates of 100.4–100.7% for saxagliptin and 100–105% for dapagliflozin, falling within the ICH-recommended range of 90–110% with RSD values below 2%. Similarly, lobeglitazone demonstrated consistent recovery across all tested concentrations, confirming the method's accuracy. For metformin and pioglitazone, recovery data highlighted precise and reproducible quantification, aligning with regulatory standards. These findings validate the method's reliability for simultaneous estimation of these drugs in pharmaceutical formulations.

Accuracy studies for metformin, pioglitazone, and lobeglitazone

The accuracy studies for metformin, pioglitazone, and lobeglitazone evaluated drug recovery at 80%, 100%, and 120% standard concentrations using their marketed formulations. Metformin demonstrated recovery rates ranging from 104.68% to 108.36%, exceeding the 100 ± 10% range recommended by ICH guidelines. Pioglitazone showed recovery rates between 92.28% and 96.59%, indicating slightly lower values within the acceptable range. Lobeglitazone exhibited recovery rates from 91.24% to 102.13%, confirming compliance with ICH standards. The data highlights the reliability of the developed HPLC method for quantifying these drugs in marketed formulations.

Table 6: Accuracy Data for Metformin, Pioglitazone, and Lobeglitazone

Drug	Content	Concentration	Average Peak Area	Drug Recovery (%)	ICH Recovery Range
Metformin	500 mg	80%	14135321	104.68	100 ± 10% (90–110%)
		100%	18289514.5	108.36	
		120%	21259362.5	104.96	
Pioglitazone	15 mg	80%	245515.5	96.59	100 ± 10% (90–110%)
		100%	293217	92.28	
		120%	355790	93.31	
Lobeglitazone	0.5 mg	80%	5121881.5	102.13	100 ± 10% (90–110%)
		100%	6380544	101.78	
		120%	6863174.5	91.24	

Drug recovery studies from Human Plasma

The quantification of metformin (MTF) and evogliptin (EVG) in plasma/urine is critical for therapeutic drug monitoring and pharmacokinetic studies, yet no bioanalytical method has been reported. Two plasma extraction techniques, protein precipitation (PPT) and solid-phase extraction (SPE), were evaluated. SPE using C18, HLB, and SCX sorbents was ineffective for highly polar MTF (recovery <40%) due to impurities, while moderately polar EVG showed better recovery (>70%) with C18 and HLB. PPT using methanol (MeOH) was less effective, leading to incomplete protein removal and operational issues in HPLC and LC-MS/MS. However, PPT with acetonitrile (MeCN) proved economical and effective, forming dense precipitates and reducing interferences.

Chromatographic methods, including SCX, HILIC, and mixed-mode, were tested for simultaneous quantification of MTF and EVG in plasma from spiked and patient samples. No significant differences in recovery, sensitivity, or interference were observed across techniques, confirming the reliability of MeCN-based PPT for bioanalysis.

Recovery Data Analysis of Metformin (MTF) and Pioglitazone (PGZ) from Human Plasma

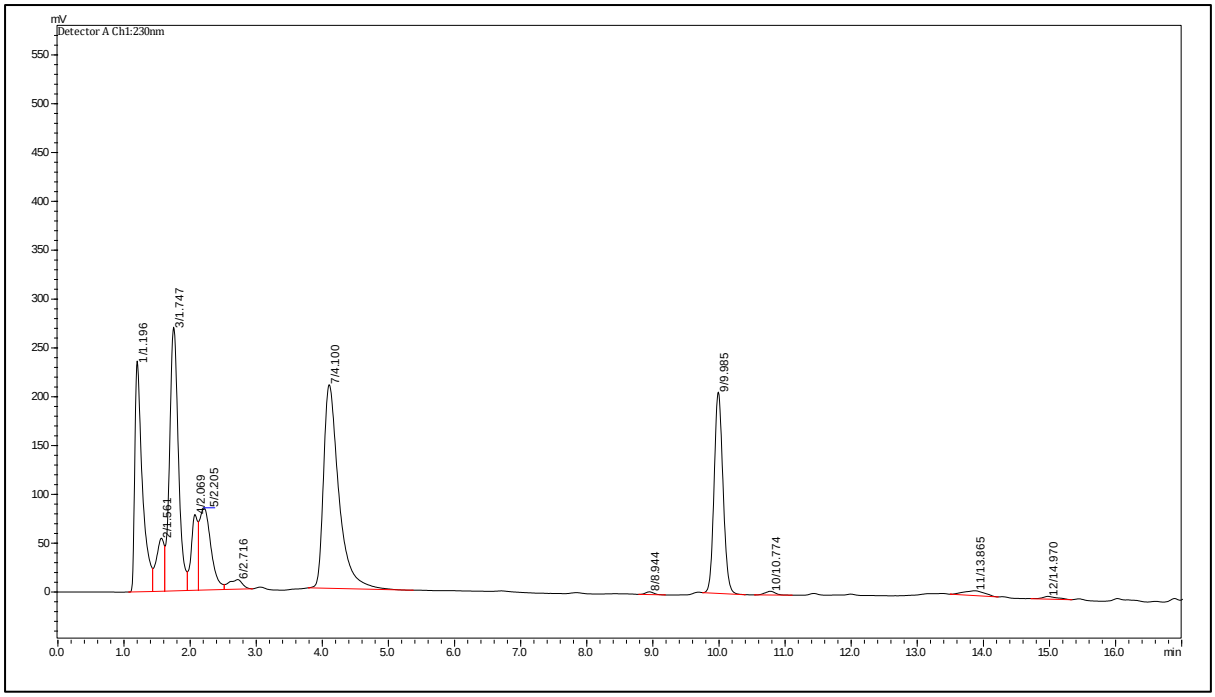

Figure 14: Plasma drug recovery of metformin and pioglitazone

Conclusion

The comparative evaluation of reverse-phase chromatography (RPC) and mixed-mode chromatography (MMC) for the simultaneous estimation of the antidiabetic drugs lobeglitazone, pioglitazone (PGZ), and metformin (MTF) revealed critical insights into their analytical performance. RPC showed efficient separation and quantification of pioglitazone and lobeglitazone, with good resolution and peak symmetry. However, the extreme hydrophilicity of metformin resulted in poor retention and low recovery in RPC, highlighting its limitations in handling highly polar compounds. In contrast, mixed-mode chromatography, specifically with HILIC-1 stationary phases, demonstrated superior reproducibility and recovery for both moderately polar and hydrophilic drugs. EVG consistently achieved recoveries ≥50%, while MTF's recoveries improved marginally in MMC compared to RPC, though they remained low overall. MMC's ability to separate a wide range of compounds effectively, coupled with its better handling of matrix interferences, underscores its potential as a robust analytical approach for such complex drug combinations. Overall, the study establishes MMC as a more versatile and reliable method for the simultaneous quantification of lobeglitazone, pioglitazone, and metformin, particularly when dealing with compounds of varying polarities. Future work could focus on further optimizing MMC conditions to enhance metformin recovery and extend the application of this technique to other challenging analytical scenarios.

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All authors have contributed equally.

CONFLICT OF INTEREST:

The authors declare that there is no conflict of interest regarding the publication of this research.

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