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INTEGRATED LC–MS/MS BIOANALYSIS FOR THE SIMULTANEOUS QUANTIFICATION OF METFORMIN HCL, PIOGLITAZONE HCL, AND TENELIGLIPTIN HBR HYDRATE IN HUMAN PLASMA

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ABSTRACT
Background: Combination therapy is widely prescribed in Type II diabetes mellitus to maintain effective glycemic control. The rising use of multidrug regimens demands selective and reliable bioanalytical methods capable of simultaneously quantifying multiple antidiabetic agents in human plasma for pharmacokinetic and bioequivalence studies. **Methodology:** A rapid, sensitive, and cost-effective LC–MS/MS method was developed and validated in accordance with ICH M10, USFDA, and EMA guidelines for the simultaneous estimation of metformin hydrochloride, teneligliptin hydrobromide hydrate, and pioglitazone hydrochloride. The assay enabled triple-drug quantification within a single 7-minute chromatographic run, showing an estimated 12–40% reduction in analysis time versus previously reported 8–15-minute single- or dual-analyte methods. Separation was achieved on a Cosmosil CN column (150 × 4.6 mm, 5 µm) using 10 mM ammonium acetate and acetonitrile (40:60 %v/v). Plasma samples were prepared by protein precipitation followed by liquid–liquid extraction, and detection was performed in positive electrospray ionization multiple-reaction-monitoring mode. **Results and Discussion:** Strong linearity was obtained for all analytes ($r^2 > 0.995$). LLOQs were 10.0 ng/mL for metformin, 1.25 ng/mL for teneligliptin, and 5.0 ng/mL for pioglitazone. Metformin-D6 served as the internal standard for metformin, while saxagliptin was used as the internal standard for teneligliptin and pioglitazone to ensure appropriate normalization across chemical classes. Precision remained below 10% CV, recovery was consistent, and stability stayed within ±15% under tested conditions. Reduced runtime and unified multi-analyte detection improved analytical throughput and minimized solvent consumption without compromising regulatory compliance. **Conclusion:** The validated LC–MS/MS method provides a reliable, resource-efficient platform for concurrent quantification of combined antidiabetic drugs in pharmacokinetic, bioequivalence, and clinical studies.

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INTRODUCTION

Diabetes mellitus (DM) is a chronic metabolic disease characterized by high blood glucose levels due to either insufficient insulin secretion, insufficient insulin action, or both. It includes multiple clinical types, such as type 1 DM, type 2 DM, gestational diabetes, and secondary diabetes resulting from hormonal imbalances or prolonged drug therapy, and is linked to serious microvascular and macrovascular complications [1]. The most prevalent kind of diabetes is type 2 diabetes (T2DM) & often necessitates combination drug therapy to achieve optimal glycemic control. Fixed-dose combinations that target different metabolic pathways are increasingly used, especially in patients who do not achieve adequate control with monotherapy or dual therapy [1].

Metformin hydrochloride

(IUPAC name: *N, N-dimethylimidodicarbonimidic diamide hydrochloride*) is widely used as the first-line antidiabetic agent due to its capacity to enhance peripheral insulin sensitivity and prevent the liver from producing glucose [2]. The chemical structure of metformin hydrochloride is shown in Figure 1(A).

Teneligliptin hydrobromide hydrate

(IUPAC name: *(2S,4S)-4-[3-(3-methyl-1-phenyl-1H-pyrazol-5-yl) propyl]-2-(2,4,5-trifluorophenyl) pyrrolidine-1-carbonitrile hydrobromide hydrate*) enhances glycemic control by elevating endogenous incretin levels, thereby promoting glucose-dependent insulin secretion and suppressing glucagon release [3]. Its chemical structure is illustrated in Figure 1(B).

Pioglitazone hydrochloride

Peroxisome proliferator-activated receptor-γ (PPAR-γ) is activated by 5-(4-[2-(5-ethylpyridin-2-yl) ethoxy] benzyl) thiazolidine-2,4-dione hydrochloride, thereby improving insulin sensitivity in adipose tissue and skeletal muscle [4]. Its chemical structure is presented in Figure 1(C).

The triple-drug fixed-dose combination of metformin hydrochloride, teneligliptin hydrobromide hydrate, and pioglitazone hydrochloride was introduced in India in 2022 to offer a comprehensive treatment strategy for T2DM. This combination exerts synergistic effects by simultaneously decreasing hepatic glucose production, raising insulin secretion, and lowering insulin resistance [5–7]. Precise measurement of these drugs in human plasma is essential for pharmacokinetic studies, bioequivalence testing, and therapeutic drug monitoring, particularly in combination therapy. Liquid chromatography–tandem mass spectrometry (LC–MS/MS) is considered the analytical method of choice due to its superior sensitivity, reliable detection, selectivity, and suitability for complex biological matrices [8-9].

Although several LC–MS/MS methods exist for individual antidiabetic agents or certain dual combinations, no validated bioanalytical method has been reported for the simultaneous determination of metformin hydrochloride, teneligliptin hydrobromide hydrate, and pioglitazone hydrochloride in human plasma [10–18]. This gap limits the ability to perform comprehensive pharmacokinetic and bioequivalence studies for this clinically important fixed-dose combination.

Accordingly, the investigation described in this work aims to develop and validate a rapid, sensitive, and robust LC–MS/MS method for the simultaneous quantification of metformin hydrochloride, teneligliptin hydrobromide hydrate, and pioglitazone hydrochloride in human plasma. The method is designed following ICH M10, FDA, and EMA guidelines for bioanalytical validation standards, facilitating its use in pharmacokinetic, bioequivalence, and therapeutic drug monitoring studies [10-18].

LC–MS/MS Applications in Diabetes Mellitus

LC–MS/MS has revolutionized the bioanalysis of antidiabetic drugs by allowing high-throughput, highly selective, and sensitive quantification of compounds with diverse physicochemical properties. It is particularly advantageous for agents such as metformin, gliptins, and thiazolidinediones, which vary significantly in polarity, molecular weight, and ionization characteristics [10–19]. The ability of LC–MS/MS to simultaneously quantify multiple analytes makes it an essential tool in modern diabetes pharmacotherapy, where combination regimens are increasingly utilized.

MATERIALS & METHODS

Plasma Source and Ethical Compliance

Human plasma used in this study was collected from healthy adult volunteers at the Rajkot Voluntary Blood Bank and Research Centre, Gujarat, India, in accordance with the ethical principles of the Declaration of Helsinki (2013 revision), and informed consent was obtained from all subjects. Only drug-free plasma was employed for ex vivo bioanalytical method development; no administration of Metformin hydrochloride,

Teneligliptin hydrobromide hydrate, or Pioglitazone hydrochloride was performed on any human or animal subjects. Before analysis, plasma samples were carefully inspected to ensure they were free from hemolysis, lipemic interference, and visible impurities.

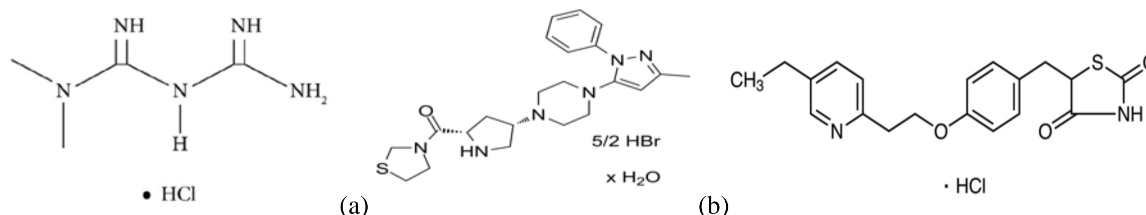


Figure 1: Chemical structure (A) Metformin HCl (B) Teneligliptin HBr Hydrate (C) Pioglitazone HCl

Active Compounds and Reagents

Reference standard active pharmaceutical ingredients of metformin hydrochloride, teneligliptin hydrobromide hydrate, and pioglitazone hydrochloride were supplied by Dhamtec Pharma and Consultants (Navi Mumbai, Maharashtra, India). Methanol (HPLC grade), ammonium acetate (AR grade), and ethyl acetate (HPLC grade) were purchased from Spectrochem, whereas acetonitrile (HPLC gradient grade) was obtained from Rankem. Hydrochloric acid (AR grade) was acquired from Merck. Ultrapure LC–MS grade water produced by a Milli-Q purification system was used for all analytical procedures.

Blank human plasma treated with K3EDTA anticoagulant was sourced from the Department of Pharmaceutical Sciences, Saurashtra University, Rajkot. The plasma was verified to be free from interfering medications and preserved under controlled frozen storage conditions until further use. Saxagliptin and Metformin-D6, employed as internal standards, were likewise obtained from the Department of Pharmaceutical Sciences, Saurashtra University, Rajkot.

Internal Standard Selection

Metformin-D6 was chosen as the internal standard for metformin because its isotopically labelled form behaves almost identically to the analyte during extraction, chromatography, and ionization, enabling effective correction of matrix effects and instrumental variation. For teneligliptin and pioglitazone, saxagliptin was applied as a structural internal standard owing to its comparable polarity, consistent retention time, and stable response in positive electrospray mode. Although saxagliptin is not an isotopic analogue and belongs to a different therapeutic class, validation results showed uniform signal normalization with acceptable accuracy and precision across all quality control levels, indicating no observable quantitative bias.

LC-MS/MS System and Analytical condition

An analytical approach was designed, optimized, and validated for the intended application using a quadrupole-based tandem mass spectrometer coupled to an HPLC system. Mass spectrometric detection was performed using an electrospray ionization (ESI) source in positive-ion mode. Quantification was achieved using multiple reaction monitoring (MRM), with optimized precursor-to-product ion transitions selected based on compound-specific fragmentation behaviour. The monitored transitions were m/z 130.25 → 71.05 for metformin hydrochloride, m/z 427.20 → 243.20 for teneligliptin hydrobromide hydrate, and m/z 357.20 → 134.10 for pioglitazone hydrochloride. The internal standards were monitored at m/z 316.30 → 180.25 (ISTD-1) and m/z 136.30 → 77.10 (ISTD-2). A dwell time of 100 ms was applied for each transition, and collision energies were optimized within the range of –25 to –30 eV to achieve maximum sensitivity and reproducible fragmentation. Figure 2 illustrates Illustrative product ion mass spectra & optimized MRM transitions of (A) Metformin HCl, (B) Teneligliptin HBr hydrate, and (C) Pioglitazone HCl, confirming the suitability of the selected transitions for selective and sensitive quantification.

Chromatographic Condition

Chromatographic separation was carried out as part of the formulation and confirmation of an LC coupled to tandem mass spectrometry approach for a triple-drug combination containing MetforminHCl, TeneligliptinHBr hydrate & Pioglitazone HCl. Chromatographic resolution was obtained on a Cosmosil CN analytical cartridge (150×4.6 mm, packed with 5 µm particles). Chromatographic separation was carried out using a solvent system composed of 10 mM ammonium acetate in purified water and acetonitrile (40:60 %v/v), delivered at a flow rate of 0.5 mL/min. The analytical column was kept at 35 ± 0.3°C, while

the autosampler temp. was control- led at 10 ± 3°C throughout the analysis. An injection sample was introduced at a volume of 20 µL, and detection was performed using an MS/MS detector with a 7min. acquisition time. run time overall.

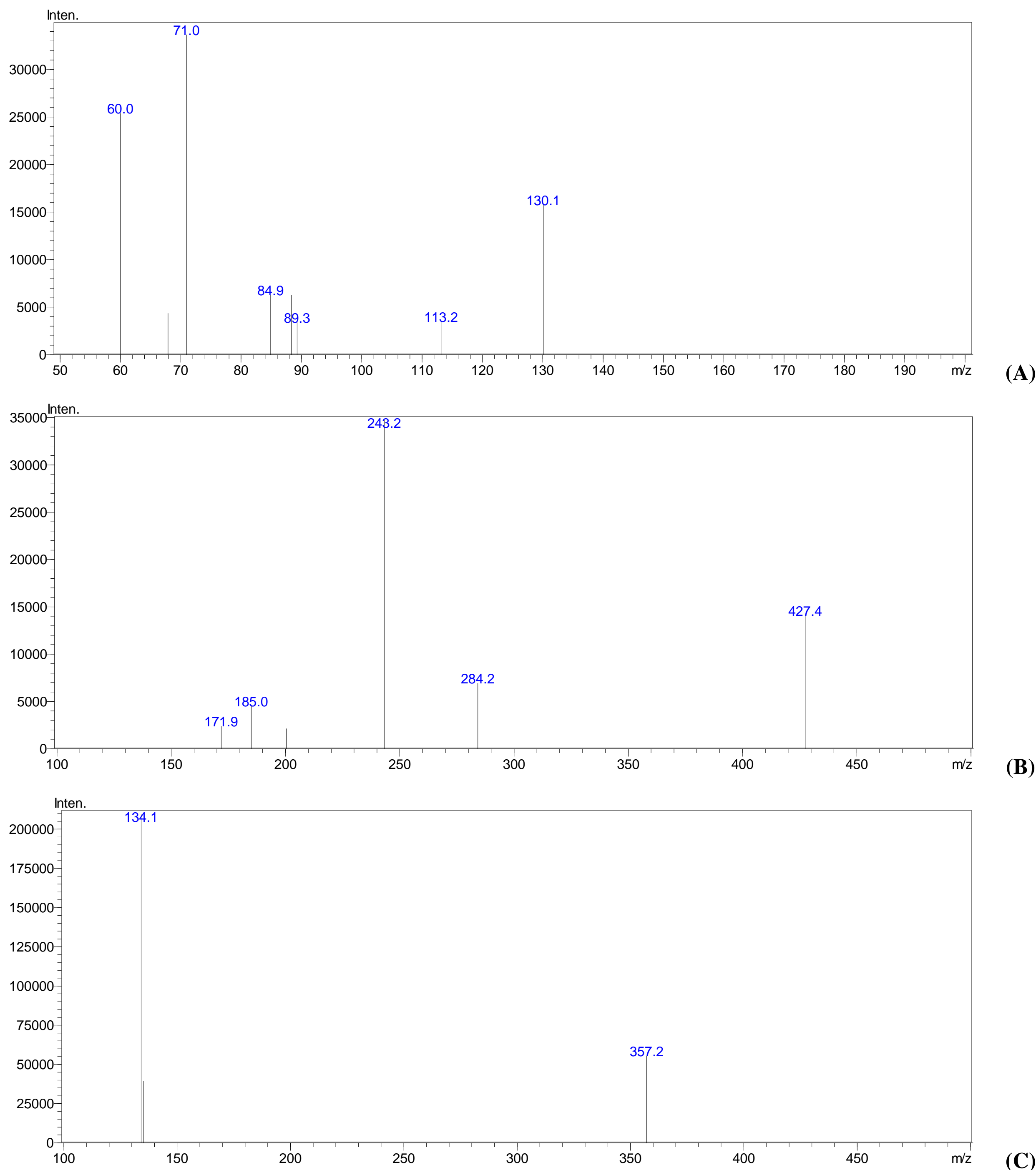


Figure 2: Mass spectra of (A) Metformin HCl, (B) Teneligliptin HBr Hydrate, (C) Pioglitazone HCl

Preparations of Solution

Calibration and Quality Control Sample Preparation

The calibration standards were constructed through serial dilution of a mixed Mid-level stock solution containing metformin hydrochloride, teneligliptin hydrobromide hydrate, and pioglitazone hydrochloride in methanol to obtain eight calibration spiking solutions. To create calibration standards across the concentration ranges of 10–2000 ng/mL for metformin, 1.25–250 ng/mL for teneligliptin, and 5–1000 ng/mL for pioglitazone, including the lower limit of quantification (LLOQ), spiking solutions were added to drug-free K3EDTA human plasma. Quality control (QC) samples

were prepared independently using separately prepared spiking solutions at four concentration levels: LLOQ QC, low QC (LQC), medium QC (MQC) and high QC (HQC) representing the lower, middle, and upper regions of the calibration curve. All calibration standards and QC samples were freshly prepared and processed using the same extraction procedure as study samples to ensure accuracy, precision and method consistency throughout the analytical range.

Sample preparation in Human Plasma

Plasma aliquots (200 µL) were prepared using a sequential hybrid extraction consisting of protein precipitation followed by liquid–liquid extraction (PPT–LLE). This approach was selected to address the contrasting physicochemical behaviour of the target analytes within a single workflow. Protein precipitation was first employed to efficiently recover the highly polar metformin while simultaneously reducing protein-related interferences. Subsequently, liquid–liquid extraction with ethyl acetate was introduced to improve the extraction efficiency of the comparatively lipophilic analytes, teneligliptin and pioglitazone, and to further enhance sample cleanliness. Owing to its strong hydrophilic and ionic properties, metformin predominantly remained in the aqueous phase during the LLE step, thereby limiting the likelihood of analyte loss. Following the addition of internal standards (50 µL), acetonitrile (400 µL) was added for protein precipitation, after which ethyl acetate (1 mL) was used for extraction. The mixture was vortex-mixed and centrifuged at 10,000 rpm for 10 min. at 10 ± 2 °C. The separated organic layer was carefully transferred, evaporated under vacuum at 40 ± 5 °C, and the dried residue was reconstituted in 100 µL of reconstitution solvent prior to LC–MS/MS analysis. This optimized sequential strategy provided balanced recovery for all three analytes while effectively reducing matrix-associated variability and supporting reproducible quantification.

Method Validation

The LC–MS/MS method was validated in accordance with ICH M10, US FDA, and EMA bioanalytical method validation guidelines to confirm its reliability and suitability for quantitative analysis in human plasma. Validation parameters included assessment of calibration model performance, selectivity, accuracy, precision, recovery, matrix effect, carryover, dilution integrity, and reinjection reproducibility. Matrix effects were evaluated using multiple independent human plasma lots to address potential inter-individual variability, as recommended by regulatory agencies. Analyte stability was systematically investigated under a comprehensive range of conditions, including bench-top (BT), freeze–thaw (FT), long-term matrix (LTM), short-term extract (SE), dry extract (DE), autosampler (AQ), and stock solution stability under short-term (STSS) and long-term (LTSS) storage. Validation experiments were conducted in a predefined, sequential manner, beginning with assessment of the calibration curve, followed by precision and accuracy evaluations, matrix effect studies, and stability testing under relevant storage and processing conditions. This structured validation approach ensured regulatory compliance and confirmed the method's applicability for routine bioanalytical and pharmacokinetic investigations.

RESULTS AND DISCUSSION

Linearity & System Suitability

Linearity was evaluated by analyzing calibration standards prepared in human plasma across the concentration ranges of 10–2000 ng/mL for metformin hydrochloride, 1.5–250 ng/mL for teneligliptin hydrobromide hydrate, and 5–1000 ng/mL for pioglitazone hydrochloride. Calibration curves were constructed using eight non-zero calibration levels and were fitted using a weighted ($1/x^2$) least-squares linear regression model based on analyte-to-internal-standard peak area ratios. The regression coefficients (R^2) were 0.9998 for metformin, 0.9996 for teneligliptin, and 0.9998 for pioglitazone, demonstrating an acceptable linear relationship between analyte concentration and instrument response. Back-calculated concentrations of the calibration standards met the predefined acceptance criteria, with accuracy within 80–120% and precision (%CV) ≤ 15%, in accordance with ICH M10 and FDA bioanalytical validation requirements. Representative calibration curves are presented in Figure 3 (A–C). The calibration results for Metformin hydrochloride, Teneligliptin hydrobromide hydrate, and Pioglitazone hydrochloride, as mentioned in Table 1, demonstrated a consistent, linear response across their respective concentration ranges. All back-calculated concentrations agreed closely with nominal values, with accuracy remaining within ±15% across all calibration levels. The strong linear relationship between analyte response and concentration confirms the method's reliability. Overall, these results validate the suitability of the method for accurate and reproducible quantification of the selected antidiabetic drugs in bioanalytical studies.

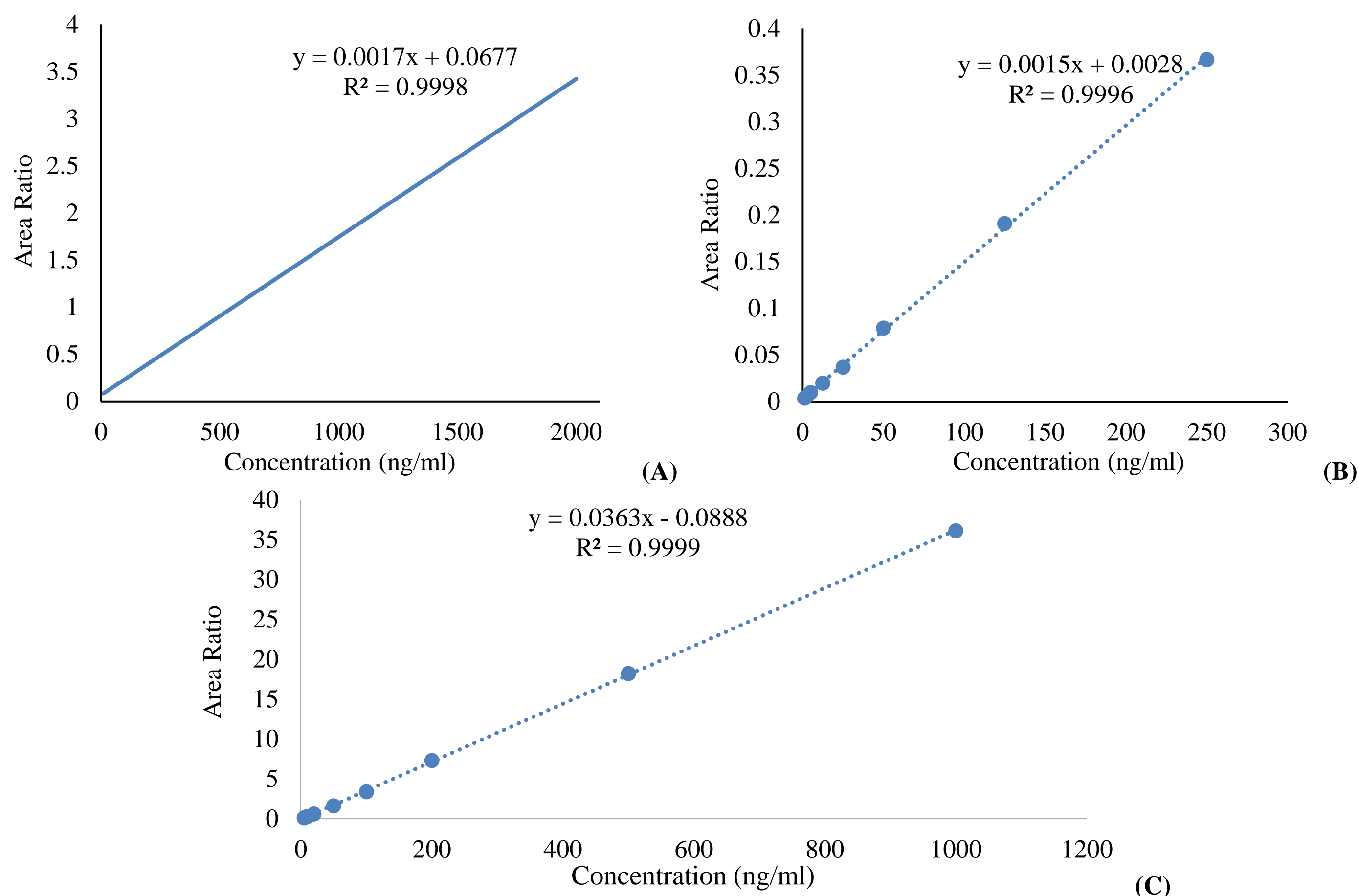


Figure 3: Calibration Curve (A) Metformin HCl (B) Teneligliptin HBr Hydrate (C) Pioglitazone HCl

Table 1: Summary of system suitability or linearity data for Metformin HCl, Teneligliptin HBr Hydrate & Pioglitazone HCl

Compound	Calibration Level	Nominal Conc.(ng/mL)	Measured Conc.(ng/mL)	Accuracy(%)
Metformin Hydrochloride	STD 1	2000	2000.000	100.0
Metformin Hydrochloride	STD 2	1000	1000.000	100.0
Metformin Hydrochloride	STD 3	400	398.376	99.6
Metformin Hydrochloride	STD 4	200	198.828	99.4
Metformin Hydrochloride	STD 5	100	101.371	101.4
Metformin Hydrochloride	STD 6	40	39.514	98.8
Metformin Hydrochloride	STD 7	20	20.298	101.5
Metformin Hydrochloride	STD 8	10	10.278	102.8
Teneligliptin HBr Hydrate	STD 1	250	250.000	100.0
Teneligliptin HBr Hydrate	STD 2	125	125.000	100.0
Teneligliptin HBr Hydrate	STD 3	50	49.831	99.7
Teneligliptin HBr Hydrate	STD 4	25	24.512	98.0
Teneligliptin HBr Hydrate	STD 5	12.5	12.615	100.9
Teneligliptin HBr Hydrate	STD 6	5	5.100	102.0
Teneligliptin HBr Hydrate	STD 7	2.5	2.482	99.3
Teneligliptin HBr Hydrate	STD 8	1.25	1.261	100.9
Pioglitazone Hydrochloride	STD 1	1000	998.742	99.87
Pioglitazone Hydrochloride	STD 2	500	501.263	100.25
Pioglitazone Hydrochloride	STD 3	200	199.813	200
Pioglitazone Hydrochloride	STD 4	100	98.857	100
Pioglitazone Hydrochloride	STD 5	50	50.114	50
Pioglitazone Hydrochloride	STD 6	20	20.345	20
Pioglitazone Hydrochloride	STD 7	10	10.218	10
Pioglitazone Hydrochloride	STD 8	5	5.319	5

Accuracy and Precision

The representative HQC chromatograms of Metformin hydrochloride (a), Teneligliptin hydrobromide hydrate (b), and Pioglitazone hydrochloride (c) in Figure 4 showed sharp, symmetrical, and well-resolved peaks at consistent retention times. All analytes exhibited clean baselines with no significant interference, demonstrating excellent chromatographic selectivity and separation efficiency. The uniform peak shapes and stable responses across compounds further confirm the robustness of the LC–MS/MS method and its suitability for validated quantitative bioanalysis in accordance with ICH M10 guidelines.

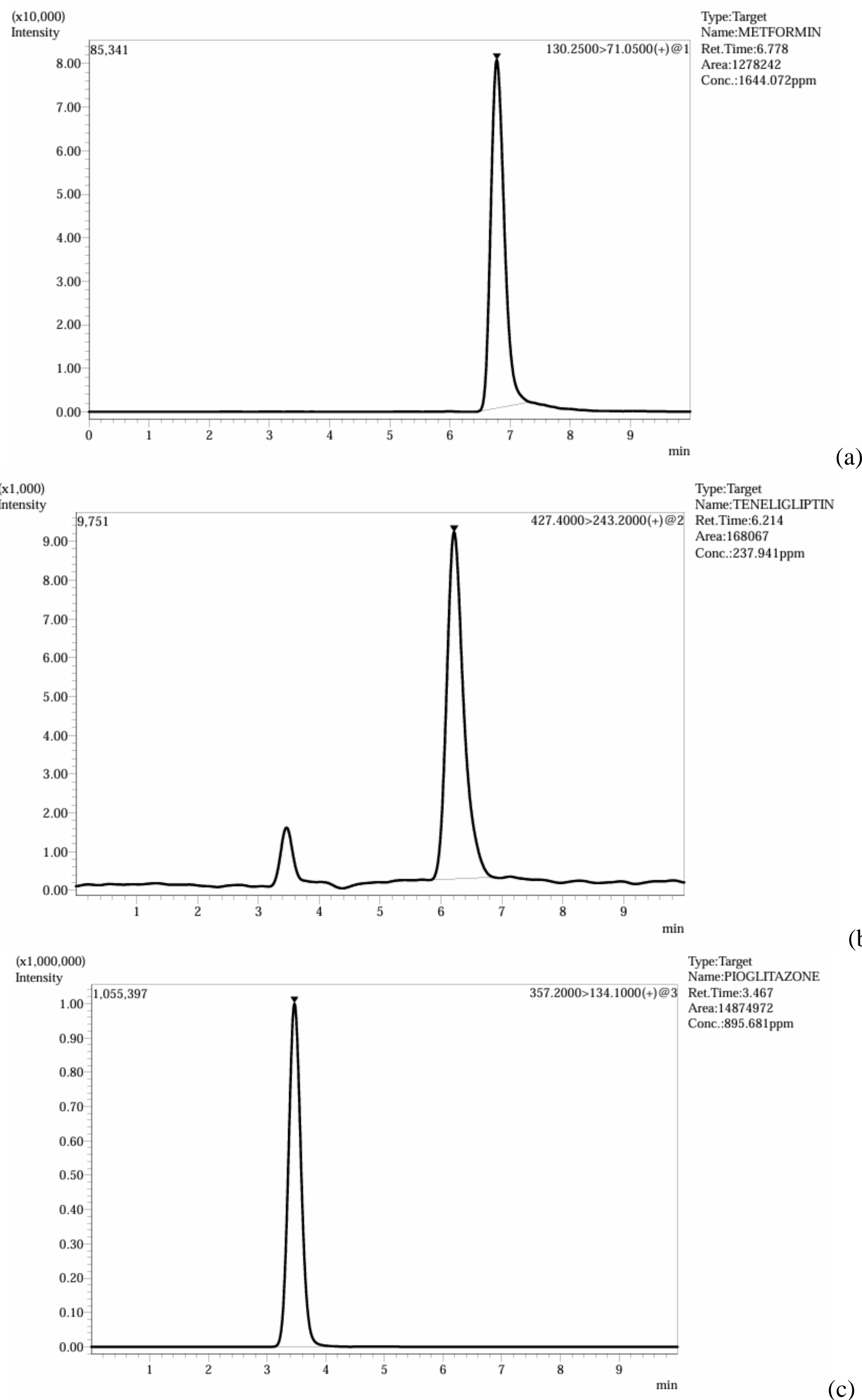
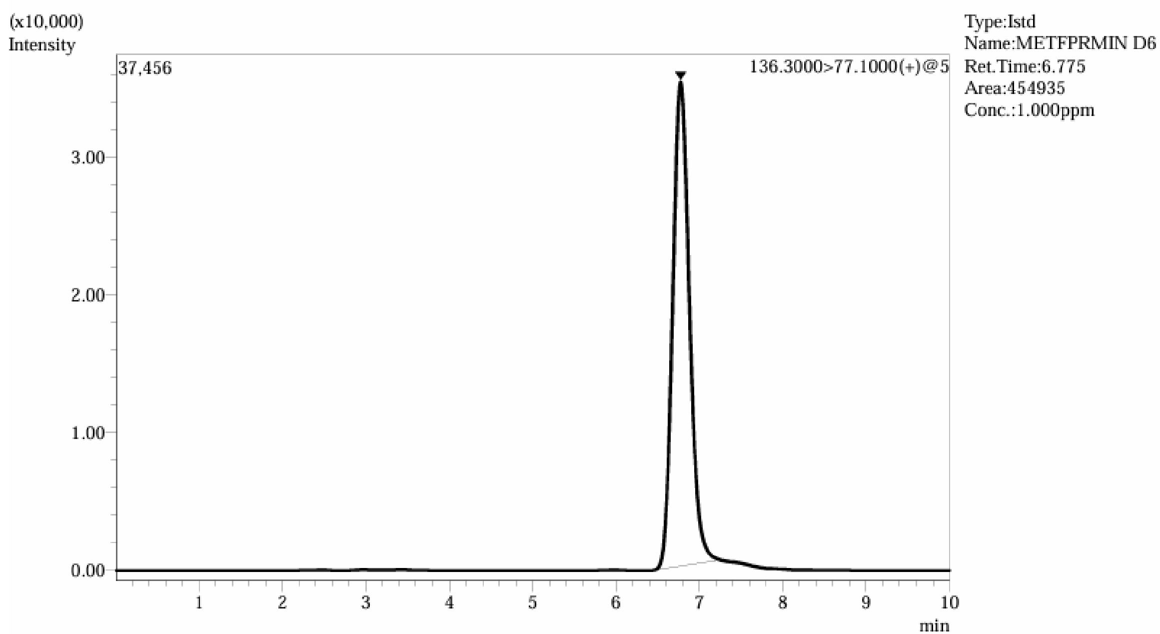
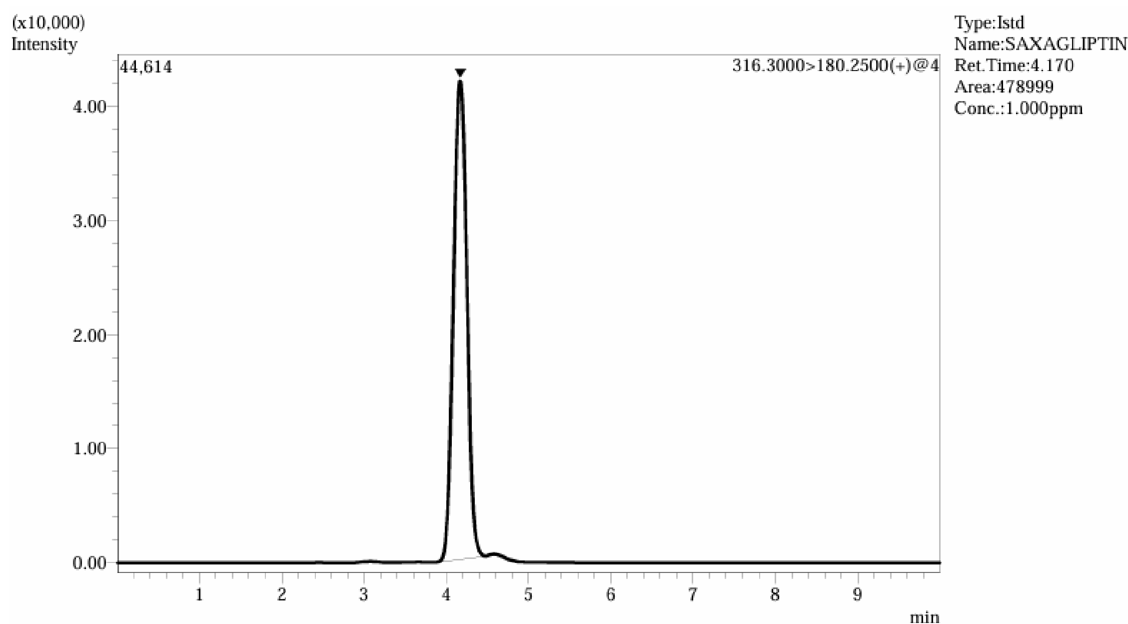


Figure 4: Representative HQC chromatogram of (a) Metformin hydrochloride (b) Teneligliptin hydrobromide hydrate (c) Pioglitazone hydrochloride

Representative chromatograms of the internal standards (Metformin-D6 and Saxagliptin) show as (a) & (b) in Figure 5, exhibiting sharp, well-resolved peaks at consistent retention times with clean baselines. The stable and reproducible internal standard responses across the chromatographic run support accurate normalization of analyte signals and further confirm the robustness and reliability of the method for simultaneous quantification of Metformin, Teneligliptin, and Pioglitazone in accordance with ICH M10 guidelines.



(a)



(b)

Figure 5: Representative Chromatogram of IS (a) Metformin D6 (b) Saxagliptin

The intra-batch accuracy and precision results for Metformin hydrochloride, Teneligliptin hydrobromide hydrate, and Pioglitazone hydrochloride demonstrated consistent and reliable method performance across all quality control levels. Accuracy values for all three analytes remained within ±15% of the nominal concentrations at the LQC, MQC, and HQC levels, confirming the method's trueness over the tested concentration ranges. Precision expressed as %CV was well controlled for all compounds, with values not exceeding 5.58%, indicating excellent repeatability of the analytical procedure. Detailed data is given in Table 2. The low variability observed across QC levels reflects stable chromatographic behaviour and robust mass spectrometric detection for each analyte. Collectively, these results satisfy the acceptance criteria specified in ICH M10

bioanalytical method validation guidelines and confirm the suitability of the developed LC–MS/MS method for accurate, precise, and reproducible quantification of the selected antidiabetic drugs in routine bioanalysis and pharmacokinetic studies.

Table 2: Accuracy and Precision

Compound	QC Level	Mean Conc. (ng/mL)	Nominal Conc.(ng/mL)	Accuracy%	Precision %CV
Metformin HCl	HQC	1800	1644.072	91.3	1.82%
Metformin HCl	MQC	500	516.728	103.3	5.58%
Metformin HCl	LQC	30	29.605	98.7	4.75%
Teneligliptin HBr hydrate	HQC	225	237.941	105.8	1.90%
Teneligliptin HBr hydrate	MQC	62.5	65.440	104.7	0.04%
Teneligliptin HBr hydrate	LQC	3.75	3.877	103.4	2.54%
Pioglitazone HCl	HQC	900	895.681	99.5	5.02%
Pioglitazone HCl	MQC	250	267.366	106.9	3.29%
Pioglitazone HCl	LQC	15	15.287	101.9	3.89%

Recovery

The extraction recovery and matrix effect of the developed LC–MS/MS method were evaluated in accordance with ICH M10. Metformin hydrochloride showed high and concentration-independent recovery (92.37–111.23%), whereas teneligliptin hydrobromide hydrate and pioglitazone hydrochloride exhibited comparatively lower absolute recovery, particularly at LQC, which may be attributed to their relatively higher lipophilicity and plasma protein binding characteristics. Nevertheless, recovery remained consistent and reproducible across all QC levels, supporting reliable quantification. Matrix effect assessment at LQC and HQC demonstrated minimal ion suppression or enhancement for all analytes (90.70–102.95%), indicating negligible interference from endogenous plasma components. Importantly, the comparatively lower recovery of teneligliptin and pioglitazone did not adversely influence method sensitivity or signal-to-noise performance at the LLOQ level. The LLOQ responses for teneligliptin (1.25 ng/mL) and pioglitazone (5 ng/mL) consistently met regulatory acceptance criteria for accuracy and precision, demonstrating that adequate detector response and reliable quantification were maintained despite reduced absolute extraction yield. As emphasized by ICH M10, consistency and reproducibility of recovery and matrix performance, rather than absolute recovery alone, confirm the robustness and suitability of the method for simultaneous bioanalysis. Data is given in Table 3.

Table 3: Recovery and Matrix Effect for Metformin HCl, Teneligliptin HBr hydrate, and Pioglitazone HCl

Compound	QC Level	Mean Recovery (%)	%CV	Matrix Effect (%)	Metrix Factor
Metformin HCl	LQC	111.23%	3.4	98.24%	0.982
Metformin HCl	MQC	101.54%	2.6	-	-
Metformin HCl	HQC	92.37%	3.1	102.95%	1.029
Teneligliptin HBr hydrate	LQC	52.7 %	4.8	93.04%	0.930
Teneligliptin HBr hydrate	MQC	64.2 %	2.9	-	-
Teneligliptin HBr hydrate	HQC	72.9 %	3.5	101.77%	1.018
Pioglitazone HCl	LQC	58.3 %	4.2	92.34%	0.923
Pioglitazone HCl	MQC	85.3 %	3.0	-	-
Pioglitazone HCl	HQC	69.7 %	3.7	90.70%	0.907

***Note**: Matrix Factor (MF) was calculated as the ratio of analyte peak response in post-extracted plasma to that in neat standard solution, expressed in decimal form in accordance with ICH M10 guidelines.*

Stability and Sensitivity Data

The stability and sensitivity of Metformin HCl, Teneligliptin HBr hydrate, and Pioglitazone HCl were systematically evaluated across bench-top, freeze–thaw, long-term, and stock extract conditions. All compounds demonstrated excellent stability, with accuracy values ranging from 90.0% to 108.8% across both low and high concentrations, confirming the robustness of the analytical method under routine laboratory handling. The LLOQ values—10 ng/mL for Metformin, 1.25 ng/mL for Teneligliptin, and 5 ng/mL for Pioglitazone—were measured with high accuracy (102–103%), highlighting the method's sensitivity for quantifying low concentrations. Minor variations observed at lower conc. did not compromise overall reliability, indicating suitability for precise therapeutic monitoring. Overall, the results confirm that the developed method is precise, accurate, and reproducible, providing reliable

quantification of all three antidiabetic agents across a wide concentration range. Its robust performance under various stress conditions, combined with sensitive LLOQs, makes it ideal for clinical pharmacokinetic studies, therapeutic drug monitoring, and regulatory-compliant bioanalytical applications. All values complied with regulatory acceptance limits: accuracy within ±15% of nominal and precision (%CV) ≤15%. LLOQ performance for three analytes also met the criteria for accuracy of 80–120% and CV ≤20%.

Table 4. Stability data of Metformin HCl, Teneligliptin HBr hydrate, and Pioglitazone HCl (Mean ± SD, n = 6)

Compound	Condition	Nominal Conc.(ng/mL)	Measured Conc.(Mean±SD)	Accuracy (%)	Comments
Metformin HCl	Bench-top	30	32.19 ± 0.091	107.3	Stable
		1800	1687.18 ± 18.5	93.7	Stable
	Freeze–Thaw	30	32.44 ± 0.098	108.1	Stable
		1800	1665.52 ± 20.3	92.5	Stable
	Long-term	30	32.62 ± 0.102	108.7	Stable
		1800	1831.62 ± 22.1	101.8	Stable
	Stock Extract	30	31.09 ± 0.088	103.6	Stable
		1800	1776.82 ± 19.5	98.7	Stable
	LLOQ (Sensitivity)	10	10.3 ± 0.3	103	Passed
Teneligliptin HBr hydrate	Bench-top	3.75	3.90 ± 0.045	104.0	Stable
		225	239.40 ± 5.2	106.4	Stable
	Freeze–Thaw	3.75	3.83 ± 0.041	102.1	Stable
		225	238.22 ± 5.0	105.9	Stable
	Long-term	3.75	3.42 ± 0.038	91.2	Stable
		225	237.54 ± 4.9	105.6	Stable
	Stock Extract	3.75	4.08 ± 0.047	108.8	Stable
		225	236.18 ± 4.8	105.0	Stable
	LLOQ (Sensitivity)	1.25	1.28 ± 0.05	102	Passed
Pioglitazone HCl	Bench-top	15	15.37 ± 0.078	102.5	Stable
		900	843.87 ± 12.6	93.8	Stable
	Freeze–Thaw	15	15.29 ± 0.082	102.0	Stable
		900	824.62 ± 14.1	91.6	Stable
	Long-term	15	15.78 ± 0.088	105.2	Stable
		900	823.33 ± 13.9	91.5	Stable
	Stock Extract	15	14.57 ± 0.081	97.1	Stable
		900	809.99 ± 14.5	90.0	Stable
	LLOQ (Sensitivity)	5	5.1 ± 0.2	102	Passed

The chromatograms mentioned as (a), (b) & (c) in Figure 6 are consistent with the std. required for bioanalytical validation. HQC chromatograms of Metformin, Teneligliptin, and Pioglitazone exhibited sharp, symmetrical, and well-resolved peaks with no endogenous interference, confirming robust chromatographic performance, method specificity, and reliable quantification at high concentrations. Representative chromatograms at HQC level for Metformin hydrochloride, Teneligliptin hydrobromide hydrate, and Pioglitazone hydrochloride mentioned as (a), (b) & (c) in Figure 7.

Matrix effect evaluation

The matrix effect was evaluated using post-extracted spiked plasma samples, as recommended by the FDA and EMA bioanalytical method validation guidelines. Representative chromatograms of Metformin hydrochloride, Teneligliptin hydrobromide hydrate, and Pioglitazone hydrochloride exhibited sharp, symmetrical, and well-resolved peaks at their respective retention times with no significant interference from endogenous plasma components, which is clearly visible in (a), (b), and (c) in Figure 8 for Metformin hydrochloride, Teneligliptin hydrobromide hydrate, and Pioglitazone hydrochloride, respectively. The absence of ion suppression or enhancement at the analyte elution regions, along with consistent signal response and peak shape across all analytes, confirms minimal matrix influence.

These findings demonstrate the effectiveness of the sample preparation and chromatographic conditions in controlling matrix effects, ensuring accurate, precise, and reproducible quantification in compliance with regulatory acceptance criteria. Matrix effects were within acceptable regulatory limits.

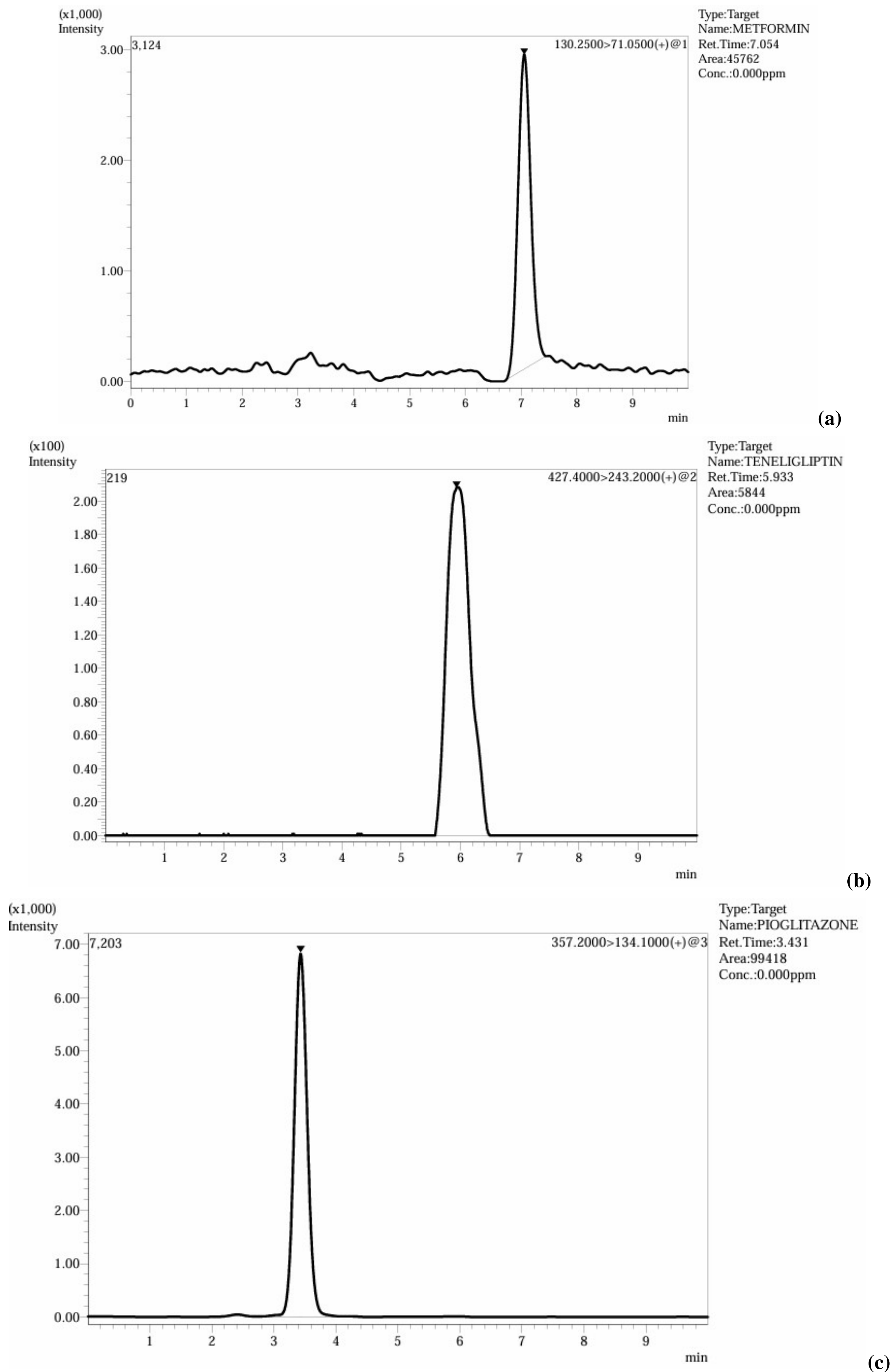
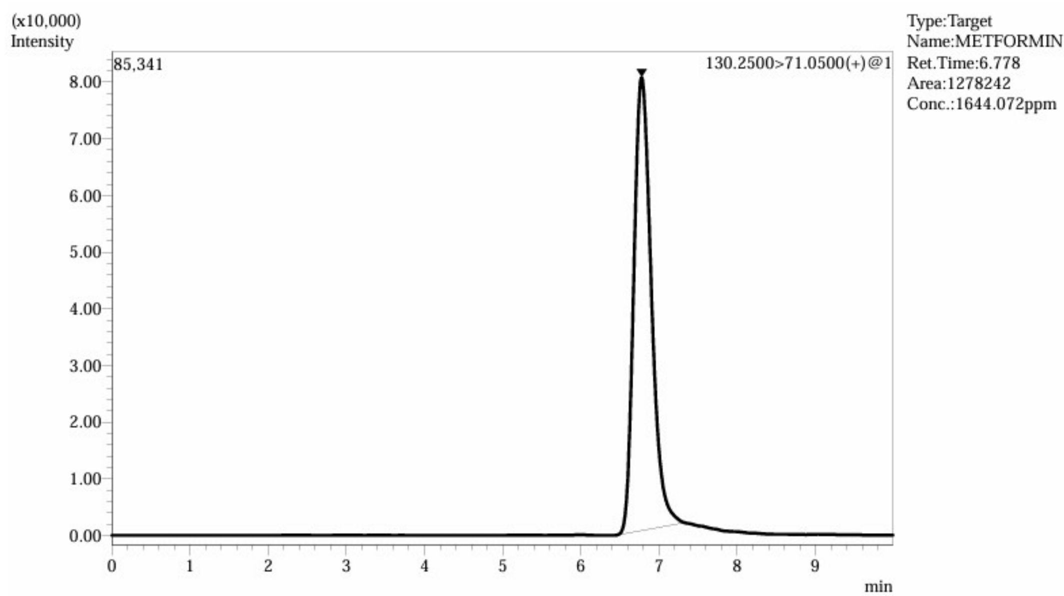
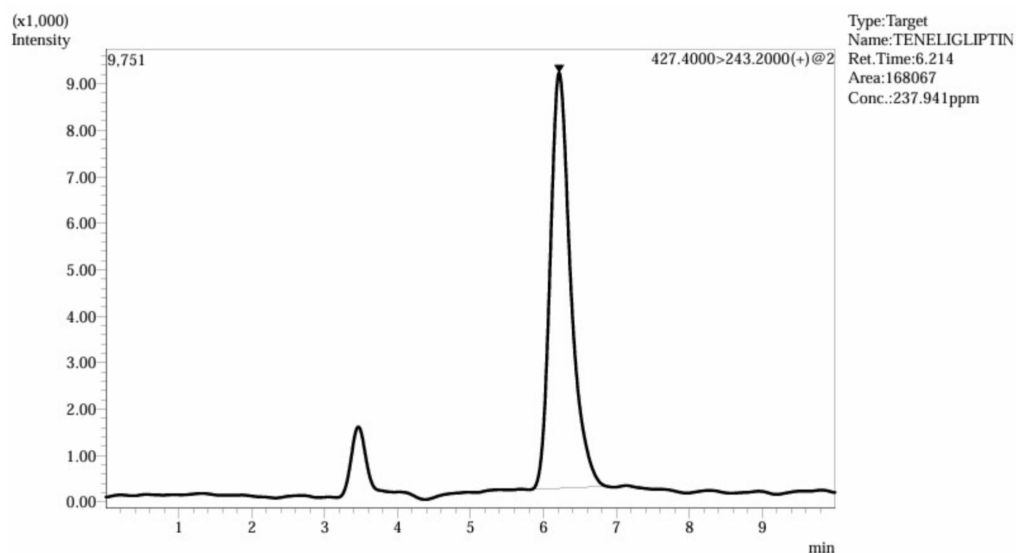


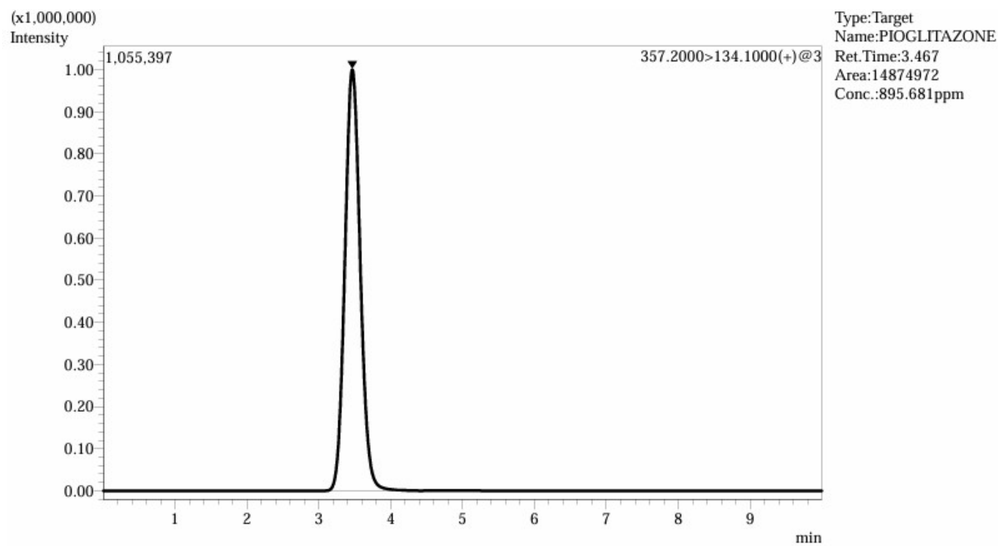
Figure 6: LLOQ chromatograms of (a) Metformin HCl (b) Teneligliptin HBr hydrate (c) Pioglitazone HCl



(a)



(b)



(c)

Figure 7: Representative chromatogram of high-quality control (HQC) samples for (a) Metformin HCl (b) Teneligliptin HBr hydrate (c) Pioglitazone HCl

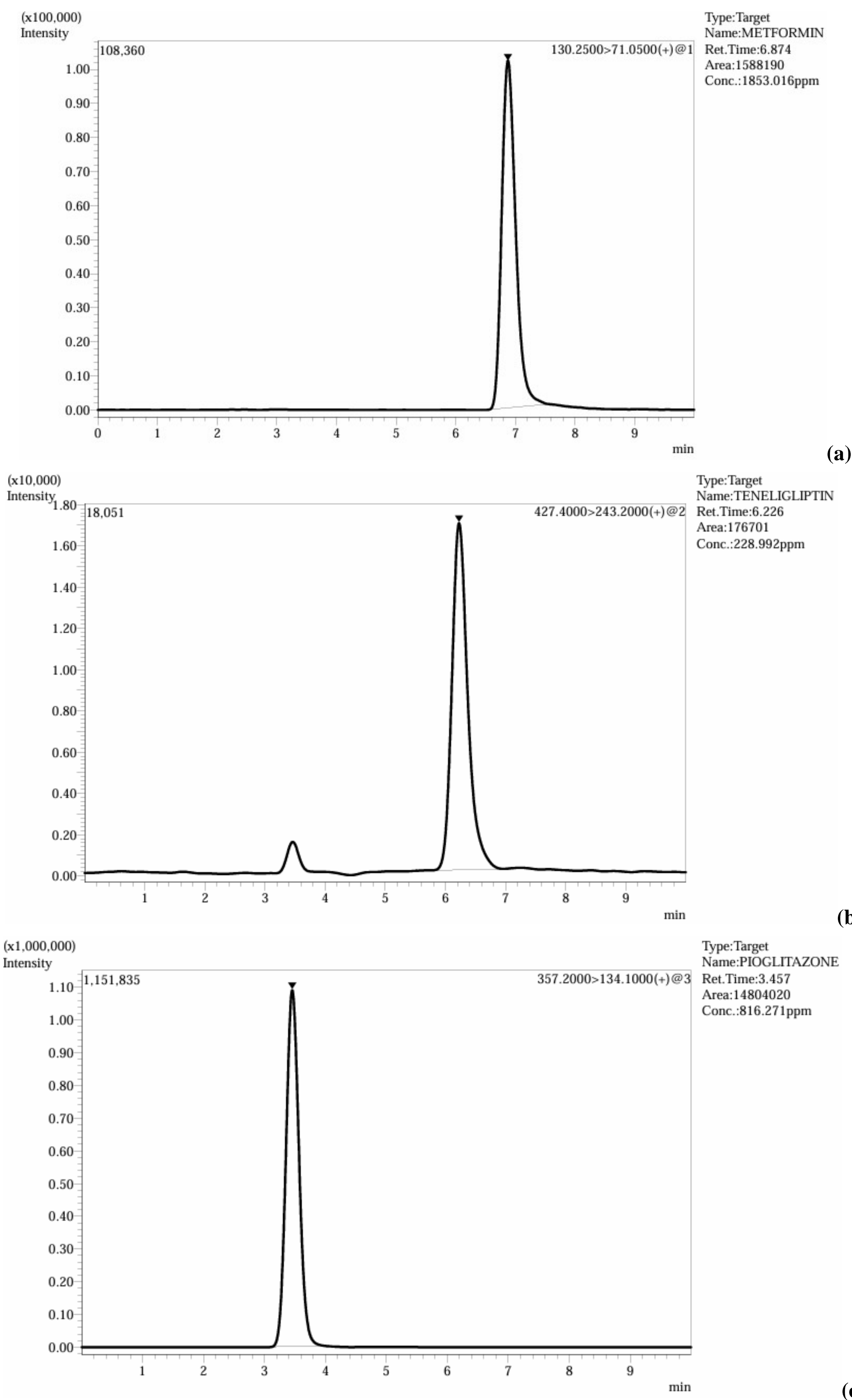


Figure 8: Representative chromatograms of post-extracted spiked plasma samples showing consistent peak shape and no significant matrix interference for (a) Metformin HCl (b) Teneligliptin HBr hydrate (c) Pioglitazone HCl

Retention Time and Specificity

Figure 9 shows representative MRM chromatograms of metformin hydrochloride, teneligliptin hydrobromide hydrate, pioglitazone hydrochloride, and their corresponding internal standards in human plasma. The absence of endogenous or cross-analyte interference at the respective retention times demonstrates the method's specificity and selectivity, in accordance with **ICH M10 Section 5.2**. The selected MRM transitions exhibited high selectivity for all analytes, with well-separated, reproducible retention times. The close elution of Metformin and its isotopically labelled internal standard (Metformin-D_6), along with the appropriate retention of Saxagliptin as ISTD, supports effective compensation for matrix effects and analytical variability, confirming method suitability for regulated bioanalysis. Figure 10 shows the absence of endogenous responses at the analyte retention times in blank plasma chromatograms, which confirms the selectivity of the developed bioanalytical method

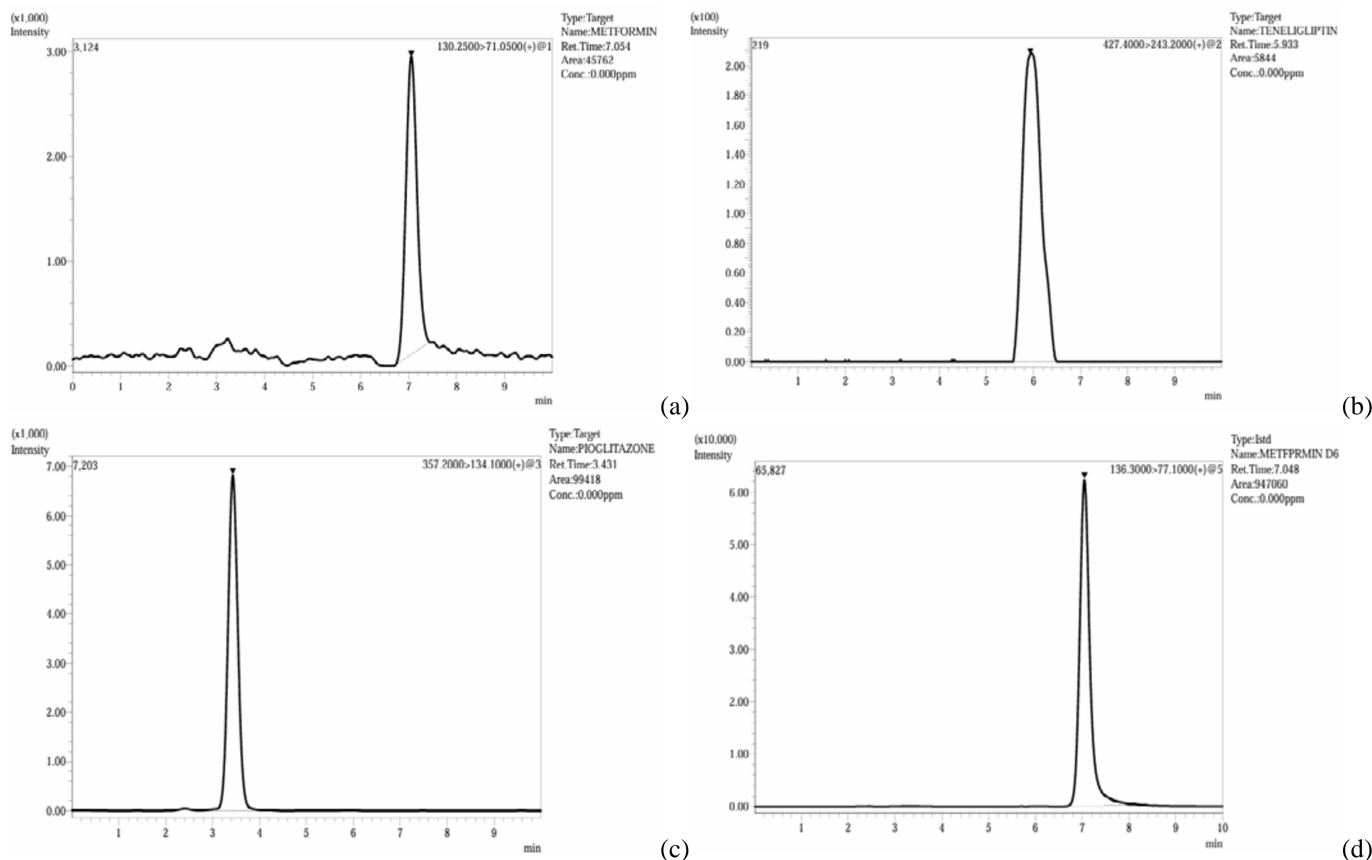
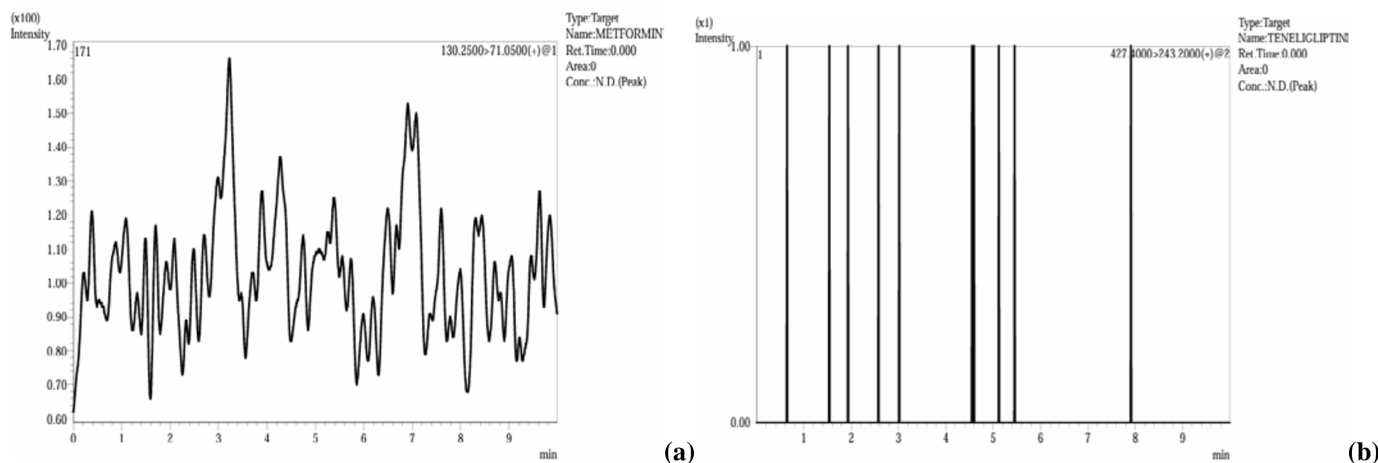


Figure 9: Combined LC-MS/MS MRM chromatograms for simultaneous quantification of (a) Metformin HCl, (b) Teneligliptin Hydrobromide hydrate, (c) Pioglitazone HCl, & their respective internal standards (d) (Saxagliptin and Metformin D6)



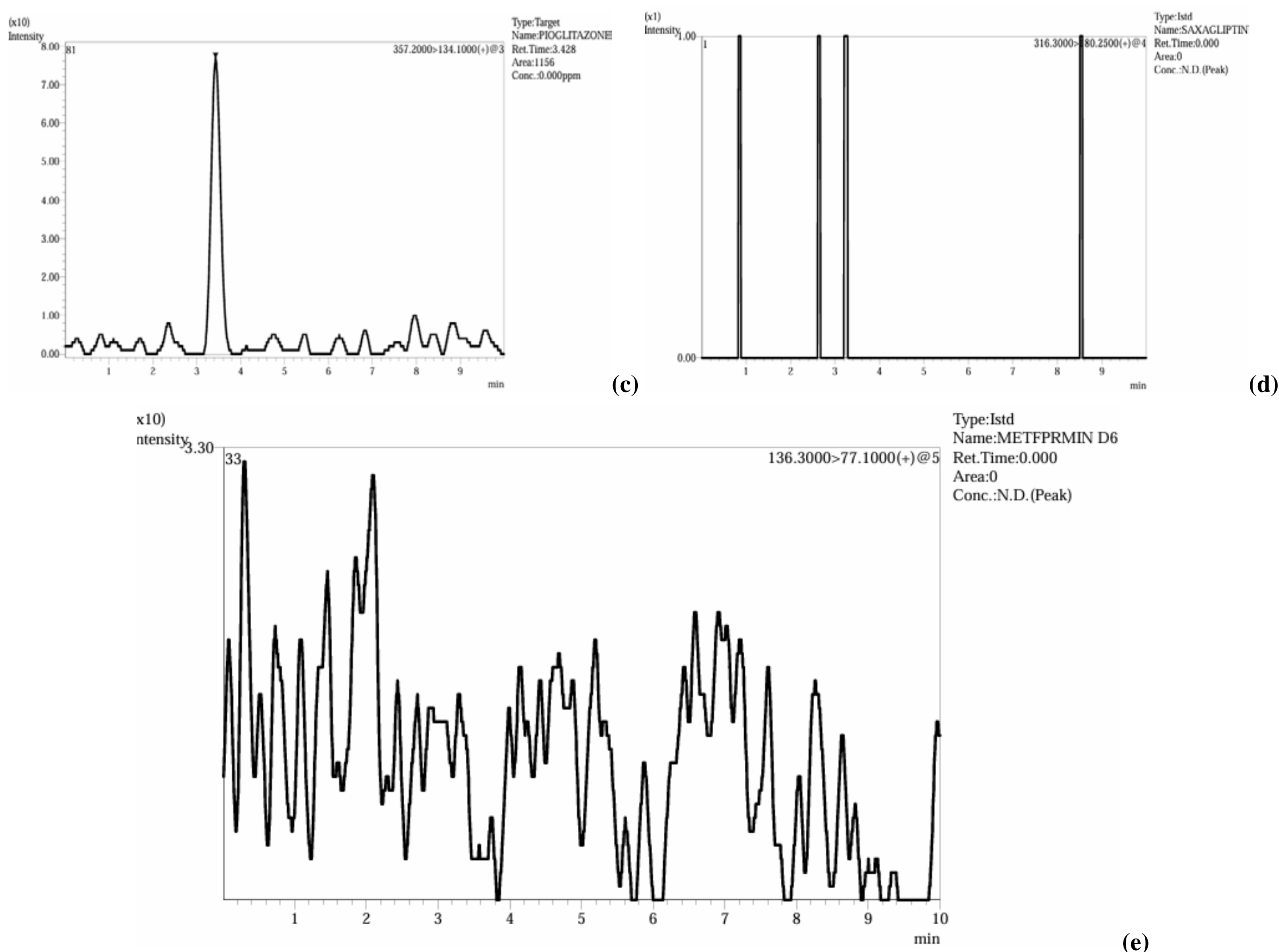


Figure 10: Representative blank plasma chromatograms showing absence of interference at analyte retention times for (a) Metformin HCl, (b) Teneligliptin HBr hydrate, (c) Pioglitazone HCl. Internal standards are also shown: (d) Saxagliptin (e) Metformin D6

Table 5: Retention time summary

Compound	Mean RT (min)	m/z Transition	Collision Energy (eV)
Metformin HCl	7.054	130.25 → 71.05	**–25**
Teneligliptin HBr	5.933	427.40 → 243.20	**–30**
Pioglitazone HCl	3.431	357.20 → 134.10	**–30**
Saxagliptin (ISTD)	4.174	316.30 → 180.25	**–25**
Metformin-D6 (ISTD)	7.048	136.30 → 77.10	**–25**

Dilution Integrity

Dilution integrity was evaluated at 1/2 and 1/10 dilution levels for Metformin Hydrochloride, Teneligliptin Hydrobromide Hydrate, and Pioglitazone Hydrochloride. For all analytes, the precision (%CV) across dilution levels ranged from 0.10% to 2.94%, meeting the acceptance criterion of ≤15%.

The mean accuracy values remained within ±15% of nominal concentrations for all diluted samples, demonstrating consistent assay performance following dilution. These results confirm that diluting samples beyond the calibration range does not affect the method's accuracy or precision, supporting its suitability for the analysis of high-concentration pharmacokinetic samples.

Carryover

Carryover was evaluated by injecting blank plasma samples immediately after ULOQ injections. No detectable analyte peaks were observed at the respective retention times, and responses were below 15% of the corresponding LLOQ, fulfilling the ICH M10 acceptance criteria.

Table 6: Data of Dilution Integrity

Analyte	Dilution Factor	Mean Concentration (ng/mL)	%CV	%Mean Accuracy
Metformin HCl	1/2 (1000 → 500)	935.0366	0.39	93.503
Metformin HCl	1/10 (1000 → 100)	204.528	0.431	102.264
Teneligliptin HBr Hydrate	1/2 (125 → 62.5)	131.784	1.386	105.427
Teneligliptin HBr Hydrate	1/10 (125 → 12.5)	24.0171	2.943	96.068
Pioglitazone HCl	1/2 (500 → 250)	503.06	0.103	100.612
Pioglitazone HCl	1/10 (500 → 50)	103.032	0.632	103.032

Table 7: Carryover Data

Compound	Injection Sequence	Response in Blank After ULOQ	% of LLOQ Response	Acceptance Limit (ICH M10)
Metformin HCl	Blank after ULOQ	No detectable peak at the analyte retention time	<10%	≤15%
Teneligliptin HBr Hydrate	Blank after ULOQ	No detectable peak at the analyte retention time	<10%	≤15%
Pioglitazone HCl	Blank after ULOQ	No detectable peak at the analyte retention time	<10%	≤15%

Comparative Discussion and Novelty

Table 8: Comparative performance of existing LC–MS/MS methods for Metformin hydrochloride, Teneligliptin hydrobromide hydrate and Pioglitazone versus the present validated method

Study	Analytes Covered	Simultaneous Triple Analysis	LLOQ (ng/mL)	Run Time (min)	Internal Standard	Regulatory Compliance	Key Advantage of Present Method
Al Bratty et al., 2017	Metformin + Gliptins	No	≥10	10–12	Non-isotopic	FDA/EMA	No pioglitazone; longer run time
Mohamed et al., 2019	Metformin + Canagliflozin	No	10	9–10	Non-isotopic	FDA	Dual-drug only
Wattamwar et al., 2020	Metformin + Canagliflozin	No	5	8–10	Non-isotopic	FDA	No gliptin or TZD
El-Zaher et al., 2019	Metformin + Saxagliptin / Dapagliflozin	No	10	10–15	Non-isotopic	FDA	Dual combination
Kusuma Kumari et al., 2021	Pioglitazone	No	5	12	Non-isotopic	FDA	Single-analyte method
Praveen et al., 2020	Pioglitazone + Alogliptin	No	~10	9–12	Non-isotopic	FDA	No metformin
Present Method	**Metformin + Teneligliptin + Pioglitazone**	**Yes**	**10 / 1.25 / 5**	**~7.0**	**Isotopic + Structural IS**	**ICH M10, FDA, EMA**	**Shorter run time with triple-drug analysis**

Comparative Discussion

As per the above comparative table 8, reported LC–MS/MS methods have quantified metformin, gliptins, or pioglitazone primarily as single analytes or in dual-drug combinations, with chromatographic run times generally ranging from 8 to 15 minutes and LLOQ values typically between 5 and 10 ng/mL, depending on the analyte and matrix [11–19]. Although these methods demonstrated acceptable analytical performance, none addressed the simultaneous determination of metformin, teneligliptin, and pioglitazone within a single assay. The present method enables concurrent quantification of all three analytes with LLOQs of 10 ng/mL for metformin, 1.25 ng/mL for teneligliptin, and 5 ng/mL for pioglitazone, achieving sensitivity comparable to or exceeding previously reported methods while reducing total run time to approximately 7 minutes. Calibration curves showed consistent linearity across the validated concentration ranges, meeting regulatory acceptance criteria. Accuracy and precision remained within ±15% at all quality

control levels, including LLOQ, consistent with or comparable to the performance reported for individual or dual-analyte methods [11–19]. Stability of all analytes was confirmed under bench-top, autosampler, freeze–thaw, and long-term storage conditions, demonstrating assay reliability throughout routine bioanalytical workflows. While stability data have been reported for individual compounds in earlier studies [11–19], the present work confirms stability under simultaneous multi-analyte conditions. Overall, the method provides a regulatory-compliant analytical option with acceptable sensitivity, robust quantitative performance, and improved analytical efficiency.

Novelty of the Study

This study reports a fully validated LC–MS/MS method for the simultaneous determination of metformin HCl, teneligliptin HBr hydrate, and pioglitazone HCl in human plasma. Previously published methods have focused on these agents individually or in limited combinations, without addressing this specific triple-drug regimen [11–19]. The developed method demonstrates robust performance, achieving low LLOQs, excellent linearity, and satisfactory accuracy & precision, all within a short chromatographic runtime. The inclusion of both an isotopically labelled internal standard and a structural internal std. further strengthens analytical reliability by reducing matrix-related variability. Validation performed in accordance with current ICH M10, USFDA & EMA guidelines confirms its suitability for pharmacokinetic, bioequivalence, and clinical studies involving combination antidiabetic therapy**.**

Furthermore, published pharmacokinetic data indicate that therapeutic plasma concentrations of these three agents are significantly higher than the validated LLOQs. Metformin reaches a peak plasma concentration (C_max) of approximately 1248 ng/mL after a 1000 mg dose, with levels consistently above 10 ng/mL throughout the dosing interval [20]. Teneligliptin attains C_max values of 187-382 ng/mL following 20–40 mg administration, with trough concentrations exceeding 20 ng/mL [21]. Pioglitazone shows a C_max of approximately 1117 ng/mL after a 30 mg dose, with plasma levels of 300–400 ng/mL at later time points [22]. These findings clearly demonstrate that the present LC–MS/MS method is sufficiently sensitive to accurately quantify clinically relevant plasma concentrations, underscoring its applicability for pharmacokinetic, bioequivalence, and therapeutic drug monitoring studies. In addition to its analytical performance, the method is relatively environmentally friendly. The short 7-minute chromatographic runtime reduces overall solvent consumption, while the selection of minimal, readily available organic solvents limits chemical waste. These features align with current trends in green analytical chemistry, highlighting that the method is not only sensitive and robust but also sustainable and resource-efficient.

Table 8: Summary of all the parameters

Validation Parameter	Regulatory Acceptance Criteria	Observed Results (Present Study)	Compliance
Selectivity	No significant interference at analyte or IS retention times	No endogenous interference observed at retention times of metformin, teneligliptin, pioglitazone, Metformin-D_6, or Saxagliptin across multiple plasma lots	Compliant
Linearity	Back-calculated concentrations within ±15% (±20% at LLOQ); $r^2 \geq 0.99$	Linear response over 10–2000 ng/mL (metformin), 1.25–250 ng/mL (teneligliptin), and 5–1000 ng/mL (pioglitazone) with $r^2 \geq 0.999$	Compliant
Accuracy & Precision	Accuracy 85–115% (80–120% at LLOQ); Precision ≤15% CV (≤20% at LLOQ)	Intra- and inter-day accuracy ranged from 91.3–106.9%; precision ≤5.58% CV across LQC, MQC, and HQC levels	Compliant
Recovery	Consistent and reproducible recovery	Metformin showed high recovery (92.37–111.23%); teneligliptin and pioglitazone showed lower but reproducible recovery across QC levels	Compliant
Matrix Effect	IS-normalized matrix factor variability ≤15%	Minimal ion suppression or enhancement observed; matrix factor ranged from 90.70–102.95%	Compliant
Sensitivity (LLOQ)	Accuracy 80–120%; Precision ≤20% CV; S/N ≥10	LLOQ achieved at 10 ng/mL (metformin), 1.25 ng/mL (teneligliptin), and 5 ng/mL (pioglitazone) with acceptable accuracy and precision	Compliant
Stability	Mean concentration within ±15% of nominal	All analytes are stable under bench-top, freeze–thaw, long-term, autosampler, and stock-solution conditions	Compliant
Dilution Integrity	Accuracy and precision within ±15%	Samples diluted 1/2 and 1/10 met acceptance criteria with %CV ≤2.94%	Compliant

CONCLUSION

This work presents the successful development and validation of a sensitive and selective LC–MS/MS bioanalytical method for the simultaneous determination of metformin HCl, teneligliptin HBr hydrate, and pioglitazone HCl in human plasma. The method consistently met all critical validation criteria, including accuracy, precision, linearity, recovery, matrix effect control, and stability, in full accordance with ICH M10, USFDA, and EMA regulatory guidelines. The strategic use of protein precipitation combined with liquid–liquid extraction enabled effective sample cleanup and reliable analyte recovery, despite the contrasting physicochemical characteristics of the three compounds, thereby supporting reproducible, high-throughput analysis. Although the validation was limited to human plasma and did not encompass inter-laboratory testing or real-world clinical samples, the method provides a solid analytical framework for future extension to other biological matrices and special patient populations. In the context of contemporary diabetes management, where combination therapies are increasingly adopted to enhance glycemic control and individualize treatment, the availability of a single, validated assay for this commonly prescribed triple-drug regimen is highly relevant. By addressing the shortcomings of previously reported single- or dual-analyte methods, this approach provides a reliable tool for pharmacokinetic evaluation, bioequivalence studies, therapeutic drug monitoring, and translational research, ultimately contributing to more informed, evidence-based antidiabetic therapy. The method provides a practical and regulatory-compliant analytical tool for the pharmacokinetic evaluation, therapeutic monitoring, and bioequivalence assessment of combination antidiabetic therapy. Its robust validation in accordance with international regulatory frameworks ensures reliable, accurate, and reproducible measurement of drug exposure, facilitating decision-making in clinical development and translational research settings.

FINANCIAL ASSISTANCE

NIL

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTION

Sejal Pandya was responsible for the conception and design of the study, execution of all experimental work, comprehensive data analysis and interpretation, and preparation of the manuscript. Hitesh Vekaria provided academic oversight and strategic direction for the research and critically reviewed the manuscript for scientific rigor and intellectual content. Both authors reviewed and approved the final manuscript for submission.

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