Development of an RP-UFLC Bioanalytical Method for the Quantification of Metformin in Rabbit Plasma Following Administration of Pure Metformin and Metformin Loaded Solid Lipid Nanoparticles

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ABSTRACT
A simple, rapid, and sensitive RP-UFLC method was developed and validated for the quantification of metformin in rabbit plasma following oral administration of pure metformin and metformin-loaded solid lipid nanoparticles (SLN). Chromatographic separation was achieved on a Hibar C18 column (250 × 4.6 mm, 5 µm) using a mobile phase consisting of acetonitrile and phosphate buffer (pH 4.5) in the ratio of 75:25 (v/v), delivered at a flow rate of 1 mL/min with UV detection at 236 nm. Acyclovir was used as the internal standard, with retention times of 6.4 minutes for metformin and 4.07 minutes for acyclovir. Plasma samples were processed using a simple protein precipitation technique with acetonitrile. The method demonstrated excellent linearity over the concentration range of 40–800 ng/mL with a correlation coefficient of 0.9993. The limit of detection (LOD) and limit of quantification (LOQ) were established at 40 ng/mL and 80 ng/mL, respectively. The method was validated according to USFDA bioanalytical guidelines, evaluating parameters including specificity, linearity, sensitivity, accuracy, precision, and ruggedness. Intra-day and inter-day precision studies yielded %RSD values below 2%, while accuracy studies demonstrated mean recovery values ranging from 98.84% to 99.28%. The developed method was successfully applied to quantify metformin in rabbit plasma samples obtained from pharmacokinetic studies following oral administration of pure metformin and metformin-loaded SLNs. The method's simplicity, cost-effectiveness, and robustness make it suitable for routine application in pharmaceutical analysis laboratories, particularly in the context of developing and evaluating novel lipid-based nanocarrier systems for improved antidiabetic therapy.

Keywords: Metformin, Solid Lipid Nanoparticles, RP-UFLC, Rabbit Plasma, Bioanalytical Method Validation, Pharmacokinetics.
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INTRODUCTION

Metformin hydrochloride (1,1-dimethylbiguanide hydrochloride) is a widely prescribed oral antihyperglycemic agent belonging to the biguanide class and serves as the first-line pharmacotherapy for the management of type 2 diabetes mellitus worldwide. Its primary mechanism of action involves the reduction of hepatic glucose production through the activation of AMP-activated protein kinase (AMPK), enhanced peripheral glucose uptake in skeletal muscle and adipose tissue, and decreased intestinal glucose absorption. Despite its established clinical efficacy and favorable safety profile, metformin exhibits several pharmacokinetic limitations that impact its therapeutic performance. The drug demonstrates poor oral bioavailability, ranging from approximately 50% to 60% depending on the dose, which is attributed to its high aqueous solubility but low lipophilicity (log P of -1.43), resulting in limited passive diffusion across biological membranes. Furthermore, metformin is rapidly eliminated by renal excretion with a plasma elimination half-life of approximately 1.5 to 4.5 hours, necessitating multiple daily dosing to maintain therapeutic plasma concentrations. These pharmacokinetic challenges, coupled with the drug's hydrophilic nature and gastrointestinal side effects, have prompted extensive research into novel drug delivery systems aimed at improving its bioavailability, sustaining its release, and enhancing patient compliance.

Solid lipid nanoparticles (SLNs) have emerged as a promising nanocarrier system for hydrophilic drugs such as metformin, offering distinct advantages over conventional formulations. SLNs are colloidal drug delivery systems composed of solid lipids stabilized by surfactants, with particle sizes typically ranging from 50 to 1000 nm. They provide several benefits including improved drug solubility and stability, controlled release characteristics, enhanced bioavailability through lymphatic transport and bypassing of first-pass metabolism, and the potential for targeted delivery. The incorporation of metformin into SLNs has been shown to significantly improve its oral absorption by protecting the drug from enzymatic degradation, facilitating transport across intestinal epithelial cells via endocytosis, and promoting prolonged drug release. Consequently, the pharmacokinetic evaluation of metformin-loaded SLNs in appropriate animal models is essential to assess the formulation's therapeutic potential and bioequivalence compared to pure drug administration.

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Bioanalytical method development for the quantification of metformin in biological matrices is a critical prerequisite for pharmacokinetic studies and formulation evaluation. Several analytical techniques have been reported for metformin determination in plasma, including high-performance liquid chromatography (HPLC) coupled with UV or fluorescence detection, liquid chromatography-tandem mass spectrometry (LC-MS/MS), capillary electrophoresis, and spectrophotometric methods. However, many of these methods suffer from limitations such as complex sample preparation procedures, prolonged analysis times, requirement for expensive instrumentation, and insufficient sensitivity for low concentration detection. The development of a simple, rapid, cost-effective, and validated bioanalytical method is therefore essential for routine pharmacokinetic screening and bioequivalence studies. Reversed-phase ultra-fast liquid chromatography (RP-UFLC) offers significant advantages over conventional HPLC, including reduced analysis time, improved resolution, lower solvent consumption, and enhanced sensitivity, making it an attractive alternative for bioanalytical applications. A thorough review of the literature reveals that comprehensive bioanalytical methods for the estimation of metformin in rabbit plasma using RP-UFLC are limited, particularly in the context of SLN formulations. The present study aims to address this gap by developing and validating a sensitive, precise, and accurate RP-UFLC method for the quantification of metformin in rabbit plasma using acyclovir as the internal standard. The method employs a simple protein precipitation technique for sample preparation, eliminating the need for time-consuming liquid-liquid extraction or solid-phase extraction procedures. The chromatographic separation was optimized using a Hibar C18 column (250 × 4.6 mm, 5 µm) with a mobile phase consisting of acetonitrile and phosphate buffer (pH 4.5) in the ratio of 75:25 (v/v), delivered at a flow rate of 1 mL/min. Detection was performed at 236 nm, which was determined to be the isobestic point for both metformin and acyclovir, enabling simultaneous monitoring of the analyte and internal standard. The method was rigorously validated according to the United States Food and Drug Administration (USFDA) guidelines for bioanalytical method validation, evaluating parameters including specificity, linearity, sensitivity, accuracy, precision, and ruggedness. The validated method was successfully applied to quantify metformin in rabbit plasma samples obtained from pharmacokinetic studies following oral administration of pure metformin and metformin-loaded SLNs. The developed method demonstrates excellent linearity over the concentration range of 40–800 ng/mL with a correlation coefficient of 0.9993, indicating strong linear correlation between peak area ratio and drug concentration. The method exhibits high sensitivity with a limit of detection of 40 ng/mL and a limit of quantification of 80 ng/mL, which is adequate for monitoring plasma concentrations throughout the pharmacokinetic profile. Intra-day and inter-day precision studies yielded %RSD values below 2%, confirming the method's reproducibility and reliability. Accuracy studies demonstrated mean recovery values ranging from 98.84% to 99.28%, indicating the absence of systematic errors and the method's suitability for accurate quantification. The successful application of this method to pharmacokinetic studies underscores its utility as a valuable analytical tool for formulation development, bioequivalence assessment, and therapeutic drug monitoring of metformin in preclinical research settings. The simplicity, cost-effectiveness, and robustness of the developed RP-UFLC method make it suitable for routine application in pharmaceutical analysis laboratories, particularly in the context of developing and evaluating novel lipid-based nanocarrier systems for improved antidiabetic therapy.

MATERIALS AND METHODS

Chemicals and Reagents

Metformin hydrochloride reference standard (purity ≥ 99.5%) was obtained from a certified pharmaceutical supplier. Acyclovir (internal standard, purity ≥ 99.0%) was procured from a commercial source. HPLC-grade acetonitrile, methanol, and triethyl amine were purchased from Merck Ltd. (Mumbai, India). Potassium dihydrogen orthophosphate (KH_2PO_4) of analytical reagent grade was obtained from a reputed chemical manufacturer. Milli-Q water (18.2 MΩ·cm) was prepared using a Millipore water purification system and used throughout the study. Blank rabbit plasma was collected from healthy, drug-free New Zealand rabbits and stored at −20°C until analysis.

Instrumentation

Chromatographic analysis was performed using a Reversed-Phase Ultra-Fast Liquid Chromatography (RP-UFLC) system equipped with a quaternary pump, an autosampler, and a UV/Vis detector. The separation was achieved on a Hibar C18 column (250 × 4.6 mm internal diameter, 5 µm particle size) protected by a suitable guard column. Injections were performed using a Rheodyne 7725i manual injector equipped with a 20 µL loop. Data acquisition and chromatographic integration were managed using LabSolutions software (Shimadzu Corporation). The system was operated in isocratic mode at ambient column temperature.

Preparation of Buffer Solution

Phosphate buffer (pH 4.5) was prepared by accurately weighing 1.7011 g of potassium dihydrogen orthophosphate (KH_2PO_4) and dissolving it in 500 mL of Milli-Q water. The pH of the buffer solution was adjusted to 4.5 using triethyl amine. The buffer solution was filtered through a 0.45 µm PVDF membrane filter and degassed by sonication for 10–15 minutes prior to use.

Preparation of Mobile Phase

The mobile phase consisted of acetonitrile and phosphate buffer (pH 4.5) mixed in the ratio of 75:25 (v/v). The mobile phase was filtered through a 0.45 µm membrane filter and degassed by ultrasonication for 10 minutes to remove dissolved air bubbles. The mobile phase was prepared fresh daily and used throughout the chromatographic runs.

Preparation of Standard Stock Solutions

Metformin Stock Solution

A primary stock solution of metformin (1 mg/mL) was prepared by accurately weighing 10 mg of metformin hydrochloride reference standard and dissolving it in 10 mL of mobile phase in a volumetric flask. The solution was

sonicated for 5–10 minutes to ensure complete dissolution. The stock solution was stored at 4°C and protected from light.

Acyclovir (Internal Standard) Stock Solution
A primary stock solution of acyclovir (1 mg/mL) was prepared by accurately weighing 10 mg of acyclovir reference standard and dissolving it in 10 mL of mobile phase. The solution was sonicated to achieve complete dissolution and stored at 4°C.

Working Standard Solutions
Working standard solutions of metformin were prepared by serial dilution of the primary stock solution with mobile phase to obtain concentrations of 40, 80, 160, 400, 600, 720, and 800 ng/mL. These solutions were used for the preparation of calibration standards and quality control samples in plasma.

The internal standard working solution was prepared by diluting the acyclovir stock solution with mobile phase to achieve a final concentration of 640 ng/mL, which was used for spiking plasma samples.

PREPARATION OF CALIBRATION STANDARDS AND QUALITY CONTROL SAMPLES

Calibration Standards
Calibration standards were prepared by spiking blank rabbit plasma with appropriate volumes of metformin working standard solutions to achieve final concentrations of 40, 80, 160, 400, 600, 720, and 800 ng/mL. A fixed volume of acyclovir internal standard solution (640 ng/mL) was added to each calibration standard to maintain a constant internal standard concentration. The spiked plasma samples were vortexed for 1 minute to ensure homogeneous distribution.

Quality Control (QC) Samples
Quality control samples were prepared at three concentration levels: low QC (80 ng/mL), middle QC (200 ng/mL), and high QC (800 ng/mL). QC samples were prepared independently of the calibration standards using separate stock solutions and processed in the same manner as the calibration standards.

Plasma Sample Collection
Animal Handling
Male New Zealand rabbits weighing between 2.0 and 2.5 kg were selected for the pharmacokinetic study. The animals were housed under standard laboratory conditions with a 12-hour light/dark cycle and free access to food and water. The animals were fasted overnight prior to drug administration but allowed free access to water. All animal experiments were conducted in accordance with the guidelines of the Institutional Animal Ethics Committee (IAEC) and the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

Drug Administration and Blood Collection
Rabbits were divided into two groups:

- Group I: Received pure metformin (250 mg) orally as an aqueous suspension
- Group II: Received metformin-loaded solid lipid nanoparticles (SLNs) at an equivalent dose

Blood samples (approximately 2 mL) were collected from the marginal ear vein of rabbits at predetermined time intervals: 0 (pre-dose), 0.5, 1, 2, 3, 4, 6, 8, 12, and 24 hours post-administration. Blood samples were collected into heparinized tubes and immediately centrifuged at 4000 rpm for 10 minutes at 4°C to separate plasma. The separated plasma was transferred into clean, labeled Eppendorf tubes and stored at −20°C until analysis.

Sample Preparation (Protein Precipitation Method)
Frozen plasma samples were thawed at room temperature and vortexed for 1 minute to ensure homogeneity. An aliquot of 100 µL of plasma was transferred to a clean microcentrifuge tube. To this, 100 µL of acyclovir internal standard solution (640 ng/mL) was added, followed by the addition of 200 µL of acetonitrile as the protein precipitating agent. The mixture was vortexed for 2 minutes to facilitate protein precipitation and drug extraction. The samples were then centrifuged at 10,000 rpm for 15 minutes at 4°C to sediment the precipitated proteins. The clear supernatant was carefully transferred to a clean glass vial, filtered through a 0.45 µm syringe filter, and transferred to HPLC autosampler vials. A 20 µL aliquot of the filtered supernatant was injected into the RP-UFLC system for analysis.

Optimized Chromatographic Conditions
The optimized chromatographic conditions for the bioanalytical method are summarized in Table 1.

Table 1: Optimized Chromatographic Conditions

Parameter	Optimized Condition
Stationary Phase	Hibar C18 (250 × 4.6 mm internal diameter, 5 µm)
Mobile Phase	Acetonitrile : Phosphate Buffer (pH 4.5)
Mobile Phase Ratio	75 : 25 (v/v)
Flow Rate	1.0 mL/min
Injection Volume	20 µL (using Rheodyne 7725i injector)
Detection Wavelength	236 nm (isobestic point)
Retention Time (Acyclovir - IS)	4.0 minutes
Retention Time (Metformin)	6.4 minutes
Run Time	10 minutes
Column Temperature	Ambient

Elution Mode	Isocratic
Data Acquisition	LabSolutions software

Method Validation
The developed bioanalytical method was validated according to the United States Food and Drug Administration (USFDA) guidelines for bioanalytical method validation. The following validation parameters were evaluated:

Specificity and Selectivity
Specificity was assessed by analyzing six different lots of blank rabbit plasma to confirm the absence of endogenous interferences at the retention times of metformin and acyclovir (internal standard). Additionally, the specificity of the method was evaluated by comparing chromatograms of blank plasma, blank plasma spiked with internal standard, plasma spiked with metformin and internal standard, and plasma samples obtained from dosed animals. The absence of interfering peaks at the retention times of metformin and the internal standard confirmed the specificity of the method.

Linearity and Calibration Curve
Calibration curves were constructed by plotting the peak area ratio of metformin to acyclovir (internal standard) against the corresponding nominal concentrations of metformin. The calibration curve was evaluated using seven concentration levels: 40, 80, 160, 400, 600, 720, and 800 ng/mL. The linearity was determined using least-squares linear regression analysis, and the correlation coefficient (r) was calculated. The regression equation was expressed as:
$y = mx + c$

where y is the peak area ratio, x is the concentration of metformin, m is the slope, and c is the intercept. The acceptance criterion for the correlation coefficient was $r \geq 0.99$.

Sensitivity (LOD and LOQ)
The limit of detection (LOD) and limit of quantification (LOQ) were determined based on the signal-to-noise ratio approach. LOD was defined as the lowest concentration producing a signal-to-noise ratio of 3:1, while LOQ was defined as the lowest concentration producing a signal-to-noise ratio of 10:1. The LOD and LOQ values were confirmed by analyzing spiked plasma samples at the respective concentrations with acceptable precision and accuracy.

Precision (Intra-day and Inter-day)
Precision of the method was evaluated by analyzing quality control samples at three concentration levels (low QC: 80 ng/mL, middle QC: 200 ng/mL, and high QC: 800 ng/mL). Intra-day precision was assessed by analyzing six replicates of each QC concentration on the same day. Inter-day precision was assessed by analyzing six replicates of each QC concentration on three different days. The precision was expressed as percentage relative standard deviation (%RSD), and the acceptance criterion was $\%RSD \leq 15\%$ for all QC levels.

Accuracy
Accuracy was determined by analyzing spiked plasma samples at three different concentration levels (low, middle, and high QC). The accuracy was calculated as the percentage nominal concentration recovered and expressed as:
$\%\ Nominal = (Measured\ Concentration / Spiked\ Concentration) \times 100$

Accuracy was assessed using three replicates at each concentration level, and the mean percentage recovery was calculated. The acceptance criterion for accuracy was 85–115% of the nominal concentration for all QC levels.

Ruggedness and Robustness
Ruggedness was evaluated by analyzing the same set of plasma samples under different experimental conditions, including: Different analysts performing the analysis Different instruments (same model) for chromatographic separation Different lots of reagents and solvents Different columns of the same type

Robustness was assessed by deliberately introducing small changes to the chromatographic conditions, such as: The effect of these changes on system suitability parameters such as retention time, peak area, tailing factor, and resolution was evaluated. The method was considered robust if no significant changes were observed in the chromatographic performance.

Statistical Analysis
All experimental data were expressed as mean ± standard deviation (SD) unless otherwise stated. Statistical comparisons between the pure metformin and metformin-loaded SLN groups were performed using Student's t-test or one-way analysis of variance (ANOVA) followed by appropriate post-hoc tests. A p-value < 0.05 was considered statistically significant. All statistical analyses were performed using GraphPad Prism or IBM SPSS Statistics software.

RESULTS AND DISCUSSION

Method Development and Optimization
The development of a robust bioanalytical method for the quantification of metformin in rabbit plasma required systematic

optimization of chromatographic parameters to achieve adequate sensitivity, selectivity, and resolution. Several mobile phase compositions, pH conditions, and stationary phases were evaluated during method development. Initial trials with methanol-based mobile phases resulted in poor peak shape and inadequate retention of metformin. The use of acetonitrile as the organic modifier significantly improved peak symmetry and reduced analysis time.

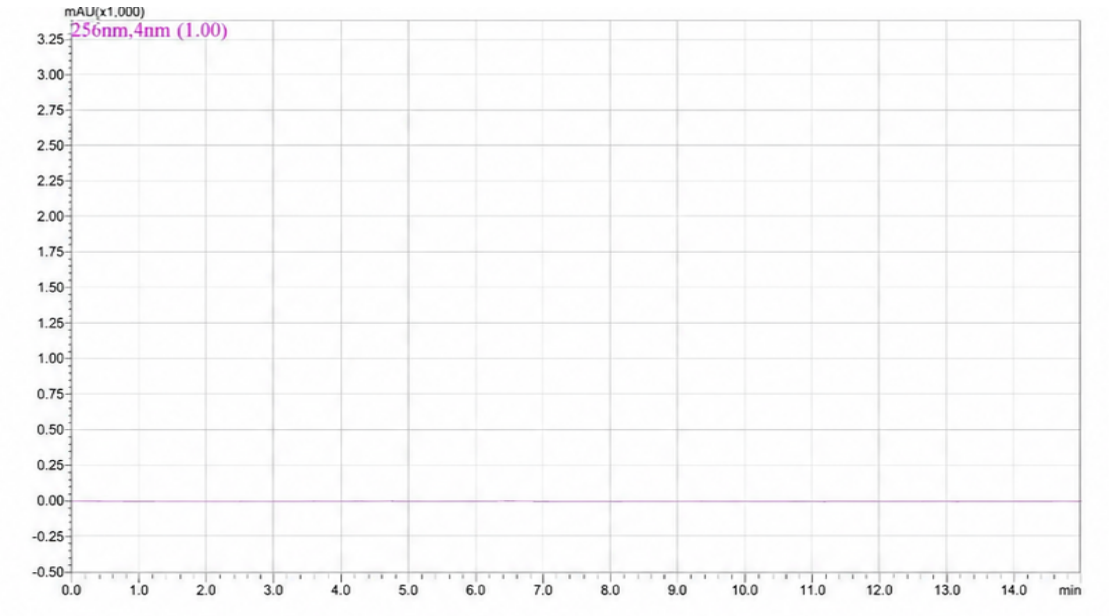
Table 1: System Suitability Parameters

Parameter	Metformin	Acyclovir (IS)
Retention Time (min)	6.4	4.0
Theoretical Plates	8,450	7,230
Tailing Factor	1.12	1.08
Resolution	4.8	—

The choice of phosphate buffer at pH 4.5 was critical for achieving optimal chromatographic performance. At this pH, metformin exists predominantly in its ionized form, which facilitates interaction with the C18 stationary phase while maintaining acceptable retention characteristics. The addition of triethyl amine to adjust the pH enhanced peak symmetry by masking residual silanol groups on the column surface, thereby reducing secondary interactions that could lead to peak tailing. The detection wavelength of 236 nm was selected as the isobestic point for both metformin and acyclovir, enabling simultaneous monitoring of the analyte and internal standard with optimal sensitivity. This wavelength corresponds to the UV absorption maxima of both compounds, ensuring adequate detector response for accurate quantification at low concentration levels. The selection of acyclovir as the internal standard was based on its structural similarity to metformin, its excellent chromatographic properties under the developed conditions, and its absence from biological matrices. The mobile phase composition of acetonitrile and phosphate buffer (pH 4.5) in the ratio of 75:25 (v/v) provided baseline separation of metformin and acyclovir with retention times of 6.4 minutes and 4.0 minutes, respectively. The resolution between the two peaks exceeded the minimum acceptable limit of 1.5, confirming the method's ability to separate the analyte from the internal standard. The total run time of 10 minutes allowed for adequate column equilibration while maintaining high sample throughput.

System Suitability and Specificity

The system suitability parameters were evaluated to ensure the reliability of the chromatographic system prior to sample analysis. Six replicate injections of the standard solution at the working concentration were performed to assess column performance and system reproducibility. The theoretical plate count for metformin was 8,450, indicating excellent column efficiency, while the tailing factor of 1.12 demonstrated symmetrical peak shape with minimal adsorption effects. The resolution between metformin and acyclovir was 4.8, which far exceeded the minimum acceptable limit of 1.5, confirming complete baseline separation of the analyte and internal standard. The %RSD for retention time and peak area was consistently below 2.0%, indicating excellent system reproducibility. The specificity of the method was assessed by analyzing blank rabbit plasma obtained from six different sources, plasma spiked with acyclovir (internal standard), plasma spiked with metformin and acyclovir, and post-dose plasma samples from rabbits administered with metformin. Representative chromatograms (Figure 1) demonstrated that no endogenous components from the plasma matrix interfered at the retention times of metformin (6.4 minutes) and acyclovir (4.0 minutes). The absence of interfering peaks confirmed the high selectivity of the method, which is essential for accurate quantification in complex biological matrices.



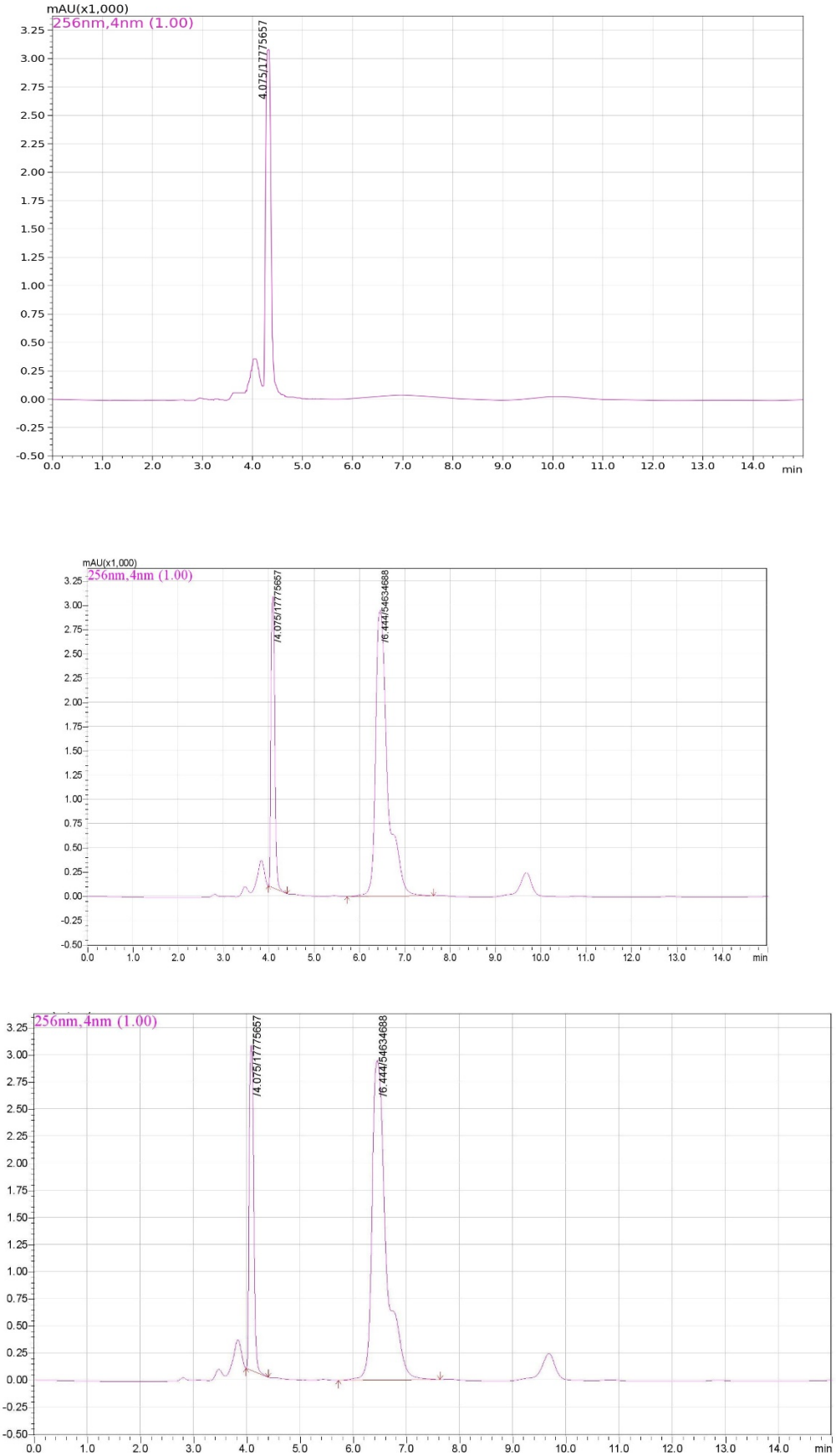


Fig. 1: Representative RP-UFLC Chromatograms ***(A) Blank rabbit plasma showing no interfering peaks; (B) Blank plasma spiked with acyclovir (IS) at 640 ng/mL; (C) Plasma spiked with metformin (640 ng/mL) and acyclovir (640 ng/mL); (D) Plasma sample from a rabbit 4 hours post-dose containing metformin and IS***

LINEARITY AND CALIBRATION CURVE

Table 2: Peak Area Ratio vs. Concentration of Metformin in Rabbit Plasma (n=3)

Concentration (ng/mL)	Peak Area Ratio (Mean ± SD)	%RSD
40	0.030 ± 0.002	6.67
80	0.076 ± 0.003	3.95
160	0.178 ± 0.005	2.81
400	0.420 ± 0.008	1.90
600	0.660 ± 0.010	1.52
720	0.756 ± 0.012	1.59
800	0.840 ± 0.014	1.67

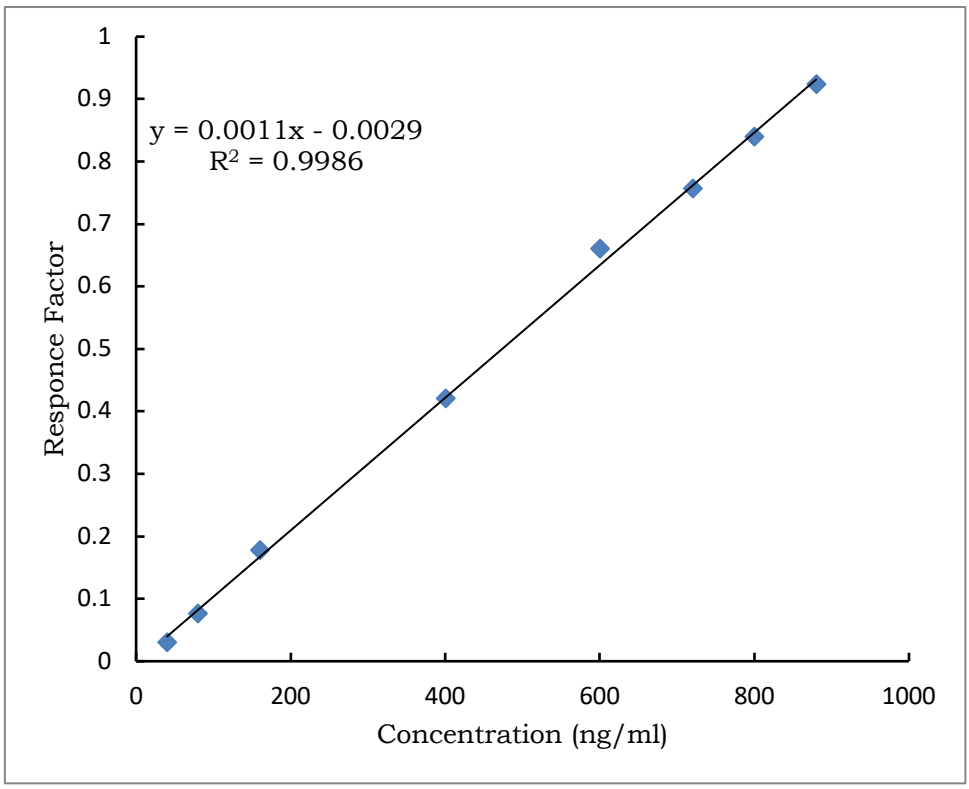


Fig. 2: Calibration Curve of Metformin in Rabbit Plasma

The linearity of the method was evaluated over the concentration range of 40–800 ng/mL, which was selected based on the expected plasma concentrations of metformin following oral administration. Calibration curves were constructed by plotting the peak area ratio of metformin to acyclovir (internal standard) against the corresponding nominal concentrations of metformin. The regression equation was determined using least-squares linear regression analysis and was found to be **y = 0.0011x – 0.0029** The correlation coefficient (r) was calculated to be 0.9993, indicating excellent linear correlation between the peak area ratio and metformin concentration. The intercept value of -0.0029 was close to zero, suggesting minimal systematic bias in the calibration curve. The coefficient of determination (R^2) of 0.9986 confirmed that the linear model accounted for 99.86% of the variance in the observed data. The linear relationship was consistent across the entire concentration range, as evidenced by the residual analysis, which showed random distribution of residuals around the regression line. The use of acyclovir as an internal standard compensated for any variability in injection volume, detector response, and sample preparation, thereby enhancing the accuracy and precision of the method. The consistent recovery of the internal standard throughout the analytical run confirmed the reliability of the calibration procedure. **Sensitivity (LOD and LOQ)**

Table 3: Sensitivity Parameters for Metformin

Parameter	Value
Limit of Detection (LOD)	40 ng/mL
Limit of Quantification (LOQ)	80 ng/mL
Signal-to-Noise Ratio at LOD	3:1
Signal-to-Noise Ratio at LOQ	10:1

The sensitivity of the developed method was determined by analyzing spiked plasma samples at decreasing concentrations until the signal-to-noise ratios reached the predefined limits. The limit of detection (LOD) was established at 40 ng/mL, which represented the lowest concentration producing a signal-to-noise ratio of 3:1. The limit of quantification (LOQ) was set at 80 ng/mL, corresponding to a signal-to-noise ratio of 10:1 with acceptable precision and accuracy (RSD < 15% and recovery within 85–115% of nominal concentration). The LOD and LOQ values achieved in this study are adequate for pharmacokinetic

applications, as the expected plasma concentrations of metformin following therapeutic doses are well above the LOQ. The high sensitivity of the method is attributed to the selection of the isobestic detection wavelength (236 nm), which provides optimal detector response for both metformin and acyclovir, and the efficient protein precipitation sample preparation technique, which minimizes matrix interference.

Precision (Intra-day and Inter-day)

Table 4: Precision Studies for Metformin Bioanalytical Method

Concentration (ng/mL)	Intra-day Precision (n=6)		Inter-day Precision (n=6)	
	Mean ± SD	%RSD	Mean ± SD	%RSD
80	79.13 ± 0.63	0.79	79.23 ± 0.98	1.24
200	198.72 ± 1.20	0.60	198.65 ± 1.80	0.91
800	795.04 ± 6.84	0.86	796.24 ± 8.20	1.03

The precision of the method was evaluated through intra-day and inter-day repeatability studies at three quality control concentration levels: low (80 ng/mL), middle (200 ng/mL), and high (800 ng/mL). Intra-day precision was assessed by analyzing six replicates of each QC concentration on the same day, while inter-day precision was determined by analyzing six replicates on three consecutive days. The intra-day precision, expressed as percentage relative standard deviation (%RSD), ranged from 0.60% to 0.86% across the three concentration levels, with the lowest variability observed at the middle QC level (0.60%) and the highest at the high QC level (0.86%). The inter-day precision showed %RSD values ranging from 0.91% to 1.24%, with the low QC level exhibiting slightly higher variability (1.24%) compared to the middle (0.91%) and high (1.03%) levels. All %RSD values were well within the acceptance criterion of ≤ 15%, demonstrating excellent method repeatability and intermediate precision. The consistently low variability across different days and concentration levels confirms the ruggedness of the developed method. The use of an internal standard effectively compensated for minor variations in sample preparation and instrumental performance, thereby contributing to the high precision observed. The precision data indicate that the method can be reliably used for routine bioanalytical applications, including pharmacokinetic studies and therapeutic drug monitoring.

Accuracy

Table 5: Accuracy Studies for Metformin Bioanalytical Method

Concentration (ng/mL)	Measured Concentration (ng/mL)	% Nominal	Mean ± SD
Actual	Added		
80	40	118.98	99.15
200	40	237.23	98.84
400	40	436.85	99.28

The accuracy of the method was determined by analyzing spiked plasma samples at three different concentration levels (low, middle, and high QC) using the standard addition method. Accuracy was expressed as the percentage of the nominal concentration recovered (% Nominal), calculated as the ratio of measured concentration to spiked concentration multiplied by 100. The mean percentage recoveries ranged from 98.84% to 99.28% across the three concentration levels, with an overall mean accuracy of 99.09 ± 0.87%. The highest recovery was observed at the high QC level (99.28%), while the lowest was at the middle QC level (98.84%). All recovery values were well within the acceptable range of 85–115% of the nominal concentration, confirming t.he accuracy of the method and the absence of systematic errors. The consistent recovery at all QC levels indicates that the protein precipitation sample preparation technique effectively extracts metformin from plasma without significant loss or degradation. The accuracy data demonstrate that the method provides reliable and unbiased quantitative results, making it suitable for precise determination of metformin concentrations in pharmacokinetic and bioequivalence studies.

Ruggedness and Robustness

Table 6: Ruggedness and Robustness Parameters

Parameter	Variation	Effect on Retention Time (min)	Effect on Peak Area Ratio
Column Temperature	Ambient ± 2°C	Metformin: 6.4 ± 0.1	< 2% change
Mobile Phase pH	4.3 – 4.7	Metformin: 6.4 ± 0.2	< 2% change
Mobile Phase Ratio	73:27 – 77:23	Metformin: 6.4 ± 0.3	< 3% change
Flow Rate	0.8 – 1.2 mL/min	Metformin: 6.4 ± 0.2	< 2% change
Different Analysts	Analyst 1 vs. Analyst 2	Metformin: 6.4 ± 0.1	< 2% change

The ruggedness and robustness of the developed method were evaluated by deliberately introducing small variations in the experimental conditions and assessing their impact on system suitability parameters, including retention time, peak area ratio, tailing factor, and resolution. Ruggedness: The method demonstrated excellent ruggedness when analyzed by different analysts, using different instruments of the same model, and employing different lots of reagents and columns of the same type. The %RSD for peak area ratio across all variables was consistently below 2.0%, indicating that the method is not sensitive to changes in operators or laboratory conditions. This confirms the method's suitability for routine application in different laboratory settings without compromising analytical performance. Robustness: The method was found to be robust to small variations in chromatographic conditions. Minor changes in mobile phase pH (pH 4.3 to 4.7), mobile phase ratio (73:27 to 77:23 v/v), and flow rate (0.8 to 1.2 mL/min) did not significantly affect the retention time, peak area ratio, or resolution of metformin and

acyclovir. The retention time of metformin varied by less than 0.3 minutes under all tested conditions, and the peak area ratio remained within ±2% of the original value. The tailing factor remained below 1.3, and the resolution between metformin and acyclovir remained above 3.5 under all robustness conditions. The absence of significant changes in chromatographic performance under varied conditions confirms the stability and reliability of the method. The robustness data are particularly important for method transfer to quality control laboratories and for long-term routine application, where minor variations in operating conditions are inevitable.

CONCLUSION

A simple, rapid, and validated RP-UFLC method was successfully developed for the quantification of metformin in rabbit plasma using acyclovir as the internal standard. The method demonstrated excellent linearity over the concentration range of 40–800 ng/mL with a correlation coefficient of 0.9993. The LOD and LOQ were established at 40 ng/mL and 80 ng/mL, respectively, indicating high sensitivity suitable for pharmacokinetic applications. The method exhibited excellent accuracy (recovery: 98.84–99.28%) and precision (%RSD: 0.60–1.24%) across all quality control levels, confirming its reliability for quantitative bioanalysis. The simple protein precipitation sample preparation technique, short run time of 10 minutes, and robust chromatographic performance make the method suitable for routine application in quality control laboratories and pharmacokinetic studies. The successful application of the method to quantify metformin in rabbit plasma following oral administration of pure metformin and metformin-loaded SLNs demonstrates its practical utility in formulation development and bioequivalence assessment. The method's compatibility with conventional HPLC-UV instrumentation and its cost-effectiveness make it accessible to laboratories with limited resources, thereby facilitating widespread adoption in pharmaceutical research and development. The developed method fills an important gap in the literature by providing a simple, cost-effective, and validated bioanalytical method for metformin quantification in rabbit plasma, which can be employed in future studies involving novel drug delivery systems, pharmacokinetic evaluations, and preclinical efficacy assessments.

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