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Original Article

OPTIMIZATION OF LC-MS/MS METHOD FOR THE SIMULTANEOUS DETERMINATION OF METFORMIN AND ROSIGLITAZONE IN HUMAN PLASMA WITH BOX-BEHNKEN DESIGN

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ABSTRACT

Objective: The objective of this study was to develop a robust Liquid Chromatography – Tandem Mass Spectrometry (LC-MS/MS) methodology for the precise quantification of metformin and rosiglitazone in human plasma.

Methods: A Design of Experiments (DOE) framework was utilized, specifically employing a Box-Behnken experimental design, to optimize critical parameters such as Capillary voltage, Cone voltage, Desolvation temperature, and Collision energy. Sample preparation involved protein precipitation using acetonitrile, simplifying the procedure. Chromatography was performed with a mobile phase of 0.1% formic acid and acetonitrile (60:40 V/V) to enhance sensitivity and reproducibility. Quantification was achieved through Multiple Reaction Monitoring (MRM) of the transition's m/z 130.1 → m/z 60.1 for metformin, m/z 358.2 → m/z 134.9 for rosiglitazone, and m/z 206.3 → m/z 59.9 for phenformin. The methodology was validated according to regulatory guidelines.

Results: The developed methodology demonstrated selectivity, linearity, accuracy, precision, recovery, and stability. The calibration curve showed linearity over the concentration range of 5 ng/ml to 1000 ng/ml for metformin and 1.5 ng/ml to 300 ng/ml for rosiglitazone. Accuracy and precision were within acceptable limits across calibration and quality control standards. Assessments of extraction recovery and matrix effects confirmed the robustness of the extraction procedure, with negligible interference from plasma components. Stability studies indicated that the method maintained acceptable limits for metformin and rosiglitazone concentrations under various storage and handling conditions.

Conclusion: The validated Liquid Chromatography – Tandem Mass Spectrometry (LC-MS/MS) methodology provides a reliable and accurate platform for the quantification of metformin and rosiglitazone in human plasma. This method shows potential applications in pharmacokinetic studies and clinical research, ensuring consistent performance in routine analysis.

Keywords: Metformin, Rosiglitazone, Box-behnken design, Biological matrices, Optimization, LC-MS/MS

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INTRODUCTION

Type 2 Diabetes Mellitus (T2DM) is a worldwide health issue characterized by reduced insulin activity and high blood sugar levels [1]. Metformin and rosiglitazone are commonly prescribed oral antidiabetic drugs that have different mechanisms of action. Metformin reduces hepatic glucose production and boosts insulin sensitivity in peripheral tissues by activating AMP-Activated Protein Kinase (AMPK), inhibiting hepatic gluconeogenesis, and enhancing glucose uptake [2, 3]. It also enhances insulin-mediated glucose utilization in muscle cells by promoting Glucose transporter type 4 (GLUT4) translocation [4]. Rosiglitazone, a thiazolidinedione, acts as a selective agonist of Peroxisome Proliferator-Activated Receptor Gamma (PPAR-γ) in adipose tissue, muscle, and liver cells. This activation enhances insulin sensitivity by upregulating insulin-responsive genes involved in glucose uptake and utilization [5, 6]. Additionally, rosiglitazone reduces hepatic glucose output by suppressing gluconeogenesis and promoting glycogen synthesis [7].

Liquid Chromatography coupled with Tandem Mass Spectrometry (LC-MS/MS) has emerged as a versatile and sensitive analytical technique for drug quantification in biological samples. LC-MS/MS offers high specificity, selectivity, and sensitivity, allowing for simultaneous quantification of multiple analytes within complex matrices [8, 9]. The precise quantification of pharmaceutical agents within biological matrices is essential for elucidating their pharmacokinetic profiles and optimizing therapeutic regimens [10, 11]. Therefore, a sensitive, reliable, and rapid method to simultaneously determine metformin and rosiglitazone in human plasma is required.

Various techniques have been utilized for the separate quantification of metformin and rosiglitazone, including High-Performance Liquid Chromatography (HPLC) with Ultra-Violet (UV) detection [12-15]. Additionally, individual LC-MS/MS methods have been developed for both compounds in human and rat plasma [16-19]. However, only two studies have employed Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) for their simultaneous determination in human plasma. Notably, no reports exist regarding the concurrent quantification of metformin and rosiglitazone using a systematically optimized method based on design of experiments.

The Box-Behnken design is a statistical experimental design methodology widely employed for optimizing analytical methods. This design enables the systematic exploration of multiple factors and their interactions with a minimal number of experiments, thereby reducing time, resources, and experimental error [20]. By systematically varying factors such as chromatographic conditions, mobile phase composition, and mass spectrometry parameters, the Box-Behnken design facilitates the development of robust and efficient LC-MS/MS methods.

This study introduces a newly developed LC-MS/MS method, systematically optimized using statistical design of experiments, for the simultaneous quantification of metformin and rosiglitazone concentrations in human plasma. This method is rigorously validated following the regulatory guidelines to ensure its suitability for pharmacokinetic studies in clinical practice.

MATERIALS AND METHODS

Materials and solvents

For this study, Metformin was sourced from Lee Pharma, Hyderabad, India, while Rosiglitazone was obtained from Jigs Chemicals Ltd, Ahmedabad, India. Phenformin was acquired from Rx Innovations, Hyderabad, India. Human plasma was procured from the local Blood bank. Analytical grade Methanol, acetonitrile, and water were purchased from Sigma Aldrich, Bengaluru, India, ensuring high-quality reagents for the experimental procedures.

Solutions preparation

Stock solutions and working solutions

For the preparation of calibration standards for metformin and rosiglitazone, a primary stock solution with a concentration of 1 mg/ml was initially prepared with water and methanol. From these individual stock solutions, a series of working standard concentrations were generated through serial dilution. The working standard concentrations are 100 ng/ml, 200 ng/ml, 500 ng/ml, 1000 ng/ml, 4000 ng/ml, 8000 ng/ml, 12000 ng/ml, and 20000 ng/ml for metformin calibration standards. The working standard concentrations of rosiglitazone for the calibration standards were as follows: 30 ng/ml, 60 ng/ml, 300 ng/ml, 600 ng/ml, 1200 ng/ml, 2400 ng/ml, 3600 ng/ml, and 6000 ng/ml. These concentrations were achieved by diluting the stock solution, accordingly, ensuring a range of concentrations suitable for the calibration and quantification of metformin in subsequent analytical procedures.

Working solutions for different Quality Control (QC) levels were derived through serial dilution from the respective primary stock solutions of metformin and rosiglitazone. The QC standards included the Lower Limit of Quantification (LLOQ QC), Lower Quality Control (LQC), Medium Quality Control (MQC), and High-Quality Control (HQC). Specifically, the concentrations of working solutions for the QC standards were as follows: 50 ng/ml, 300 ng/ml, 10000 ng/ml, and 16000 ng/ml for metformin and 15 ng/ml, 90 ng/ml, 3000 ng/ml, and 4800 ng/ml for rosiglitazone.

A primary stock solution of phenformin in water with 1 mg/ml concentration was diluted further with redistilled water to create the working internal standard solution, achieving a concentration of 1 µg/ml of phenformin.

Calibration and quality control sample preparation

Calibration curves and quality control samples were prepared by spiking the blank plasma with 10 µl of above mentioned working standard solutions of metformin and rosiglitazone. The final concentrations of calibration standard for metformin are: 5 ng/ml (LLOQ), 10 ng/ml, 25 ng/ml, 50 ng/ml, 200 ng/ml, 400 ng/ml, 600 ng/ml, and 1000 ng/ml (ULOQ) and for rosiglitazone are: 1.5 ng/ml (LLOQ), 3 ng/ml, 15 ng/ml, 30 ng/ml, 60 ng/ml, 120 ng/ml, 180 ng/ml, and 300 ng/ml (ULOQ). The final concentrations of quality control samples for metformin are: 2.5 ng/ml (LLOQ-QC), 15 ng/ml (LQC), 500 ng/ml (MQC), and 800 ng/ml (HQC) and for rosiglitazone are: 0.75 ng/ml (LLOQ-QC), 4.5 ng/ml (LQC), 150 ng/ml (MQC), and 240 ng/ml (HQC).

Sample preparation

For the sample preparation, 200µl of plasma sample was initially combined with 20µl of the Internal Standard (IS) solution. Following this, protein precipitation was conducted by adding 0.5 ml of acetonitrile to the mixture. The resulting mixture was then subjected to vortex-mixing for 2 min to ensure thorough mixing. Subsequently, centrifugation was performed to separate the protein precipitate, after which 10µl of the supernatant was extracted and injected into the LC-MS/MS system for analysis.

LC-MS/MS instrumentation and conditions

The analysis was performed on a Shimadzu triple quadrupole LC-MS/MS (Shimadzu LCMS-8040). The analytical column was Luna C18 (50 mm×2.0 mm × 5 µm.) and maintained at 35 °C. The mobile phase consisted of 0.1% formic acid-acetonitrile (60:40, v/v) at an isocratic flow rate of 0.4 ml/min. The injection volume was 10µl. Mass spectrometer settings in positive-ion mode (ESI+) were capillary voltage at 4000 V, cone voltage at 30V desolvation temperature at 440 °C, collision energy at 32eV, collision gas (N2) at medium, curtain gas at 20, ion source gases at 40 and 60. Data acquisition and processing were performed with the Lab Solution software.

Design of experiments

The current investigation employs a Design of Experiments (DOE) framework to formulate a robust LC-MS/MS methodology for the precise quantification of metformin and rosiglitazone within biological matrices. The selection of the Box-Behnken experimental design for method development is predicated upon its notable merits encompassing optimal resource utilization, equilibrium of factor levels, heightened resilience to extraneous influences, and proficiency in the discernment of quadratic effects. This design facilitates the comprehensive exploration of factor permutations, concomitantly mitigating procedural variance, thus accommodating the constraints inherent in a restricted experimental domain. The application of response surface modelling inherent to this design furnishes predictive insights into response patterns, thereby affording the identification of paramount parameter configurations that optimize the methodology.

Within this paradigm, four pivotal experimental variables emerged as determinative, thereby being established as critical independent factors: specifically, Capillary voltage (A), Cone voltage (B), Desolvation temperature (C), and Collision energy (D). The establishment of the operational scope of these variables was undertaken by anchoring them within empirically derived minimum and maximum levels. Specifically, Capillary voltage was delimited within the range of 1.5 kV to 4.5 kV, Cone voltage spanned from 10V to 35V. Desolvation temperature encompassed the interval of 350 °C to 550 °C, and the Collision energy was confined within 10 eV to 20 eV.

Given the paramount significance of analyte quantification within the ambit of bioanalytical methodology employing Mass spectrometric detection, the present investigation adopts the response area of metformin (R1), rosiglitazone (R2) and the internal standard (R3) as pivotal response variables meriting optimization efforts. A total of 29 different experiments were conducted according to the Box-Behnken design. The data table containing the factors at various levels and their measured responses after the experimental runs were enumerated in table 1.

Method validation

The developed methodology was validated by assessing its selectivity, stability, specificity, linearity, matrix effect, precision, recovery, and accuracy.

RESULTS

Selection of internal standard

After an assessment, phenformin was chosen as the internal standard, for quantifying metformin and rosiglitazone in plasma using LC-MS/MS. Phenformin showed optimal chromatographic behaviour, minimal interference to the analytes, and shared similar physical and chemical characteristics with the target substances enabling reliable and precise measurement, in samples over a broad range of concentrations.

Sample preparation

Metformin extraction from biological fluids using the liquid-liquid extraction method was considered impracticable due to its strong polarity. Solid-Phase Extraction (SPE) was deemed ineffective for high throughput analysis due to cost limitations. A protein precipitation technique was devised in this study to simplify sample preparation, minimizing the necessity for additional concentration steps, and making the procedure more straightforward. Three protein precipitation agents, namely acetonitrile, methanol, and acetone, were assessed for their extraction efficiency. Acetonitrile showed excellent protein precipitation efficiency with minimal drug sample loss. This approach was also effective when used with rosiglitazone samples. The straightforward single-step acetonitrile protein precipitation method was chosen for further experimentation.

Chromatography and mass spectrometry

The 10 mmol ammonium formate buffer was chosen after a thorough assessment because of its significant improvements in analyte response, peak shape, and intensity compared to other options such as an ammonium acetate buffer or a 0.1% formic acid buffer. The 0.1% formic acid showed better ionization efficiency, leading to improved sensitivity and reproducibility in LC-MS/MS analysis. The buffer choice also showed enhanced peak shapes,

resulting in higher resolution and accuracy in quantifying target analytes, ensuring reproducible and accurate results in the analytical process. Therefore, 0.1% formic acid and acetonitrile are finalized as the mobile phase. MS/MS parameters were carefully optimized using Design of experiments. Positive ionization had yielded greater signal intensity and signal to noise ratio than negative ionization. So, the experimental optimization performed in positive ionization mode with selected critical mass parameters. Quantification was performed using Multiple Reaction Monitoring (MRM) of the transitions of m/z 130.1 → m/z 60.1 for metformin, m/z 358.2→m/z 134.9 for rosiglitazone and m/z 206.3 → m/z 59.9 for phenformin. The product ion spectra of metformin, rosiglitazone, and phenformin are shown in fig. 1.

Experimental optimization

Effectiveness of experimental design in response variable analysis

The Fit Summary, ANOVA, and Fit Statistics results (table 2) jointly indicate the efficacy of the experimental design in assessing response variables. The model for Metformin is well-fitted, as shown by significant linear and quadratic terms and a high Adjusted R^2 value of 0.9951. Rosiglitazone shows significant significance for the quadratic component ($p<0.0001$) and overall model ($p<0.0001$), indicating a solid fit and predictive capacity. The Internal Standard (R3) demonstrates substantial effects from linear, quadratic, and cubic components, with a high Adjusted R^2 of 0.9945 and Predicted R^2 of 0.9865, suggesting a robust model fit and predictive capability.

Table 1: Summary data of design of experiment run and responses

Run	Factor 1	Factor 2	Factor 3	Factor 4	Response 1	Response 2	Response 3
	A: Capillary voltage (kV)	B: Cone voltage (V)	C: Desolvation temperature (C)	D: Collision energy (eV)	Response area of metformin	Response area of rosiglitazone	Response area of IS
1	3	10	450	40	13024	13256	30902
2	3	10	350	30	11517	13286	27326
3	3	10	550	30	12298	13256	29178
4	4.5	22.5	350	30	15158	20770	35964
5	1.5	22.5	550	30	9955	10131	23620
6	3	22.5	350	20	12353	18690	29310
7	3	22.5	450	30	13981	27402	33021
8	4.5	35	450	30	18249	17328	43300
9	4.5	22.5	550	30	16445	16503	39018
10	3	22.5	450	30	14146	24485	33623
11	3	35	450	20	13717	14766	32546
12	3	22.5	550	20	12826	15078	30432
13	3	35	450	40	16720	14924	39672
14	1.5	35	450	30	11374	10969	26986
15	1.5	10	450	30	9449	9008	22420
16	3	22.5	450	30	14036	23769	33302
17	3	22.5	450	30	14234	25362	33772
18	4.5	10	450	30	15301	17190	36304
19	3	35	350	30	14278	19275	33876
20	3	22.5	450	30	14113	24465	33059
21	1.5	22.5	450	40	10857	7124	25760
22	3	35	550	30	14487	16306	34372
23	3	22.5	550	40	14751	13608	35000
24	1.5	22.5	450	20	10615	10227	25186
25	4.5	22.5	450	40	17875	14384	42412
26	4.5	22.5	450	20	15631	15195	37088
27	3	10	450	20	12661	12208	30040
28	3	22.5	350	40	13354	14519	31684
29	1.5	22.5	350	30	9867	7380	23410

Table 2: Summary of ANOVA and fit statistics of response variables analysis

Source	Response area of metformin		Response area of rosiglitazone		Response area of IS		Model significance
	F-value	p-value	F-value	p-value	F-value	p-value	
Model	**407.21**	**<0.0001**	**23.48**	**<0.0001**	**364.39**	**<0.0001**	**significant**
A-Capillary voltage	4346.59	<0.0001	76.15	<0.0001	3895.55	<0.0001	
B-Cone Voltage	691.48	<0.0001	8.30	0.0121	619.71	<0.0001	
C-Desolvation temperature	58.38	<0.0001	2.87	0.1122	52.34	<0.0001	
D-Collision Energy	250.82	<0.0001	2.45	0.1397	224.79	<0.0001	
AB	10.22	0.0065	0.3507	0.5632	9.18	0.0090	
AC	14.04	0.0022	5.20	0.0388	12.57	0.0032	
AD	39.14	<0.0001	0.5543	0.4689	35.08	<0.0001	
BC	3.20	0.0955	0.9114	0.3559	2.86	0.1130	
BD	68.06	<0.0001	0.0836	0.7767	61.00	<0.0001	
CD	8.34	0.0119	0.7698	0.3951	7.48	0.0161	
A^2	41.03	<0.0001	134.01	<0.0001	32.87	<0.0001	
B^2	3.35	0.0884	69.85	<0.0001	1.97	0.1822	
C^2	178.05	<0.0001	47.66	<0.0001	151.42	<0.0001	
D^2	0.5980	0.4522	99.39	<0.0001	1.13	0.3048	
Lack of Fit	3.33	0.1290	1.28	0.4393	1.61	0.3418	not significant
Fit statistics							
	Response area of metformin		Response area of rosiglitazone		Response area of IS		
CV (%)	1.18		9.69		1.25		
R^2	0.9976		0.9592		0.9973		
Adjusted R^2	0.9951		0.9183		0.9945		
Predicted R^2	0.9870		0.8057		0.9865		
Adequate Precision	74.0355		16.4116		70.0888		

Response area of metformin (R1)

$$R1 = 14102+3045.17* A+1214.58* B+352.917* C+731.5* D+255.75* AB+299.75* AC+500.5* AD+-143* BC+660* BD+231* CD+-402.417* A^2+-115.042* B^2+-838.292* C^2+48.5833* D^2 \ldots Eq.\ 1.$$

The response variable, the Response Area of Metformin, is influenced by several factors as indicated by the coefficients in the provided equation and the perturbation plots in fig. 1. An increase in Capillary Voltage (A) results in a notable increase in the response area, with a coefficient of 3045.17 units, suggesting a positive relationship between capillary voltage and the response. Similarly, Cone Voltage (B) exhibits a positive effect, with a coefficient of 1214.58 units, indicating that higher cone voltage contributes to higher response areas. Desolvation Temperature (C) also positively influences the response variable, with a coefficient of 352.917 units, implying that higher desolvation temperatures lead to increased response areas. Additionally, Collision Energy (D) shows a positive impact, with a coefficient of 731.5 units, indicating that higher collision energy is associated with higher response areas. Moreover, the interaction terms (AB, AC, AD, BC, BD, CD) and quadratic terms (A^2, B^2, C^2, D^2) further elucidate the combined and curved effects (fig. 1) of these factors on the response variable, providing valuable understandings for optimizing experimental conditions to achieve desired response outcomes.

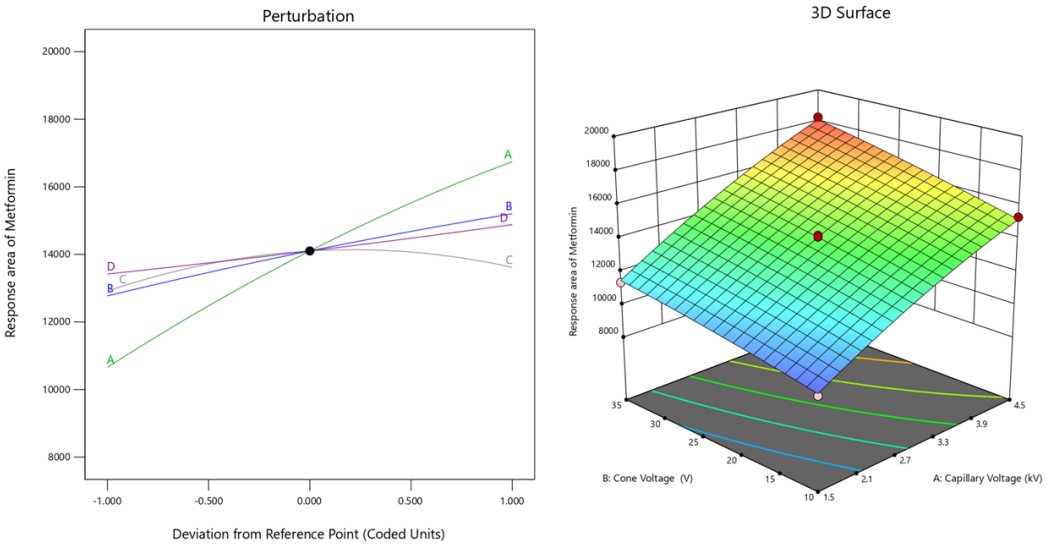

Fig. 1: Perturbation and 3D surface plots displaying the individual and interaction effects of critical process parameters on response area of metformin

Response area of rosiglitazone (R2)

$$R2 = 25096.6+3877.58* A+1280.33* B+-753.167* C+-695.75* D+-455.75* AB+-1754.5* AC+573* AD+-734.75* BC+-222.5* BD+675.25* CD+-6996.38* A^2+-5051.01* B^2+-4172.26* C^2+-6025.13* D^2 \ldots Eq.\ 2.$$

The effect of factors on the response variable can be understood by examining the coefficients provided in the model equation and perturbation plots in fig. 2. Positive coefficients, like those for Capillary Voltage (A) and Cone Voltage (B), suggest that higher levels of these factors lead to an increase in the response variable, indicating their positive effect on Rosiglitazone's response area. Conversely, negative coefficients, such as those for Desolvation Temperature (C) and Collision Energy (D), indicate that higher levels of these factors result in a decrease in the response variable, signifying their negative effect. Additionally, the significant quadratic terms (A^2, B^2, C^2, D^2) suggest nonlinear relationships between these factors (3D Surface plot-fig. 2) and the response variable, highlighting the importance of considering higher-order terms in the model for accurate predictions.

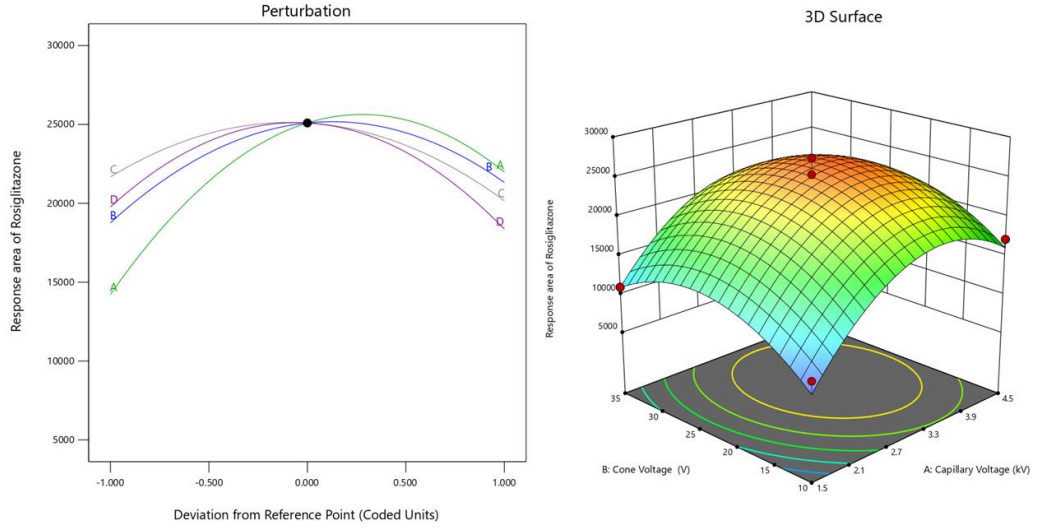

Fig. 2: Perturbation and 3D surface plots displaying the individual and interaction effects of critical process parameters on response area of rosiglitazone

Response area of internal standard (R3)

$$R3 = 33355.4+7225.33* A+2881.83* B+837.5* C+1735.67* D+607.5* AB+711* AC+1187.5* AD+-339* BC+1566* BD+548.5* CD+-902.783* A^2+-221.033* B^2+-1937.53* C^2+167.717* D^2 \quad \text{...Eq. 3.}$$

Analyzing the coefficients in the provided model equation and perturbation plot (fig. 3) reveals the effects of several factors on the Response Area of Internal Standard (R3). Capillary Voltage (A) demonstrates a strong positive effect, with a coefficient of 7225.33 indicating that increasing Capillary Voltage leads to a proportional increase in the Response Area. Similarly, Cone Voltage (B) and Desolvation Temperature (C) exhibit positive effects, as higher levels of these factors result in increased Response Area, with coefficients of 2881.83 and 837.5, respectively. Collision Energy (D) also shows a positive effect, with a coefficient of 1735.67 indicating that higher Collision Energy levels lead to increased Response Area. Moreover, interaction terms such as AD demonstrate how combined effects of two factors influence the response variable. Furthermore, the quadratic terms reveal the curvature of the relationship (3D Surface Plot – fig. 3) between each factor and the Response Area, indicating nonlinear effects.

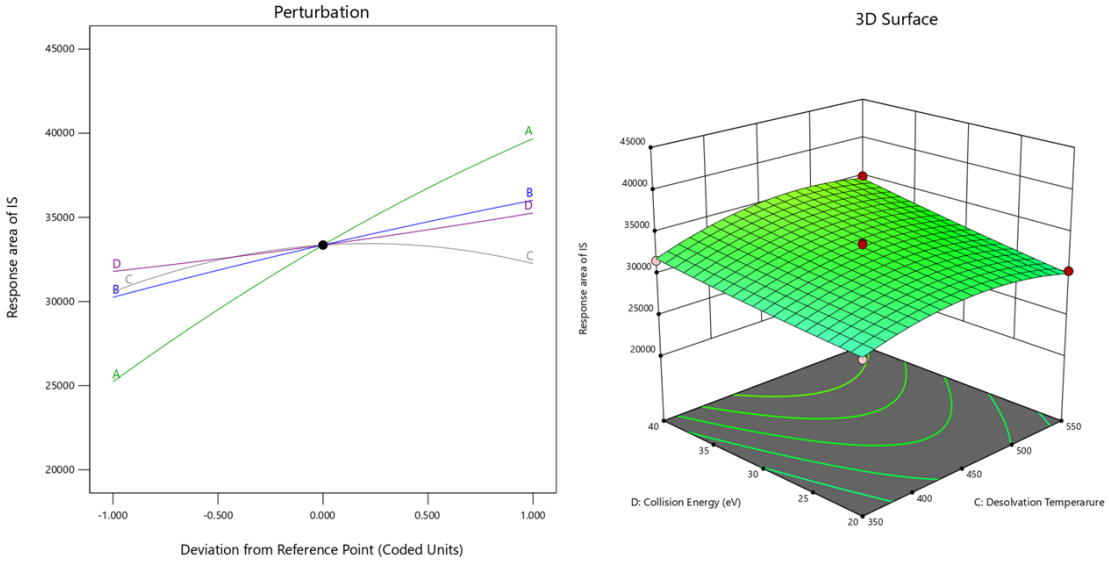

Fig. 3: Perturbation and 3D Surface plots displaying the individual and interaction effects of critical process parameters on Response area of internal standard phenformin

The DOE analysis offered valuable insights into optimizing experimental conditions to maximize the Response Areas of Metformin, Rosiglitazone, and the Internal Standard. By integrating numerical and graphical optimization, desirable constraints were input into the model to yield multiple solutions. The final output, the Method Optimal Design Region (MODR), depicted in fig. 4, highlights the region where the experimental conditions are optimized for the desired responses.

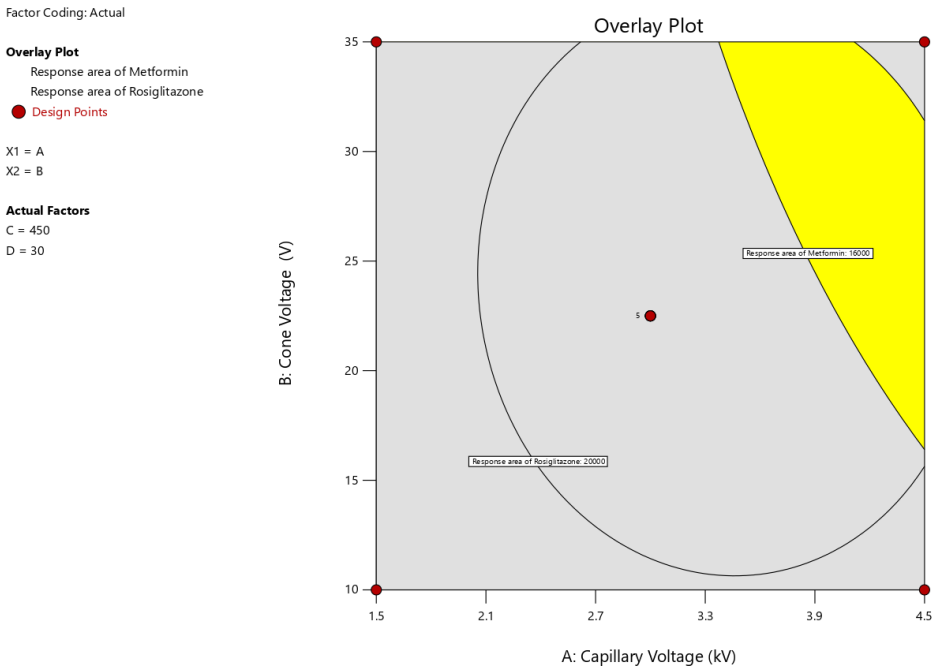

Fig. 4: Method optimal design region (MODR) highlighting the optimised region for experimental conditions

Method validation

Selectivity

Selectivity of the method was established by displaying the non-interference of endogenous substances at retention times of the metformin, rosiglitazone, and phenformin. Six different batches of blank, zero, and spiked plasma sample chromatograms were assessed to establish the selectivity. The representative chromatograms of blank and spiked samples were given in the fig. 5. Which displayed no interference of endogenous substance with the analytes and internal standard.

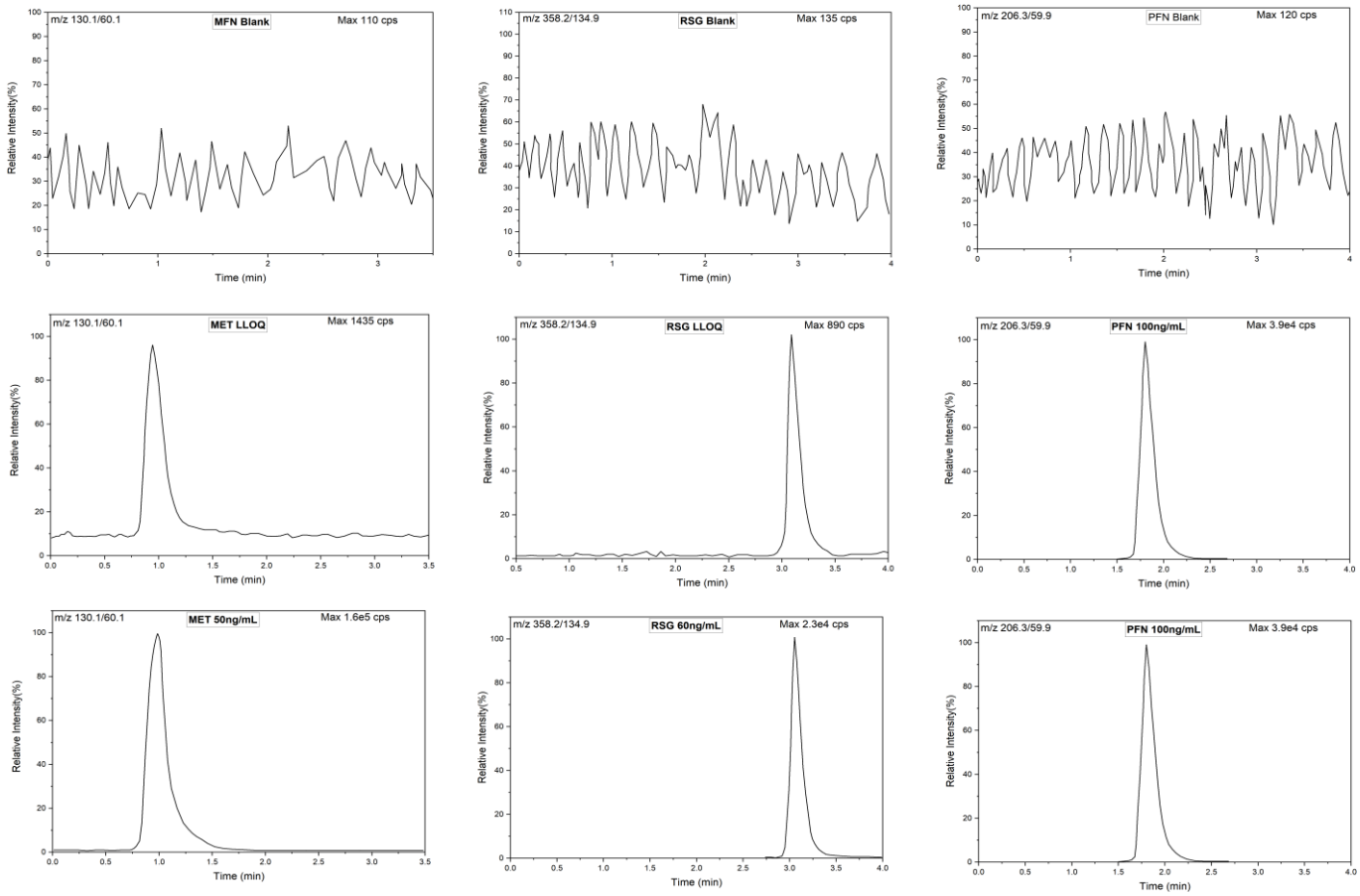

Fig. 5: MRM chromatograms of blank, Metformin (LLOQ, 50 mg/ml), rosiglitazone (LLOQ, 60ng/ml), phenformin (100ng/ml) sample

Linearity

Linearity of the method was established by evaluating the area of the peaks of calibration curves of metformin and rosiglitazone. The developed method displayed linearity response over 5 – 1000 mg/ml for metformin and 1.5 – 300ng/ml for Rosiglitazone. All the back-calculated concentrations of calibration standards fall below the acceptance criteria of ±15%.

The LLOQ for metformin and Rosiglitazone were 5ng/ml and 1.5ng/ml, respectively. At these concentration levels, the precision and accuracy were 18.9% and 95.3% for metformin, and 17.82% and 101.2% for rosiglitazone, respectively.

Accuracy and precision

The precision (% CV) and accuracy of the method for metformin and rosiglitazone in human plasma were evaluated across a concentration range using calibration and QC standards. The summary of accuracy and precision study results were enumerated in table 3. Precision for metformin calibration standards ranged from 9.74% to 18.94% and from 9.37% to 19.57% for QC standards. For rosiglitazone, precision for calibration standards ranged from 8.98% to 17.82%, and from 9.64% to 18.74% for QC standards.

For metformin, accuracy ranged from 94.7% to 105.4% for calibration standards and from 95.6% to 104.4% for QC standards. For rosiglitazone, accuracy ranged from 96.8% to 106.8% for calibration standards and from 100.9% to 102.8% for QC standards. Most values fall within the acceptable range of 85% to 115%, indicating good accuracy of the method across the concentration range evaluated.

Extraction recovery

The extraction recovery of LQC, MQC, and HQC levels of metformin and rosiglitazone samples was evaluated to assess the efficiency of the extraction method. The mean recovery percentages of metformin at LQC, MQC, and HQC levels were 84.9%, 86.7%, and 89.5%, respectively. Similarly, for rosiglitazone, the mean recoveries were 88.4%, 92.1%, and 86.2%, respectively. The % CV values for both metformin and rosiglitazone at the test levels were less than 10%, which falls within the acceptance criteria, and determines the establishment of extraction recovery of the method. The extraction recovery results indicate that the extraction method effectively recovers the analyte from plasma samples across different concentration levels demonstrating the robustness and reliability of the extraction procedure.

Matrix effect

The behaviour of the developed method remained consistent despite the presence of plasma components, as indicated by the % CV of five replicates at LQC, MQC, and HQC levels, which were all below 8% for both metformin and rosiglitazone. This suggests that interference from plasma was negligible.

Table 3: Summary data of precision and accuracy experiments for metformin and rosiglitazone in human plasma

Metformin				Rosiglitazone			
Concentration added (ng/ml)	**Concentration added (ng/ml) (Mean±SD) (n=5)**	**Precision (%)**	**Accuracy (%)**	**Concentration added (ng/ml)**	**Concentration added (ng/ml) (Mean±SD) (n=5)**	**Precision (%)**	**Accuracy (%)**
Calibration standards							
5	4.94±0.73	18.94	95.3	1.5	1.59±0.21	17.82	101.2
10	10.13±1.95	15.69	100.3	3	3.08±0.61	16.33	96.8
25	25.37±2.89	14.07	99.07	15	15.23±2.54	12.38	100.04
50	50.08±5.76	13.19	103.9	30	30.74±3.97	11.94	99.5
200	198.36±19.41	11.94	101.4	60	58.77±7.95	12.71	106.8
400	403.52±53.18	12.06	98.3	120	118.63±12.26	9.37	103.7
600	607.85±49.37	10.48	100.8	180	183.51±9.74	10.05	98.6
1000	1029.44±91.43	9.74	105.4	300	307.11±23.18	8.98	99.5
Quality control standards							
2.5	2.67±0.58	19.57	94.7	0.75	0.76±0.18	18.74	95.6
15	15.08±2.04	16.32	98.5	4.5	4.58±0.84	16.75	104.4
500	511.23±45.62	9.37	100.5	150	157.36±11.21	9.64	102.8
800	807.52±63.19	10.15	103.8	240	249.52±14.28	10.62	100.9

*All the values are expressed in mean±SD, where n=5

Stability

The stability of plasma samples containing metformin and rosiglitazone was assessed across various conditions. Freeze-thaw stability was determined by subjecting aliquots of QC samples (LQC, MQC, and HQC) to three cycles and comparing measured concentrations with nominal values. Long-term stability was evaluated by storing aliquots at-20 °C for 20 and 45 days, followed by analysis and comparison of concentrations. Short-term stability was assessed by exposing QC samples to ambient temperature for 4 h and analyzing the samples thereafter. Post-preparation stability was evaluated by storing QC samples in an autosampler at 25 °C for 8 h and comparing concentrations with nominal values. Additionally, the stability of stock solutions for metformin, rosiglitazone, and phenformin was examined after 4 h at 25 °C and 30 days at 4 °C.

The results from the stability tests enumerated in table 4 suggest that the experimental concentrations of Metformin and Rosiglitazone at low (LQC), medium (MQC), and high (HQC) quality control levels observed to be within acceptable limits (±15%). This indicates robust stability throughout the extraction and analytical procedures. Therefore, the method shows suitability for routine analysis.

Table 4: Stability of metformin and rosiglitazone in plasma sample at different stability conditions

Parameter	Metformin			Rosiglitazone		
	Accuracy percentage (Mean±SD)			Accuracy percentage (Mean±SD)		
	LQC	**MQC**	**HQC**	**LQC**	**MQC**	**HQC**
Freeze-thaw stability	103.5±1.74	99.5±3.22	103.9±6.48	97.4±3.97	99.8±4.23	102.4±3.09
Short-term stability	98.2±1.09	104.7±4.68	106.2±5.95	103.6±4.22	98.1±6.37	100.7±1.98
Long-term stability	107.1±2.06	94.2±8.61	101.8±5.43	108.1±5.38	105.8±4.76	105.3±4.06
Post-preparative stability	102.9±1.95	101.9±2.55	102.5±3.14	101.7±2.29	103.2±2.95	100.8±3.07
Stability of Stock Solutions						
Parameter	Accuracy percentage (Mean±SD)					
	Metformin		Rosiglitazone		Phenformin	
25 °C for 4 h	100.4±1.36		101.3±2.01		99.3±1.12	
4 °C for 30 d	96.6±2.81		104.6±1.75		98.6±1.05	

*All the values are expressed in mean±SD

DISCUSSION

The method development and validation for quantifying metformin and rosiglitazone in plasma using LC-MS/MS revealed several critical findings that underline its robustness and reliability. The selection of phenformin as the internal standard was pivotal due to its optimal chromatographic behaviour and minimal interference with metformin and rosiglitazone. This choice ensured precise and reliable measurements across various concentrations, highlighting the importance of selecting an internal standard with similar physical and chemical properties to the analytes.

Given metformin's strong polarity, traditional liquid-liquid extraction was impractical, and solid-phase extraction was cost-prohibitive for high-throughput analysis. Instead, the protein precipitation technique, particularly with acetonitrile, proved to be a highly effective and straightforward method for sample preparation. This method minimized sample loss and avoided additional concentration steps, demonstrating its practicality and efficiency for both metformin and rosiglitazone.

The selection of a 10 mmol ammonium formate buffer and a 0.1% formic acid/acetonitrile mobile phase significantly enhanced analyte response, peak shape, and intensity. The preference for positive ionization mode due to its higher signal intensity and signal-to-noise ratio further optimized the sensitivity and reproducibility of the LC-MS/MS analysis. The use of MRM transitions specific to each analyte and the internal standard allowed for precise quantification and reduced interference.

The DOE approach provided a comprehensive understanding of the factors affecting the response areas of metformin, rosiglitazone, and the internal standard. For metformin, factors like capillary voltage, cone voltage, desolvation temperature, and collision energy positively influenced the response area. Similarly, for rosiglitazone, positive and negative coefficients indicated the need to balance a range of factors. The analysis of the internal standard response area highlighted the significant positive effects of capillary voltage, cone voltage, desolvation temperature, and collision energy. The MODR identified through DoE optimization ensured the method was robust and reproducible under varied conditions.

The validation of the method demonstrated excellent selectivity, linearity, accuracy, precision, extraction recovery, minimal matrix effect, and stability. The method effectively differentiated the analytes from endogenous substances, showed a linear response

over a wide concentration range for both analytes, and exhibited high precision and accuracy across the tested concentration ranges, with values within acceptable limits. High recovery rates and low % Coefficient of Variation (%CV) values confirmed the method's efficiency and consistency, and minimal interference from plasma components ensured reliable quantification. Stability tests confirmed the robustness of the method under various conditions, making it suitable for routine analysis. The comprehensive optimization and validation processes ensured the method's robustness and reproducibility, making it a valuable tool for accurate measurement of these analytes in biological samples.

CONCLUSION

The study presents a robust method for quantifying metformin and rosiglitazone in human plasma using LC-MS/MS, offering valuable understandings for pharmacokinetic studies. Our findings demonstrate the effectiveness of phenformin as an internal standard, the suitability of protein precipitation with acetonitrile for sample preparation, and the optimization of chromatography and mass spectrometry parameters. By employing the DOE, we systematically assessed numerous factors influencing the response variables, including capillary voltage, cone voltage, desolvation temperature, and collision energy. This approach allowed us to identify optimal conditions for maximizing the response areas of metformin, rosiglitazone, and the internal standard, ensuring the robustness and reproducibility of our analytical method. The method exhibits excellent selectivity, linearity, accuracy, and precision, as well as robust extraction recovery and negligible matrix effects. Stability studies further confirm the method's reliability for routine analysis. This validated LC-MS/MS methodology provides a reliable platform for quantifying metformin and rosiglitazone in human plasma, offering significant potential for routine analysis.

AUTHORS CONTRIBUTIONS

All the authors contributed significantly to this manuscript; Rubina Kauser is responsible for the conception and design of the study, acquisition of data, analysis, and interpretation of data. Sunil Kumar Chaitanya. P, Venkatesan. P, have taken part in guiding and supervising throughout the research process, drafting the article, revising it critically; have agreed to submit to the current journal; gave final approval for the manuscript to be published.

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All authors have contributed equally

CONFLICT OF INTERESTS

Declared none

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