RESEARCH PAPER

Optimization of RP-HPLC Method for Estimating Anti-Diabetic Drugs in Combined Dosage Form in Human Plasma Using Box-Behnken Design

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ABSTRACT

A Quality by Design approach was employed to develop a robust and rugged bioanalytical RP-HPLC method, validated according to ICH guidelines. This study outlines a simple, rapid, sensitive, and cost-effective method for estimating Vildagliptin and Pioglitazone hydrochloride in human plasma. Factor screening studies were conducted using Box-Behnken design, focusing on three critical parameters: mobile phase composition, flow rate, and wavelength. Design Expert software (trial version 13.0) was utilized to optimize chromatographic conditions through statistical mathematical modeling. Optimal separation was achieved on a Waters C18 column (250 mm × 4.6 mm, 5 µ) using a mobile phase consisting of Acetonitrile and 0.1% OPA (7:93) at a flow rate of 0.9 mL/min, with UV detection at 215 nm. The Box-Behnken design revealed the interplay between mobile phase, flow rate, and wavelength at three levels, and responses such as retention time, area, and theoretical plate factor were analyzed using response surface plots and statistical data. The developed bioanalytical method was validated according to ICH guidelines, demonstrating high linearity, precision, accuracy, sensitivity, and robustness compared to existing RP-HPLC methods for Vildagliptin and Pioglitazone hydrochloride.

Keywords: Quality by Design, Box–Behnken Design, Bioanalytical Method, RP-HPLC, Anti-diabetic drugs

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INTRODUCTION

Diabetes mellitus is a chronic metabolic disorder characterized by persistent hyperglycemia resulting from defects in insulin secretion, insulin action, or both. Type 2 Diabetes Mellitus (T2DM) accounts for the majority of cases worldwide and requires long-term pharmacotherapy to maintain glycemic control. Combination therapy has emerged as an effective strategy to target multiple pathophysiological mechanisms simultaneously.

In September 2022, the Central Drugs Standard Control Organization approved a fixed-dose combination of Vildagliptin (50 mg) and Pioglitazone hydrochloride (15 mg) for patients inadequately controlled on metformin monotherapy. Vildagliptin, a DPP-4 inhibitor, enhances incretin activity and insulin secretion, whereas pioglitazone, a thiazolidinedione, improves insulin sensitivity. Owing to their complementary mechanisms, simultaneous estimation of these drugs in pharmaceutical formulations and biological matrices is important for quality control and pharmacokinetic investigations.

A detailed literature survey reveals that several analytical methods have been reported for the estimation of vildagliptin and pioglitazone either individually or in combination with other drugs using UV spectrophotometry, RP-HPLC, HPTLC, and LC–MS/MS techniques. However, these methods exhibit certain limitations. Many reported RP-HPLC methods are based on conventional trial-and-error optimization, leading to limited robustness and higher method variability. Some methods demonstrate longer run times, inadequate peak resolution, or suboptimal sensitivity. Additionally, very few studies have addressed simultaneous quantification of this combination in human plasma, and none have reported a statistically optimized Quality by Design (QbD)-based RP-HPLC method with defined design space and risk assessment.

Traditional analytical method development approaches often lack systematic evaluation of critical method parameters (CMPs) and their impact on critical analytical attributes (CAAs), which may result in reduced reproducibility and regulatory challenges. In

contrast, Quality by Design (QbD) provides a structured, science-based framework that emphasizes risk assessment, statistical experimental design, and establishment of a method operable design region. This approach aligns with guidelines of the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH Q8, Q9, and Q10), ensuring enhanced method robustness, lifecycle management, and regulatory acceptability. In the context of bioanalytical estimation in plasma—where matrix interference, sensitivity, and precision are critical—application of QbD becomes particularly important to systematically control variability and achieve reliable quantification.

Therefore, the present study aims to develop and statistically optimize a robust, sensitive, and reproducible RP-HPLC method for the simultaneous quantification of vildagliptin and pioglitazone hydrochloride in human plasma using a Box–Behnken experimental design under the QbD framework. The method intends to establish a well-defined design space, evaluate the influence of critical chromatographic parameters on analytical performance, and validate the optimized method in accordance with regulatory guidelines to ensure high sensitivity, accuracy, and robustness.

MATERIALS AND METHODS

Materials

The Vildagliptin and Pioglitazone hydrochloride sample was provided by Vivan life Science Pvt. Ltd. Mumbai. The commercially available tablet formulation of Vylda-P tablets of Emcure Pharmaceuticals Ltd was used for assay. HPLC grade methanol, Acetonitrile, Water and orthophosphoric acid (AR grade) were sourced from Merck Ltd. for the study.

Instrumentation and chromatographic conditions

Chromatographic analyses were performed using an Agilent 1100 HPLC system equipped with a G1314A detector featuring a DAD source and a double reciprocating plunger pump capable of constant flow and pressure delivery. UV spectra were recorded on a UV-Visible spectrophotometer (Analytical Technologies Limited, Japan, Model UV-2080) using UV Analyst software. Chromatographic separation was achieved on a Waters C18 column (250 mm × 4.6 mm, 5 µm particle size) using reverse-phase mode. The mobile phase consisted of acetonitrile and 0.1% OPA (7:93) in isocratic elution mode, with a flow rate of 0.9 mL/min. Detection was performed using a PDA detector at 215 nm. Experimental design was facilitated by Design Expert software (trial version 13.0).

Wavelength selection for detection

VILDA and PIO standard solutions were scanned over the range of 200-400 nm, the resulting spectra showed 209 nm and 226 nm absorbance respectively. The Isosbestic point was observed at 215 nm and selected as a detection wavelength (figure 1).

Mobile phase and standard stock solution preparation

The mobile phase consisted of acetonitrile and 0.1% orthophosphoric acid (7:93, v/v). The mixture was filtered through a 0.42 µm membrane filter and sonicated for 15 minutes to ensure thorough degassing and dissolution. Standard stock solutions were prepared by dissolving approximately 50 mg of VILDA and 15 mg of PIO in 100 mL of methanol, yielding concentrations of 500 µg/mL and 150 µg/mL, respectively.

Preparation of plasma sample

The study protocol was approved by the Institutional Ethics Committee (IEC Approval No.: IEC/PHARM/2024/103). Plasma samples were collected from healthy volunteers after obtaining written informed consent in accordance with institutional and national ethical guidelines.

Plasma samples were stored at −20°C until analysis. At the time of analysis, the samples were removed from the deep freezer and kept in the room temperature and allowed to thaw. 2.0 mL of sample was pipetted into 10 ml centrifuge tube with this 0.1 ml of standard stock solution was added. The resulting solution was vortexed for 15 minutes and centrifuged at 5000 RPM for 15 minutes. Organic layer was pipette out into a separate tube and evaporated to dryness. The residue was then reconstituted with 10mL mobile phase and subjected to chromatographic analysis.

QbD-guided method development

Establishing a clear quality target profile (QTP) is crucial in method development, as it facilitates the identification of key variables that influence ideal chromatographic conditions. Critical quality attributes (CQAs) were defined based on the method's intended purpose, such as assay and drug release determination, and the chosen analytical technique, RP-HPLC. Key method parameters that significantly impact the method's performance, including percentage organic modifier in the mobile phase, detection wavelength, column temperature, flow rate, and injection volume, were identified as critical method parameters (CMPs) for the development of a robust bioanalytical RP-HPLC method.

Design of experiment (DOE)

A response surface methodology (RSM) approach was adopted for experimental design, with Box-Behnken design (BBD) chosen over factorial and central composite designs due to its efficiency in modeling interactions between three factors at three levels with minimal runs. The CMPs - mobile phase composition, flow rate, and wavelength - were optimized using BBD, with 17 experimental runs generated using Design Expert software (trial version 13.0) (Table 1 & 2). The optimized method was then validated according to ICH guidelines, evaluating specificity, linearity, accuracy, precision, robustness, limit of detection (LOD), and limit of quantification (LOQ)[23,24].

RESULT AND DISCUSSION

Method development studies

A Bioanalytical RP-HPLC method was developed through systematic optimization of chromatographic conditions, including mobile phase composition (various ratios of methanol, water, acetonitrile, and orthophosphoric acid), flow rate (isocratic and gradient modes), column type, and temperature. Preliminary investigations revealed that a mobile phase consisting of acetonitrile and 0.1% orthophosphoric acid provided optimal peak symmetry and minimal tailing (figure 2), making it a suitable choice for further development.

Application of QbD principles for optimizing RP-HPLC method

The QTP was defined based on retention time, peak area, and theoretical plates to optimize HPLC conditions. BBD was employed to further optimize parameters within the design space, with a quadratic model selected for data analysis. The optimized model parameters are presented in (Tables 3 & 4). Adequate precision was evaluated using the signal-to-noise ratio, with a ratio > 4 indicating a satisfactory signal. Model summary statistics for VILDA and PIO are shown in (Table 5), enabling navigation of the design space. The BBD responses were visualized using 2D contour plots and 3D surface plots (figure 3), illustrating the impact of independent variables on dependent variables. The optimized method, consisting of acetonitrile and 0.1% OPA (7:93) as the mobile phase, a flow rate of 0.9 mL/min, and UV detection at 215 nm, yielded appeal close to 1.0, with critical analytical attributes within the anticipated range.

Method Validation Studies

Selectivity

The results showed no interfering peaks at the retention times of VILDA, PIO, and IS in plasma samples (Table 6, figure 4), confirming the method's selectivity.

Linearity

The concentration and area of VILDA (Y=242.09x+183.75 and R2 =0.9991) and PIO (Y=605.91x+77.67 and R2 = 0.9995) were found to have linear relationship (figure 5).

Precision and Accuracy

Table 7 shows the precision experiment results in terms of intra-day and inter-day of VILDA and PIO. The accuracy was determined in terms of percentage recovery. For this, a predetermined concentration of 10 µg/ml for VILDA and 3 µg/ml for PIO was considered and an amount of 80%, 100%, and 120% of the drug was added.

Robustness study

Triplicate experiments were conducted using 20 µg/mL VILDA and 6 µg/mL PIO to evaluate method robustness. The effects of deliberate changes in flow rate, mobile phase composition, and detection wavelength on peak area were assessed (Table 8), with results expressed as mean area, standard deviation (SD), and relative standard deviation (% RSD). The % RSD values were below 2%, indicating that the method is robust.

Limit of detection and limit of quantification

The developed method demonstrated high sensitivity, with limits of detection (LOD) and quantification (LOQ) of 0.036 µg/mL and 0.111 µg/mL for VILDA, and 0.008 µg/mL and 0.025 µg/mL for PIO, respectively.

Assay of Tablet

The % assay of drug content was found 99.52 % and 99.45 % for VILDA and PIO respectively. The results of marketed formulation having brand name (Vylda-P) was shown in (Table 9) & (figure 6).

CONCLUSION

A statistically optimized and experimentally validated bioanalytical RP-HPLC method was successfully developed for the simultaneous quantification of Vildagliptin and Pioglitazone hydrochloride in bulk drug, pharmaceutical dosage forms, and human plasma.

The method demonstrated excellent linearity (R^2 > 0.999 for both analytes), high precision (%RSD < 2%), and satisfactory accuracy (recovery within 98–102%). The limits of detection and quantification were in the low microgram range, confirming adequate sensitivity for plasma-level estimation. Robustness testing under

deliberate variations showed minimal analytical variability, validating the stability of the optimized chromatographic conditions.

Importantly, this study represents the first reported QbD-driven RP-HPLC method for the simultaneous estimation of vildagliptin and pioglitazone in human plasma, where a systematic Box–Behnken experimental design was employed to define a statistically significant quadratic model ($p < 0.05$) and establish a reliable method operable design region. The significant regression coefficients and non-significant lack-of-fit confirmed strong model predictability and analytical control.

Unlike conventional trial-and-error approaches, the present strategy provided mechanistic insight into the influence of mobile phase composition, flow rate, and wavelength on chromatographic performance, thereby enhancing method robustness and regulatory defensibility.

Overall, the developed method offers a scientifically justified, reproducible, and sensitive analytical platform suitable for routine quality control and potential bioanalytical applications, while demonstrating the practical and regulatory advantages of integrating Quality by Design principles into chromatographic method development.

TABLES

Table 1. Selection of the level for BBD design

Independent variable	Low	Intermediate	High
Mobile phase (% of Organic modifier)	85	90	95
Flow rate (ml/min)	0.9	1	1.1
Wavelength (λmax)	213	215	217

Table 2. Optimization of parameters using BBD design

Std	Run	Factor 1 A: Mobile Phase (%)	Factor 2 B: Flow Rate (ml/min)	Factor 3 C: Wavelength (nm)	VILDA			PIO		
					Response 1 R1(RT-1)	Response 2 R2 (Area-1)	Response 3 R3(TP-1)	Response 4 R4(RT-2)	Response 5 R6 (Area-2)	Response 6 R7(TP-2)
11	1	90	0.9	217	6.878	1368.8	7825	14.043	415.44	7435
5	2	85	1	213	4.438	1677.2	7128	6.343	507.74	6562
10	3	90	1.1	213	5.688	1515.8	6326	11.65	450.44	6138
8	4	95	1	217	7.731	1271.6	6345	19.567	377.47	6305
14	5	90	1	215	6.283	1398.8	6920	12.894	434.79	6492
4	6	95	1.1	215	6.829	1302.0	4098	17.208	398.79	3691
15	7	90	1	215	6.283	1398.8	6920	12.894	434.79	6492
3	8	85	1.1	215	4.037	1273.1	6269	5.784	407.52	5448
2	9	95	0.9	215	7.844	1461.0	5459	19.765	450.9	5390
9	10	90	0.9	213	6.954	1859.8	5887	14.187	553.58	5506
17	11	90	1	215	6.283	1398.8	6920	12.894	434.79	6492
1	12	85	0.9	215	4.942	1558	7244	7.082	496.62	6106
6	13	95	1	213	7.719	1708.5	5731	19.518	502.52	5374
13	14	90	1	215	6.283	1398.8	6920	12.894	434.79	6492
16	15	90	1	215	6.283	1398.8	6920	12.894	434.79	6492
7	16	85	1	217	4.432	1229.6	7351	6.337	385.80	6235
12	17	90	1.1	217	5.683	1125.1	4898	11.607	346.36	4588

RT: Retention Time (min), TP: Theoretical plate

Table 3. ANOVA responses of BBD design for VILDA

Source	Sum of Squares	df	Mean Square	F-value	p-value
For Retent					

ion Time Model	21.66	9	2.41	124.18	< 0.0001	Significant
A-Mobile Phase	18.83	1	18.83	971.72	< 0.0001	
B-Flow Rate	2.40	1	2.40	123.80	< 0.0001	
C-Wavelength	0.0007	1	0.0007	0.0363	0.8543	
For Area						
Model	5.507E+05	9	61190.61	84.29	< 0.0001	Significant
A-Mobile Phase	3.53	1	3.53	0.0049	0.9464	
B-Flow Rate	1.330E+05	1	1.330E+05	183.24	< 0.0001	
C-Wavelength	3.899E+05	1	3.899E+05	537.04	< 0.0001	
For Theoretical plate						
Model	1.417E+07	9	1.575E+06	41.79	< 0.0001	Significant
A-Mobile Phase	4.779E+06	1	4.779E+06	126.79	< 0.0001	
B-Flow Rate	2.700E+06	1	2.700E+06	71.65	< 0.0001	
C-Wavelength	2.268E+05	1	2.268E+05	6.02	0.0439	

Table 4. ANOVA responses of BBD design for PIO

Source	**Sum of Squares**	**df**	**Mean Square**	**F-value**	**p-value**	
For Retention Time Model	329.68	9	36.63	311.59	< 0.0001	Significant
A-Mobile Phase	318.93	1	318.93	2712.83	< 0.0001	
B-Flow Rate	9.74	1	9.74	82.86	< 0.0001	
C-Wavelength	0.0026	1	0.0026	0.0220	0.8861	
For Area						
Model	43598.32	9	4844.26	102.09	< 0.0001	Significant
A-Mobile Phase	578.24	1	578.24	12.19	0.0101	
B-Flow Rate	12280.41	1	12280.41	258.81	< 0.0001	
C-Wavelength	29915.51	1	29915.51	630.47	< 0.0001	
For Theoretical plate						
Model	1.213E+07	9	1.348E+06	39.58	< 0.0001	Significant
A-Mobile Phase	1.612E+06	1	1.612E+06	47.34	0.0002	
B-Flow Rate	2.613E+06	1	2.613E+06	76.74	< 0.0001	
C-Wavelength	1.208E+05	1	1.208E+05	3.55	0.1017	

Table 5. Model Summary

VILDA	Source	SD	R^2	Adjusted R^2	Predicted R^2	Adequate Precision
For Retention Time	Quadratic	0.1392	0.9938	0.9858	0.9004	38.9977
For Area	Quadratic	26.94	0.9909	0.9791	0.8537	33.8458
For Theoretical plate	Quadratic	194.14	0.9817	09582	0.7076	24.1491
PIO						
For Retention Time	Quadratic	0.3429	0.9975	0.9943	0.9602	56.4122
For Area	Quadratic	6.89	0.9924	0.9827	0.8790	37.9814
For Theoretical plate	Quadratic	184.53	0.9807	0.9559	0.6916	25.4565

Table 6. Selectivity result

Concentration	Area	%RSD	Amount found	%Label Claim
Blank	0	0	0	0
20 µg/ml of VILDA	5012.50	0.023	19.95	99.77
6 µg/ml of PIO	3699.91	0.046	5.98	99.64

Table 7. Precision and Accuracy

Precision

VILDA							PIO						
Concentration	Intraday			Interday			Concentration	Intraday			Interday		
(µg/ml)	% Amt Fnd	SD	%RSD	% Amt Fnd	SD	%RSD	(µg/ml)	% Amt Fnd	SD	%RSD	% Amt Fnd	SD	%RSD
10.00	101.58	5.59	0.21	99.13	2.60	0.10	3.00	100.71	1.54	0.08	99.67	0.92	0.05
15.00	100.17	27.94	0.73	99.83	3.42	0.09	4.50	100.85	3.85	0.14	100.76	0.67	0.02
20.00	99.74	0.38	0.01	98.99	34.90	0.70	6.00	99.87	2.29	0.06	99.08	1.08	0.03

Accuracy

% Excess drug Added	VILDA Avg.% Recovered	SD	%RSD	% Excess drug Added	PIO Avg.% Recovered	SD	%RSD
80%	99.11	0.21	0.22	80%	98.52	0.24	0.25
100%	99.54	0.06	0.06	100%	99.57	0.16	0.16
120%	99.85	0.07	0.07	120%	99.99	0.22	0.22

Table 8. Robustness study

	VILDA			PIO		
	Mean Area	SD	%RSD	Mean Area	SD	%RSD

Flow rate at 0.8 ml/min	5425.21	7.10	0.13	4023.39	3.51	0.09
Flow rate at 1 ml/min	5006.85	4.78	0.10	3721.05	6.22	0.17
Ratio (8:92)	5344.1	48.70	0.91	3972.4	3.07	0.08
Ratio (6:94)	5367.66	3.63	0.07	3982.53	4.38	0.11
Wavelengtth 214 nm	5829.5	31.52	0.54	4264.5	23.58	0.55
Wavelengtth 216 nm	5026.05	27.24	0.54	3710.92	21.23	0.57

Table 9. Analysis of marketed formulation

Concentration (µg/ml)	**Area of VILDA**	**SD**	**% RSD**
20	500.46	3.182	0
Concentration (µg/ml)	**Area of PIO**	**SD**	**%**
6	3693.13	4.087	0

Table 10. Summary of Validation Parameter

Parameter	**VILDA**	**PIO**
Linearity Range (µg/ml)	5-25	1.5-7.5
Slope	242.0m	605.9m
Intercept	183.7c	77.67c
Regression	0.9991	0.9995
Accuracy (%Recovery)	99.11-99.85	98.87-99.99
Precession (%RSD)	0.01-0.73%	0.062
Assay (%)	99.52	99.45
LOD	0.0369	0.008
LOQ	0.1119	0.025

FIGURES AND GRAPHS

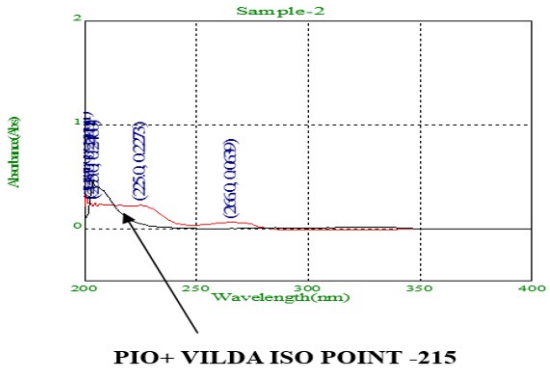


Figure 1. Isosbestic point of combination drug at 215nm

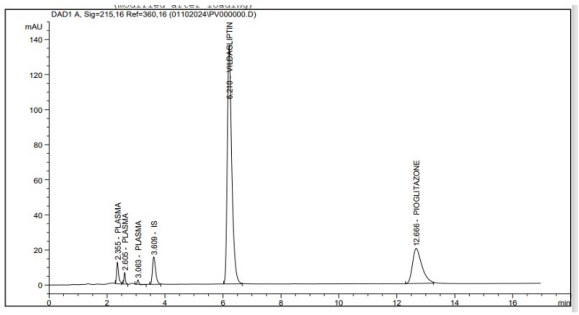


Figure 2. Representative Chromatogram of VILDA and PIO with Plasma

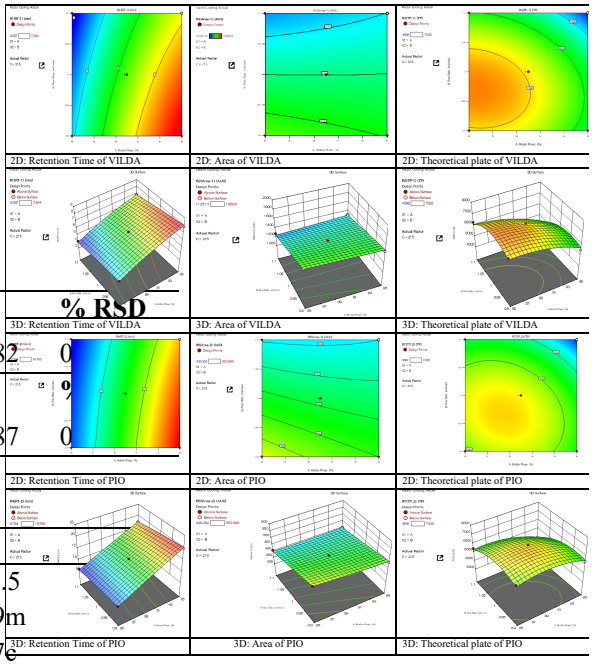


Figure 3. Effect of independent variables on the dependent variables

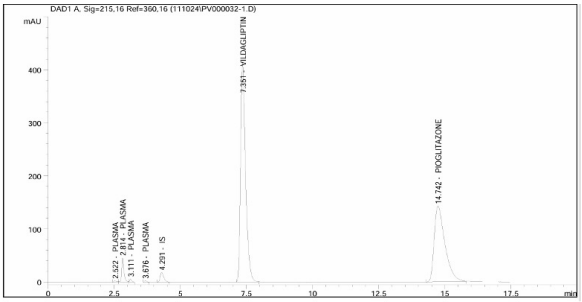
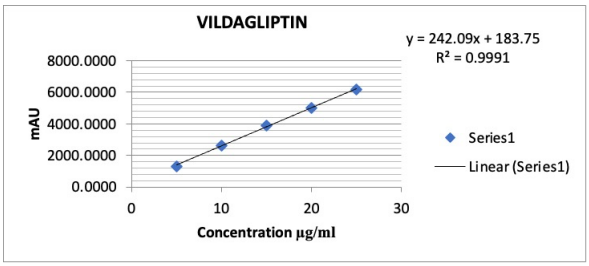


Figure 4. Selectivity Chromatogram of VILDA & PIO with Plasma



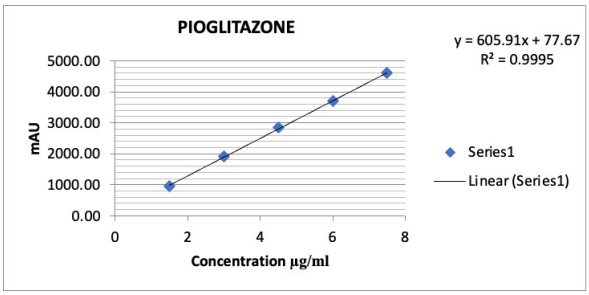


Figure 5. Calibration curve of both drug VILDA and PIO over optimised range.

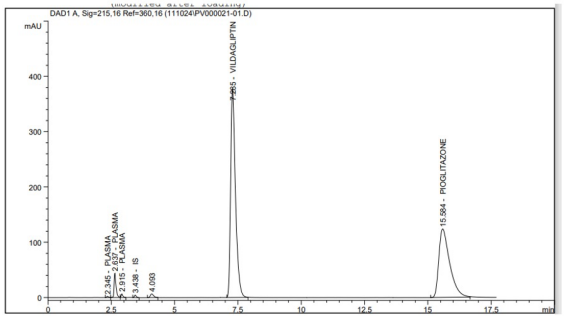


Figure 6. Representative Assay chromatogram of VILDA and PIO with plasma.

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CONFLICT OF INTEREST

The authors have no conflicts of interest regarding this investigation.

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