PHARMACIA

Pharmacia 70(1): 27–37
DOI 10.3897/pharmacia.70.e95099

Research Article

Analytical quality by design-based RP-HPLC method for quantification of pioglitazone and candesartan cilexetil in bilayer tablet and its forced degradation studies

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Received 20 September 2022 ♦ **Accepted** 25 October 2022 ♦ **Published** 9 January 2023

Citation: Kola H, Shanmugasundaram S (2023) Analytical quality by design-based RP-HPLC method for quantification of pioglitazone and candesartan cilexetil in bilayer tablet and its forced degradation studies. Pharmacia 70(1): 27–37. https://doi.org/10.3897/pharmacia.70.e95099

Abstract

Aim: The current project involves developing an RP-HPLC method for simultaneous quantification of Candesartan Cilexetil and Pioglitazone based on analytical quality by design (AQbD).

Materials and methods: When analysed in the Design Expert application, the critical method parameters were systematically refined using Central Composite Design and contours were derived for significant variables. A contour plot has been used to discover the technique operable design region that governs response variation, which is then empirically tested.

Results: Successful chromatographic separation of title analytes was achieved on kromasil C18 (150 × 4.6 mm, 5 µm) column at 30 °C with mobile phase comprising 60% 20 Mm Potassium dihydrogen orthophosphate and 40% acetonitrile (v/v), isocratic elution pattern, 0.9 mL/min flow rate, and UV detection at 220 nm. The linear model for Candesartan Cilexetil was from 4 to 24 µg/ mL and Pioglitazone at 7.5–45 µg/ mL, respectively.

Conclusion: The method met all the ICH Q2 (R1) validation criteria. The current approach aided for analysing simultaneous drugs can be expanded into quantifying drugs in biological matrix predominance with maximum recovery.

Keywords

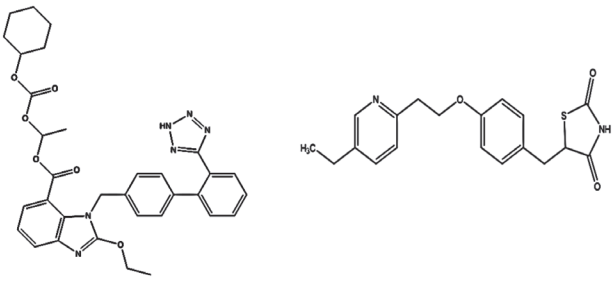
HPLC, AQbD, candesartan cilexetil, pioglitazone, bilayer tablet, degradation

Introduction

Both hypertension and diabetes have become much more inevitable around the world, furthermore, the population of adults with hypertension and diabetes is projected to increase from 972 million in 2000 to 1.56 billion in 2025, and from 171 million in 2000 to 366 million in 2030 (Wild et al. 2004; Kearney et al. 2005; Kaku et al. 2011). Because hypertension is usually associated with type 2 diabetes (Shriraam et al. 2021; Wang et al. 2021) in the same patients, many hypertensive patients are treated with a combination of antihypertensive and anti-diabetic medications. In hypertension, the combination of pioglitazone with Candesartan Cilexetil has a more favourable effect

on hypertensive cardiovascular damage (Nakamura et al. 2008; Nesti et al. 2021). It is estimated that between 60 and 65% of people who have diabetes also have hypertension, making it the most common comorbidity seen in diabetic patients (Jahan Sathi et al. 2022). Both hypertension and diabetes are independent risk factors for cardiovascular disease; however, when they occur together, the chance of the initial complaints of cardiovascular disease is significantly increased. As much as twofold increase in the relative risk of cardiovascular/renal issues is detected among hypertension individuals (Charoensri et al. 2021), and the presence of diabetes doubles the prevalence rate. (Contreras et al. 2000) Insulin resistance also plays an integral part in both hypertension and type 2 diabetes mellitus. (Arima et al. 2002; Reaven 2003; Chaud et al. 2013)

Candesartan Cilexetil is a selective angiotensin II antagonist. This is most widely used to treat all grades of hypertension (Dobrian et al. 2004; Gleiter et al. 2004). Chemical name is [1RS]-1-[cyclohexyloxycarbonyloxy] ethyl-2-ethoxy-1-{[2'-[1H-tetrazol-5-yl]biphenyl-4-yl] methyl}-1H-benzo[d]imidazole-7-carboxylate. White crystals or white crystalline powder constitute white imidazole derivatives. It is quite impossible for Candesartan Cilexetil to dissolve in water and its solubility in methanol is just on the periphery. It melts around 163 °C (Williams 2006) and pKa is 5.6 (Gleiter et al. 2004; Williams 2013). Pioglitazone is a type 2 diabetes medication that is taken orally. After being injected, pioglitazone lessens insulin resistance in the liver, thus causes a rise in insulin-dependent glucose clearance and a fall in hepatic glucose generation (Hofmann et al. 1992; Kletzien et al. 1992). The chemical name is (5RS)-5-{4-[2-(5-Ethylpyridin-2-l)ethoxy] benzyl}thiazolidine-2,4-dione monohydrochloride. It is a solid crystalline powder that is white or almost white in colour. In water, it's practically insoluble, but it's soluble in methanol. Melting point is around 193–194 °C (Williams 2013). The pKa is around 6. Fig. 1 illustrates the structures.



(a) (b)

Figure 1. Structures of (**a**) Candesartan Cilexetil; (**b**) Pioglitazone.

A detailed analysis of the literature found that a UV-Visible spectrophotometric method for simultaneous estimation of the above mentioned two drugs is available (Ware and Pekamwar 2021), along with determination of candesartan with pioglitazone and other drugs (Mirza 2018) Also a single method is reported for simultaneous estimation of drugs in human plasma by LC/MS. (Kumari Karra et al. 2012) Hence, an effort is undertaken to design and validate an analytical approach for simultaneous estimation of Candesartan Cilexetil and pioglitazone by HPLC. The suggested chromatographic method is reliable because it also uses AQbD methodology to design the chromatographic separation. The present investigation is designed and developed for simultaneous determination of Candesartan Cilexetil and Pioglitazone in bulk and in a bilayer tablet.

Method development incorporating QbD

Beyond the standard robust testing, method validation according to the ICH criteria does not give much reliability in terms of mitigating method variability. (Sandhu et al. 2016) In recent times, pharmaceutical companies are adopting QbD in analytics for trouble free compilation with FDA and ICH guidelines. (Peraman et al. 2015a) AQbD delves into the scientific understanding of technique factors and their interconnections, resulting in a region that is both robust and cost-effective. When an AQbD methodology is used in the development stage, an analytical method's flexibility is granted without the need for revalidation or regulatory assessment. (Vogt and Kord 2011; Peraman et al. 2015b; Das and Maity 2017) As a result, using Quality by Design concepts towards the construction of analytical methods has become rather prevalent in order to achieve high robustness and superior method performance. (Monks et al. 2012) In recent times, many analytical methods for single drug estimation (Alruwaili 2021; Babar and Padwal 2021; Jena et al. 2021; Patel et al. 2021; Srujani et al. 2021; Wadhwa et al. 2021) and simultaneous quantification (Gundala et al. 2019; Babar et al. 2021; Saurabh Chaudhari et al. 2022) have been developed using AQbD method. Analytical quality by design refers to the systematic and scientific creation and optimization of analytical procedures. (Palakurthi et al. 2020) The AQbD approach allows a scientific and risk assessed understanding of the major causes of variability, then the identification of CMPs through risk assessment and factor screening studies to identify the significant variables that have a massive effect on analytical performance, and then optimization of those variables through appropriate experimental designs to broaden method performance. (Rozet et al. 2012) Quality by design is well emphasized in the design and manufacture of pharmaceutical drug substance and drug product processes, as specified in ICH Q8, Q9, and Q11 (Palakurthi et al. 2020).

When using a QbD strategy, it is vital that sufficient consideration be paid to the method's intended usage so that method's objectives are articulated. The Analytical Target Profile is reflected by all this. In order to meet the ATP's standards, an acceptable technique and procedure conditions must be chosen. An exercise oriented towards understanding the method can be done based on a risk assessment (i.e., the method complexity and the possibility for robustness and ruggedness issues) to have a better understanding of the impact that key input factors may have on the method's operational qualities. As a result, a set of operational method variables can be identified. Experimentation can thus establish a functional relationship between method

input variables and method performance attributes. Insights obtained during the method's creation and early implementation is fed into risk assessment using tools like the Fishbone diagram that may be used to pick out the factors need to be studied and which need to be regulated. (Jadhav and Tambe 2013) Critical Quality Attributes (CQAs) are measurable chromatogram attributes that must be within a certain limit (or) range to ensure the method's targeted quality. CQAs are used in chromatographic techniques as resolution, theoretical plate number, tailing, and analytical peak's retention time. Critical Method Parameters are variables identified through a procedure called quality risk assessment that do have an impact on Critical Quality Attributes. Following this, based on developmental information and experiments, critical method attributes of this experiment are designated as flow rate, mobile phase ratio, and column temperature.

Materials and methods

Chemicals

Candesartan Cilexetil and Pioglitazone were obtained as gift samples from Madras Pharmaceuticals, Chennai, Tamil Nadu. Dosage form to be analysed claims 8 mg of Candesartan Cilexetil and 15 mg Pioglitazone per tablet. Ortho phosphoric acid, Potassium dihydrogen orthophosphate, sodium chloride, hydrochloric acid, hydrogen peroxide, acetonitrile (HPLC grade) were purchased from sigma-Aldrich, Chennai, India.

Instrumentation and chromatographic conditions

The technique was devised using Agilent technologies 1220 infinity II HPLC system fitted with Kromasil C_{18} (150 × 4.6 mm, 5 µm) column. Digital pH meter (MP -1PLUS) was used for pH measurements and Professional Ultra-Sonicator (ANTECH, GT SONIC) was employed to degas the mobile phase and filtered through 0.45 µm Millipore filter attached to a vacuum pump. Chromatographic separation was achieved using C_{18} column and mobile phase consisting of Buffer and acetonitrile at 60:40 v/v ratios. 20 mM Potassium dihydrogen orthophosphate is used as buffer with pH 3.5 adjusted with ortho phosphoric acid. The method development was performed in HPLC at isocratic system. 10 µL was the injection volume with a flow rate of 0.9 mL /min. UV detection was set at 220 nm for elution. The % organic phase, flow rate, and column temperature were optimized using Central Composite Design through Design Expert Trail version 11 Stat-Ease, U.S.A.

Preparing standard solution

8 mg of Candesartan Cilexetil, 15 mg of pioglitazone working standards were weighed and added to 50 mL dry volumetric flasks and are then diluted with diluent and sonicated. The flask was vigorously shaken before being filled with diluent solution to the desired volume. To obtain the needed standards, 1 mL of each of the two stock solutions was placed in a 10 mL volumetric flask and diluted to 10 mL.

Sample preparation

10 tablets each containing 8 mg of Candesartan Cilexetil and 15 mg of pioglitazone was weighed, powdered and quantity equivalent to label claim, transferred to a 100 mL volumetric flask and dissolved with diluent. The solution was filtered and 2 mL of each of the filtrate i.e., 16 µg/m L of Candesartan Cilexetil and 30 µg/m L of Pioglitazone was diluted to 10 mL with diluent and analysed.

Selection of wavelength

UV spectra of Candesartan Cilexetil and pioglitazone were acquired using a UV-VIS spectrophotometer in order to identify the analytical wavelength. Both stock solutions were scanned against a blank in the UV region between 200 and 400 nm.

Method development with QbD assistance

Analytical target profile

To begin with, Analytical Target Profiles were used to explicitly describe the objectives. HPLC should be able to separate the two drugs in an appropriate timescale by separating them simultaneously. The key aim for improving the chromatogram's quality in terms of retention times, resolution, and theoretical plates were to initially tune the chromatographic conditions, and then to successfully implement the devised method for determining Candesartan Cilexetil and Pioglitazone in tablet dosage form.

Risk assessment – Evaluation of potential dangers

Numerous aspects of HPLC, including the column design and the mobile phase, can have an impact on separation quality. The results of a method can be affected by a variety of other variables, including the temperature of the column, the detection conditions, the sample composition, and the injection volume. Hence fishbone diagram in Fig. 2 was constructed to determine the most critical method attributes (CAA) which is a systematic risk analysis. In this pictorial representation, all the aspects that affect the analytical quality were presented, and those that are crucial to quality were selected for future investigation.

Conducting experimental design

Initial method development trials were undertaken to determine the proper ranges of method variables following a risk analysis. DOE approach was employed to optimize these method variables, and a Central Composite Study design was used for this objective. This design includes centre points and axial points in addition to a full factorial design which contributes to sequential experimentation,

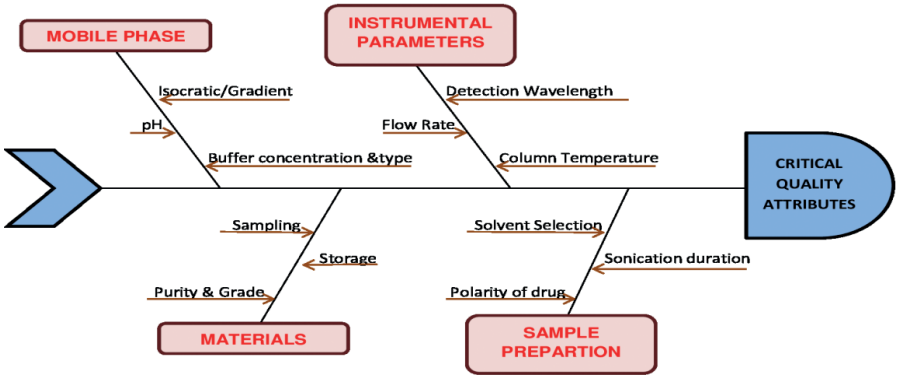


Figure 2. Fish bone diagram.

estimating quadratic or higher order trends. Central Composite Design contributes to the reproducibility and validity of experimental designs. The primary independent factors of the HPLC method were chosen as percentage of organic phase, mobile phase flow rate, and column temperature, as well as the response variables tailing factor, resolution, and number of theoretical plates. The following Table 1 lists the experimental variables.

Table 1. Levels of selected independent variables.

Variables	Level	
Selected variables	low	High
A : Flow rate (mL/min)	0.80	1.00
B: % Organic phase	35.00	45.00
C: Temperature (°C)	27.00	33.00

Analysing the results of the experiments and optimizing the method

Design expert Software was used to conduct a systematic statistical analysis of the experiment's findings. Numerical and graphical optimization followed the specification of variable constraints. Each response parameter was examined individually, and a design space was developed using statistical techniques including anticipated versus actual graphs, ANOVA (Analysis of Variance), lack of fit prediction equations and contour plots.

Validating the analytical method

Linearity

Six samples 0.25, 0.5, 0.75,1, 0.25, 0.5 mL were withdrawn to 10 mL volumetric flasks from binary standard solution and makeup to final volume with diluent until the final concentrations of Candesartan Cilexetil and pioglitazone were in the range of 4–24 µg/ mL and 7.5–45 µg/ mL, respectively. At each concentration level a 10 µl volume was injected and a linearity curve was generated by annotating the peak area against the drug concentration. Using a plot peak area vs. analyte concentration, the method's linearity was validated. This was replicated for six times.

Accuracy

Working standard solutions were analysed by HPLC using chromatographic conditions which were optimized at 50, 100, and 150% concentrations. At each level, the samples were made in triplicate, and the % recovery of the drug was determined by measuring the chromatogram's peak area.

Precision

The developed method's precision was evaluated by calculating the % RSD of within-day and in various days. Samples were triplicated thrice on the same day and also on three different days. The peak area of both drugs was calculated, as well as the % RSD.

Specificity

The purpose of the specificity testing was to show that the principal analyte peaks could be distinguished from placebo, biological matrix, and all related peaks. To ensure that there was no interference from the sample matrix, a placebo solution was also injected into the column. Experimentation with forced degradation of Candesartan Cilexetil and Pioglitazone confirmed the specificity of the devised analytical approach for these two drugs.

Limits of quantification

Limit of detection was established using the equation: LOD = 3.3 σ/S, and limit of quantitation with the help of equation: LOQ = 10 σ/S, where, σ is standard deviation of the responses where S represents slope of calibration curve.

System suitability

The method's efficiency was evaluated by running a system suitability test on chromatograms obtained by loading standard binary solution into HPLC three times. For three successive injections, system fit characteristics such as theoretical plates, tailing factor, retention time, resolution, analyte peak area were assessed.

Robustness of the method

The method's ruggedness was evaluated by deliberately altering the chromatographic conditions like % organic phase, flow rate and temperature of the column by slightly increasing or decreasing them.

Forced degradation studies

Dosage forms were subjected to various stress factors to check for degradants in accordance with the ICH Q1A (R2) guidelines resulting in drug degradation.

Results and discussion

Because of the numerous variables that influence method results, applying QbD paradigm to analytical methods is relatable. These factors include instrument settings, sample properties, procedures and model selection. The present focus is on AQbD, which enables the development of robust analytical techniques. It also aids in identifying, reducing, and controlling potential risks. For quantification of Candesartan Cilexetil and Pioglitazone simultaneously in sample matrix using HPLC, this work utilized the AQbD approach. Retention time of the analytes, resolution, and theoretical plate count were recognized as CQA. The fishbone diagram was created to better comprehend the factors that affect the quality of an analysis. As a result, the three CMA parameters chosen were % of organic phase, flow rate of mobile phase, and column temperature.

Selecting solvents and detection wavelength

Selecting the right solvents was a major hurdle for method development. The mobile phase is generally alluded to as the lifeline of the HPLC system. It is critical in transporting the analyte through the separation column and subsequently to the detector for identifying the separated components. Mobile phase is almost never a single solvent. It entails mixing water with organic solvents, aqueous buffers with polar solvents, or organic solvent mixtures at desired quantities. It is important to use different solvent mixtures in order to achieve desired polarity in the mobile phase for completely solubilize the sample, as well as to control interaction between analyte and stationary phase to attain the ideal degree of separation for separated components in the quickest possible time. Majority of RP-HPLC are performed with buffered aqueous solutions, such as a polar mobile phase, or with other polar solvents, such as methanol or acetonitrile. Polarity governs the sequence of solute elution in HPLC. More polar solutes elute first in RP-HPLC. Both the drugs were soluble in a bunch of solvents majorly used for reverse phase liquid chromatography such as acetone, dimethyl sulfoxide, acetonitrile, ethanol, and methanol and insoluble in water. Methanol and water are used in combination as a mobile phase applied in the initial trail of method development studies. Here, Candesartan Cilexetil was not eluted. Also, when pH of mobile phase was not adjusted, peak tailing and peak broadening occurred. Hence, in order to increase the retention of both the compounds onto the stationary phase, the amount of organic content in mobile phase was adjusted and water is substituted with phosphate buffer. Ortho phosphoric acid was used to deliver the pH to 3.5. Temperature affects the solubility of compounds in the mobile phase in HPLC. The retention time decreases as the column temperature goes up. Each 1 °C increase in temperature reduces retention time by 1–2%. The chromatographic separation process becomes faster and more efficient as the column temperature rises. As a result, column was kept at 30 °C throughout the study to avoid any disruptions with elution.

Candesartan Cilexetil showed maximum absorbance at 222 nm and Pioglitazone at 253 nm. An isosbestic point at 220 nm was observed which was taken as the detection wavelength. Also, the run was extended for 10 minutes to confirm that all traces of components had been removed and the column had been re-equilibrated. A chromatogram created over 7 minutes can be shown in Fig. 3, which displays the peak concentrations of both Pioglitazone and Candesartan Cilexetil.

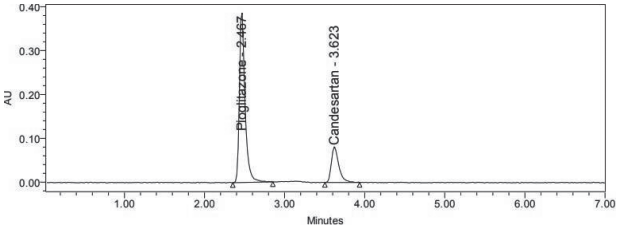


Figure 3. Chromatogram showing peaks of Pioglitazone and Candesartan Cilexetil.

Identifying analytical target profile and risk assessment

The definition of the Analytical Target Profile is a critical component of the AQbD paradigm. It provides the sum of all performance criteria needed to effectively characterize what a technique must analyze. Determining Pioglitazone and Candesartan Cilexetil simultaneously as API and in a tablet dosage form is the target and the liquid chromatographic assay method is the target method. A RP-HPLC with UV detection is selected as the analytical method. The % organic phase, flow rate of mobile phase and column temperature were chosen as the Critical Method Attributes.

Retention times of Pioglitazone and Candesartan Cilexetil, resolution, number of plates for Pioglitazone and Candesartan Cilexetil were chosen as Critical Method Parameters which were found to have effects on the chosen Critical Method Attributes. Thus, risk assessment was based on the fishbone diagram. Temperature of the column plays an important role on the analytical method and hence was chosen as essential parameter. The HPLC equipment and column require a specific flow rate. The analyte may not have enough time to interact with the stationary phase if the flow rate is higher than normal. A high flow rate reduces retention times, whereas lowering the flow rate increases resolution. This change is caused by the flow rate's effect on the number of column plates, not by the relative peak spacing. Hence, flow rate was selected as a factor for the study. Quantity of organic phase plays a tremendous role in analyte retention in RP-HPLC. This is purely based on the polarity of the mobile phase and analytes to be separated. As the stationary phase is non-polar in a reverse phase chromatography, polar of the mobile phase has to be set carefully in order to elute the analytes from the column. Thus, this was also considered as an important factor which impacts the retention times and number of theoretical plates of an analyte.

Method development and optimization of the proposed method

Narrowing down of essential parameters through risk assessment identified risk factors. These were 0.9 mL/min flow rate, 40% organic phase and column temperature of 30 °C. Finally, the optimized mobile phase was 60% volume of 20 Mm Potassium dihydrogen orthophosphate and 40% of acetonitrile. Here Design of Experiment was incorporated and a flow rate of 0.8–1.0 mL/min, 35–45% organic phase and a column temperature of 27–33 °C were set as parameters. Retention times of Pioglitazone and Candesartan Cilexetil, resolution, number of plates for Pioglitazone and Candesartan Cilexetil were observed and optimized as factors. The critical method parameters were optimized using a Central Composite experimental design with three independent variables. Design Expert software was used to simulate the experimental conditions and runs were obtained. The chromatograms from all 20 trials were analysed for retention time, resolution, and count of theoretical plates for peaks of both Candesartan Cilexetil and Pioglitazone. The model prediction equations were estimated and ANOVA was used to statistically analyse the obtained results. Table 2 displays the ANOVA findings for all factors for the quadratic response surface model.

The prediction equations for all the responses are as follows:

- For RT1 : +2.505-0.276A-0.103B-0.211 C-0.126AB-0.115AC- 0.312BC +0.126 A2-0.381B2-0.291C2
- For RT2: +3.656-0.415A+0.231 B- 0.154 C-0.236 AB-0.117 AC-0.126 BC+ 0.143 A2 +0.176 B2+0.243 C2
- For RS : +8.171-0.164 A+0.903 B-0.255 C- 0.012 AB-0.137 AC-0.121 BC- 0.191 A2-0.126 B2-0.179 C2
- For TP1 : +7648.47-29.792A-457.889B+89.448C-53.5AB-195.25AC+61.75 BC-148.087 A2-786.074 B2-322.742 C2
- For TP2 : +9014.95-93.446 A-579.562 B+86.735C-68.125 AB-265.125 AC+43.125 BC-117.601 A2-841.502 B2-355.542 C2.

The co-efficient and statistical results have been tabulated below.

The DOE model was found to be statistically significant (P value < 0.0001) for predicting all responses using ANOVA. The statistical significance of the model can be inferred from its F-values. The F-value for the lack of fit for all components indicates that the lack of fit is insignificant in contrast to the pure error. This is clearly a nice fit for the model. The regression coefficients show that the variables chosen for the study had a considerable influence on the replies. The interaction terms show how the responses alter when variables are simultaneously changed. Using contour plots, we were able to better understand the relationship between variables through Fig. 4.

Defining design space

The design space or control space defined as the method operable design (MODR) is established by the specific CMA combinations. Fig. 5 displays the contour or desirability plots for various combinations of CMAs for the optimized method.

In this design space, the selected point or a desirability region is a combination of mobile phase consisting of acetonitrile in organic phase (40%) at 30 °C column temperature with 0.9 mL/min flow rate. At this point, the predicted experimental conditions were RT1 at 2.562, RT2 at 3.635, RS at 3.521, TP1 at 6697 and TP2 at 7613. The predicted experimental conditions were confirmed experimentally with RT1 at 2.462, RT2 at 3.598, RS at 3.529, TP1 at 6458 and TP2 at 7365.The design space depicts this method operable zone where

Table 2. ANOVA results and statistical parameters of the optimized method for Pioglitazone and Candesartan Cilexetil.

CMA	RT1		RT2		RS		TP1		TP2	
co-efficients	p- value	F value	p- value	F value	p- value	F value	p- value	F value	p- value	F value
Numerical coefficient	< 0.0001	1168.90	< 0.0001	772.39	< 0.0001	34.31	< 0.0001	57.28	< 0.0001	71.60
A	< 0.0001	10408.10	< 0.0001	5131.40	0.0150	8.59	0.0050	4.751	0.0057	4.60
B	0.0181	2.06	< 0.0001	1587.35	< 0.0001	259.71	< 0.0001	112.22	< 0.0001	176.94
C	0.0377	8.528	< 0.0001	86.76	0.0011	20.71	0.00153	4.28	0.00745	3.96
AB	0.0114	2.98	0.0007	23.33	0.8680	2.991	0.00036	0.8974	0.0025	1.43
AC	0.0143	2.52	0.0049	50.26	0.0901	3.52	0.0062	11.95	0.0009	21.69
BC	0.0608	2.796	0.0063	11.87	0.0086	2.691	0.0029	1.20	0.00466	5.739
A^2	< 0.0001	97.12	< 0.0001	59.12	0.0012	2.80	0.0055	12.39	0.00197	7.69
B^2	0.0414	7.242	< 0.0001	60.09	0.0427	5.38	< 0.0001	349.00	< 0.0001	393.63
C^2	0.0414	7.242	0.0958	9.212	0.0081	10.83	< 0.0001	58.83	< 0.0001	70.27
Fit statistics										
R^2	0.9991		0.9986		0.9686		0.9810		0.9847	
Adjusted R^2	0.9982		0.9973		0.9404		0.9638		0.9710	
Predicted R^2	0.9933		0.9889		0.7654		0.8466		0.8796	
Std.Dev.	0.0100		0.0214		0.2073		159.74		161.01	
Mean	2.52		3.72		7.90		6790.20		8117.25	
C.V.%	0.3980		0.5770		2.62		2.35		1.98	
Lack of Fit										
P	0.5733		0.6500		0.9966		0.2510		0.2709	
F	1.72		1.95		0.64		1.56		1.67	

Where: A – Flow rate (mL/min); B – % Organic phase; C – Temperature in °C.

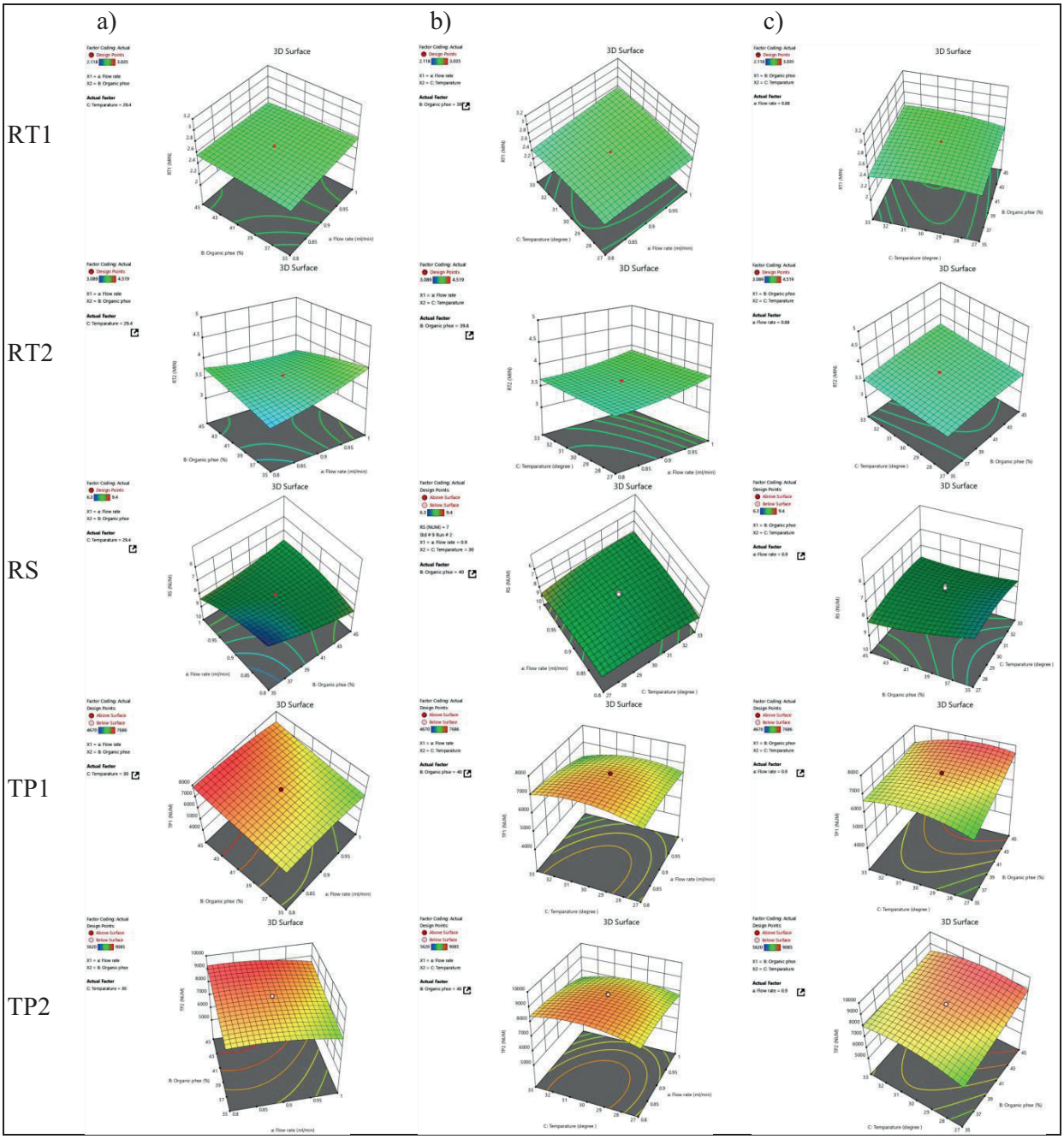


Figure 4. 3-D contour plots for dependent variables in developing the optimized model. **a**) indicates interaction of flow rate and organic phase **b**) indicates interaction of flow rate and temperature **c**) indicates interaction of organic phase and temperature.

alterations will have no effect on the analysis quality. Design space is an essential component of QbD. It was established by creating a plot by overlaying plots of independent variables with their ranges to meet the required response while keeping one variable constant. The yellow color in this plot denotes the region when all response variable requirements are met and no revalidation is necessary.

Validation of the analytical method

The validation parameters such as accuracy, precision, linearity and robustness of the optimized method were evaluated at a selected working point. The linearity equation is determined and co-efficients are shown in Table 3 analysed from Fig. 7.

The r^2 values derived by linear regression analysis were 0.9999 and 0.9997 respectively for Pioglitazone and Candesartan Cilexetil demonstrating the linearity between peak area and the drug concentration. The calculated LOD, LOQ values mentioned in Table 3 assured that the technique is sufficiently sensitive. Through accuracy and precision investigation, % recoveries ranged from 98 to 99 for Pioglitazone and from 98 to 100 for Candesartan Cilexetil. Precision in terms of % RSD were < 1% as mentioned in Table 4.

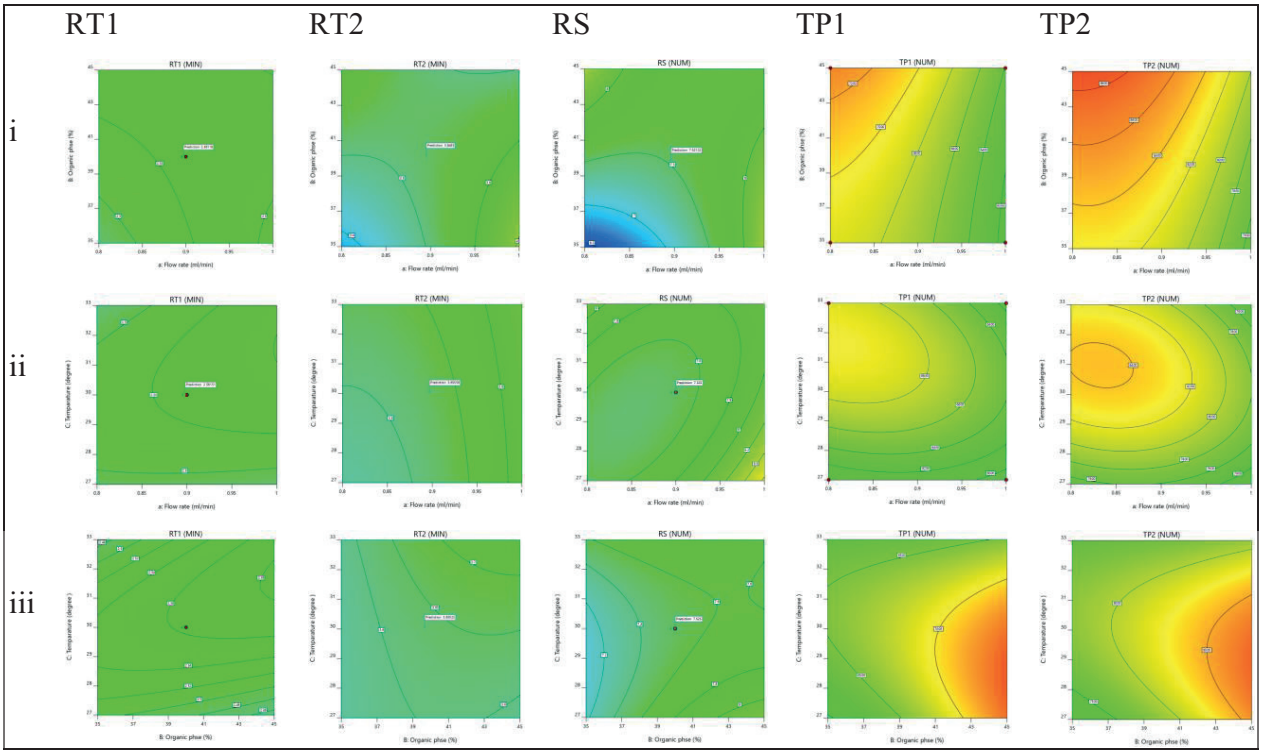


Figure 5. Contour plots for optimized method for simultaneous estimation of Pioglitazone and candesartan Cilexetil: i) Interaction between flow rate and organic phase. ii) Interaction between flow rate and temperature. iii) Interaction between organic phase and temperature.

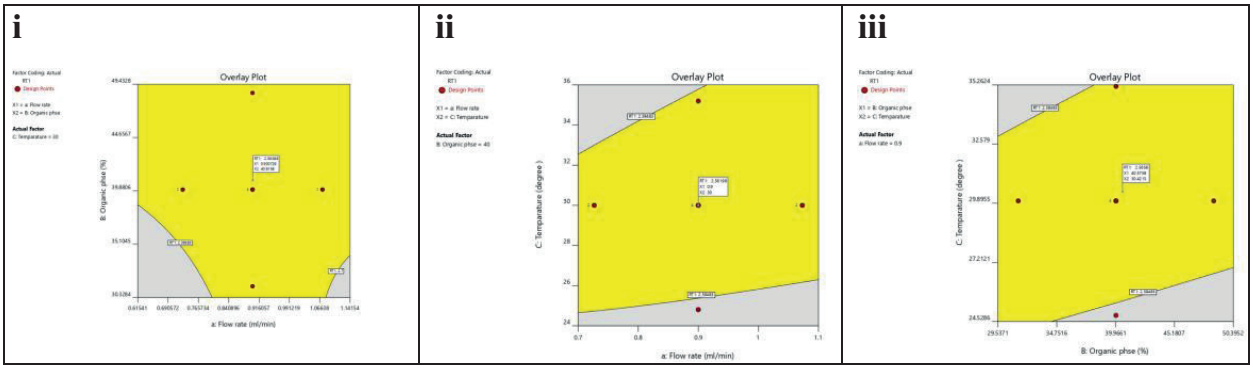


Figure 6. Overlay plots for: i) Interaction of flow rate and organic phase. ii) Interaction of flow rate and temperature. iii) Interaction of organic phase and temperature.

Table 3. Linearity data obtained for Pioglitazone and Candesartan Cilexetil.

Parameter	Pioglitazone	Candesartan Cilexetil
Linearity range(µg/mL)	7.5–45	4–24
Slope	71142	36342
Intercept	1941.9	6687.2
Co-efficient of determination(R^2)	0.9999	0.9997
LOD(µg/mL)	0.71	0.51
LOQ(µg/mL)	0.51	1.56

* n = 6.

The percentage recovery of both drugs when tested simultaneously was found to be within limits. Both Pioglitazone and Candesartan Cilexetil had percentage recoveries that were statistically acceptable. It was also revealed that the drug peaks did not interfere with one another. As a result, the approach can be said to be specific to the two drugs in combination.

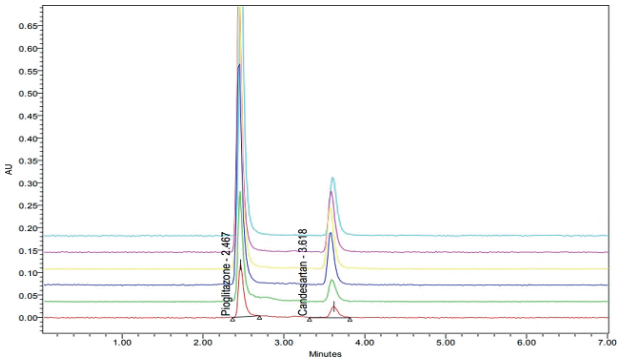


Figure 7. Linearity overlay chromatogram for Pioglitazone and Candesartan Cilexetil.

The method's ruggedness/robustness was evaluated by purposefully modifying chromatographic parameters like % mobile phase, flow rate, and column temperature.

Table 4. Accuracy, Precision studies of Pioglitazone and Candesartan Cilexetil.

	Accuracy* (% recovery)		Precision* (% RSD)		Robustness* (% RSD of peak area)		
	Inter day	Intra day	Inter day	Intra day	Factor	+	-
a) Accuracy, Precision studies of Pioglitazone							
15 µg/ mL	98.82 ±0.12	99.89±0.78	0.413	0.395	A (±0.1 mL/min)	0.610	0.483
30 µg/ mL	99.71 ±0.34	98.97±0.64	0.621	0.578	B (± 5 °C)	0.521	0.767
45 µg/ mL	99.07 ±0.19	99.22±0.81	0.892	0.644	C (± 5%)	0.837	0.818
b) Accuracy, Precision studies of Candesartan Cilexetil							
8 µg/ mL	99.66 ±0.41	99.48±0.92	0.537	0.598	A (±0.1 mL/min)	0.459	0.537
16 µg/ mL	98.51 ±0.37	100.99±0.16	0.761	0.873	B (± 5 °C)	0.564	0.943
24 µg/ mL	98.19 ±0.73	99.81±0.26	0.286	0.492	C (± 5%)	0.446	0.667

* n = 3; Where: A indicates Flow rate; B indicates column temperature; C indicates % Organic phase.

Changes in experimental conditions by increasing and decreasing the flow rate by 0.1 m L/ min, by expanding and diminishing the temperature of the column by 5 degrees and by altering the organic phase by 5% had little effect on chromatogram quality parameters as retention time, resolution, and theoretical number of plates as mentioned in Table 4. % RSD was found to be within limits. As a result, the proposed method can be used for simultaneous, quantitative analysis for the combination of Pioglitazone and Candesartan Cilexetil.

Application of the proposed approach to the prepared formulation

The outcomes of the Candesartan Cilexetil and pioglitazone assays were compared to the labelled quantities. The chromatogram obtained for analysis of the prepared formulation, which shows that there are no additional peaks, indicating that the formulation excipients employed in the tablet do not interact. Candesartan Cilexetil and pioglitazone were found to have a % assay of 100.04% and 99.50%, respectively. These values were within the % limit.

Results for stability studies

Sample chromatograms were exposed to a variety of forced degradation conditions. To induce the stress conditions, 0.1M HCL, 0.1M NaOH, H_2O_2, UV light and high temperatures were used. The solutions underwent stress conditions from 6–7 hrs. This showed an additional peak along with analytes depicted in Fig. 8 at the end of stress duration. However, chromatograms in neutral circumstances did not exhibit distinct peaks for the degradation products. When exposed to acidic deterioration, both Candesartan Cilexetil and Pioglita-

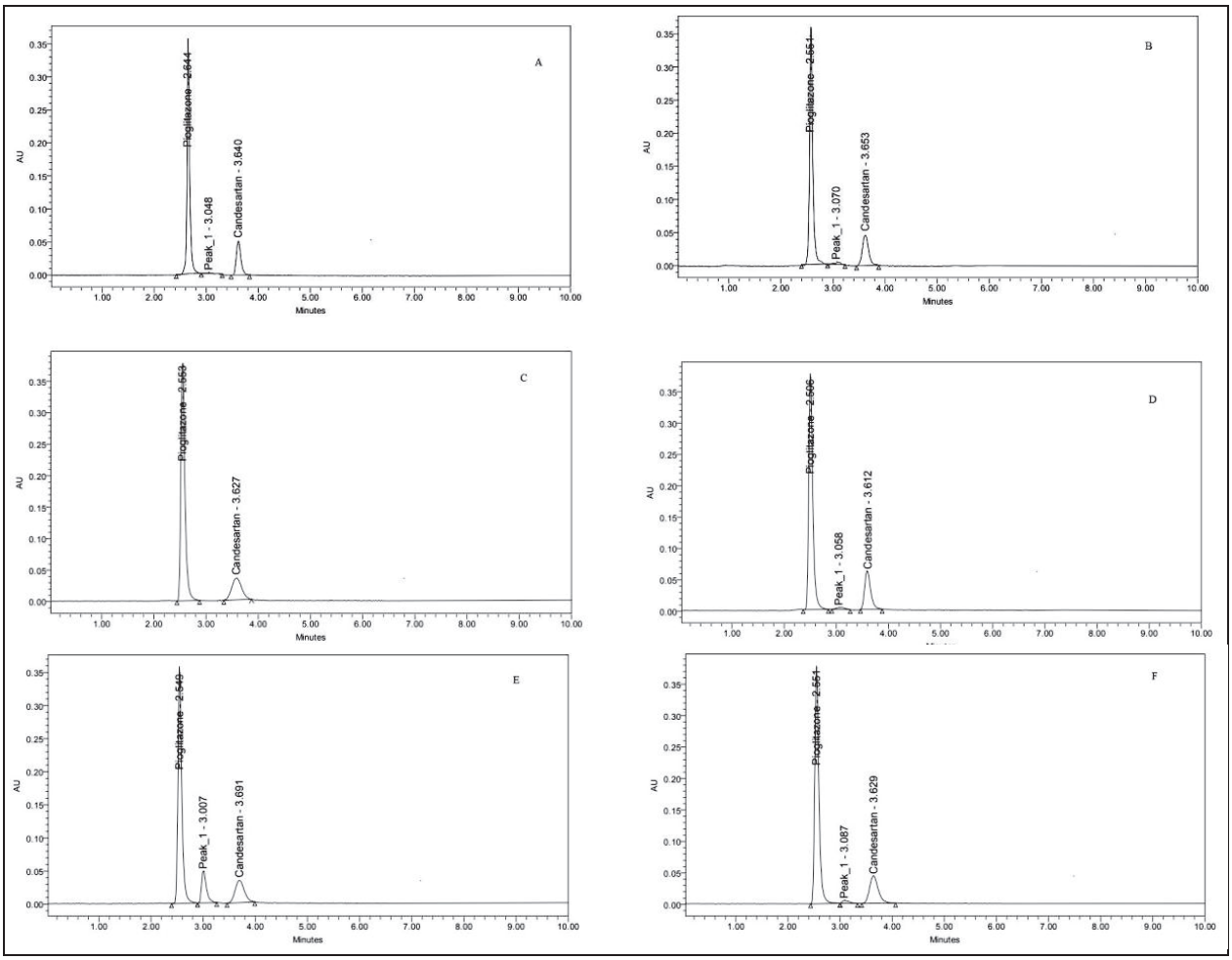


Figure 8. chromatograms of analytes subjected to varying levels of forced degradation: **A**) acidic degradation, **B**) alkali degradation, **C**) neutral, **D**) thermal degradation, **E**) peroxide degradation, **F**) UV-light degradation.

Table 5. Drug decomposition of Pioglitazone and Candesartan Cilexetil due to forced degradation studies.

Degradation conditions	% Drug decomposed		% Drug recovered	
	Pioglitazone	Candesartan Cilexetil	Pioglitazone	Candesartan Cilexetil
Acidic	4.16	5.09	95.84	94.91
Alkali	3.77	4.53	96.23	95.47
Neutral	0.35	0.24	99.65	99.76
Oxidative	2.99	2.06	97.01	97.94
UV light	1.94	1.46	98.06	98.54
Thermal	1.11	1.25	98.89	98.75

zone degraded significantly more than under any other stress state. Summary of degradation studies was reported in Table 5.

Conclusion

This study outlines the development of a systematic RP-HPLC approach for simultaneous quantification of Candesartan Cilexetil and Pioglitazone in API and tablet formulation. AQbD was incorporated to develop an accurate and reliable method which can be used without any revalidation. An optimized method was constructed within the design space and by statistical analysis; effect of the optimized method on various parameters was studied. When experimented, in the optimized method, the chromatographic peaks of both drugs were segregated with good resolution. At the confirmation location, according to the validation report, the proposed analytical method has good linearity, accuracy, precision and robustness.

Author contributions

All authors contributed to the study conception and design. All authors read and approved the final manuscript.

Acknowledgements

The authors are thankful to SRM College of Pharmacy, SRMIST, Kattankulathur, Chennai, Tamil Nadu for providing necessary facilities to carry out this work.

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