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Zalak D. Dave

zalak.dave6@gmail.com

Shri Govind Guru University

Apeksha T Patel

Navinta III INC, boco raton, Florida

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## Research Article

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# CCD-OPTIMIZED HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHIC METHOD FOR THE SIMULTANEOUS ESTIMATION OF CANDESARTAN CILEXETIL AND METFORMIN HYDROCHLORIDE IN MUPS DOSAGE FORM

Zalak D. Dave\*<sup>1</sup>, Apeksha T Patel<sup>2</sup>

\*<sup>1</sup>Assistant Professor, Department of Quality Assurance, Shri Govind Guru University, Vinzol, Post: Kankanpur, Taluka: Godhra District: Panchmahal, Gujarat-388713

<sup>2</sup>Quality Assurance Chemist, Department of Quality Assurance, Navinta III INC, Boca Raton, Florida 33487

Corresponding Author:

Zalak D. Dave\*<sup>1</sup>, Assistant Professor, Department of Quality Assurance, Shri Govind Guru University, Vinzol, Post: Kankanpur, Taluka: Godhra District: Panchmahal, Gujarat-388713

Email ID: Zalak.dave6@gmail.com

## Abstract

**Purpose:** Candesartan Cilexetil and Metformin Hydrochloride, when combined in a newer MUPS-based dosage form, present a need for an HPTLC method that can quantify both drugs at once. This work develops such a method, using Design of Experiment principles to optimize the mobile phase.

**Methods:** Central Composite Design (CCD) guided the optimization of the chromatographic conditions. Three variables were varied during this process: methanol proportion in the mobile phase, chamber saturation time, and distance traveled by solvent. Separation itself took place on aluminium plates pre-coated with silica gel 60 F254, with Methanol, CCl<sub>4</sub>, Ammonium acetate, and Ammonia combined in a 1.32:4.6:4.6:0.1 ratio to form the mobile phase. Candesartan Cilexetil was quantified between 120 and 280 ng/band, while Metformin Hydrochloride was quantified in the range of 2000–6000 ng/band; both were read at 248 nm.

**Results:** The bands were sharp and well resolved. Candesartan Cilexetil appeared at an R<sub>f</sub> of  $0.71 \pm 0.02$ , and Metformin Hydrochloride at  $0.30 \pm 0.02$  a clear separation between the two. The Calibration plots for both drugs showed strong linearity, with  $r^2$  values of 0.9971 for Candesartan Cilexetil and 0.9912 for Metformin Hydrochloride. Precision, accuracy, robustness, specificity, and the limits of detection and quantification were all evaluated in line with ICH guidelines to validate the method.

**Conclusion:** These results confirm that a novel HPTLC method for simultaneously quantifying Metformin Hydrochloride and Candesartan Cilexetil in MUPS formulations was successfully developed and validated. Application of CCD allowed systematic optimization of chromatographic variables, yielding robust and reproducible results. The method demonstrated excellent linearity, precision, accuracy, and robustness, with R<sub>f</sub> values of 0.71 (Candesartan Cilexetil) and 0.30 (Metformin Hydrochloride) under optimized conditions. The low %RSD values and compliance with ICH Q2 (R1) validation criteria confirm its reliability for quality control and routine pharmaceutical analysis. This approach not only ensures accurate quantification but also highlights the utility of experimental design strategies in method development for combination drug formulations.

## Keywords

Candesartan Cilexetil, Metformin Hydrochloride, Central Composite Design, high performance thin layer chromatography, Validation

## Introduction

Metformin hydrochloride, a biguanide derivative, is the first-line pharmacotherapy for patients with type 2 diabetes mellitus (T2DM). Its therapeutic efficacy results from the suppression of hepatic gluconeogenesis and enhancement of peripheral insulin sensitivity. Clinically, metformin is recognized for its low risk of hypoglycaemia, weight neutrality, and cardioprotective properties. Despite these advantages, metformin demonstrates incomplete gastrointestinal absorption, with an absolute bioavailability of 50–60% and a relatively short plasma half-life of 1.5 to 4.5 hours. Gastrointestinal adverse effects, such as nausea, diarrhoea, and abdominal discomfort, can decrease patient adherence, especially during dose initiation. Sustained-release formulations have been introduced to improve tolerability and patient compliance by maintaining plasma drug concentrations for 10 to 16 hours, thereby reducing dosing frequency.

Candesartan Cilexetil is an orally administered angiotensin II type 1 (AT1) receptor blocker (ARB) that functions as a potent and long-acting antihypertensive agent. As a prodrug, it undergoes rapid hydrolysis during gastrointestinal absorption to produce the active compound, Candesartan. Although it offers significant clinical benefits, its therapeutic use is restricted by poor aqueous solubility, which leads to variable bioavailability and bioequivalence. The usual dosage ranges from 4 to 32 mg per day, administered once or twice daily.

Metformin Hydrochloride is included in IP, USP, and BP, while Candesartan Cilexetil is official in the USP, BP, and JP. The combination of these two drugs, however, is not listed in any current pharmacopeia. Although various spectrophotometric, spectrofluorimetric, high-performance liquid chromatography (HPLC), and high-performance thin-layer chromatography (HPTLC) methods have been reported for their individual or combined determination with other drugs, no validated HPTLC method for their simultaneous estimation in a MUPS formulation has been documented to date.

This study presents the first validated high-performance thin-layer chromatography (HPTLC) method utilizing a multivariate design of experiments (DoE) approach, specifically Central Composite Design (CCD), for the simultaneous estimation of Metformin Hydrochloride and Candesartan Cilexetil. CCD was chosen for its ability to capture both main effects and interaction effects among chromatographic parameters within a single, efficient design — a feature that ultimately strengthens the robustness of the method. The primary aim was to establish a simple, accurate, and reproducible analytical procedure appropriate for routine quality control of pharmaceutical dosage forms.

Metformin Hydrochloride, chemically known as 3-(diamino-methylidene)-1,1-dimethylguanidine hydrochloride, is the only available biguanide and remains the first-line drug therapy for patients with Type 2 diabetes mellitus (T2DM). Its mechanism of action involves decreasing hepatic glucose output and reducing peripheral insulin resistance [10]. The primary advantages of metformin include a very low risk of hypoglycaemia, weight neutrality, and a reduced risk of cardiovascular morbidity and mortality [11]. As an oral anti-hyperglycaemic agent, metformin exhibits incomplete absorption from the gastrointestinal tract, with an absolute bioavailability of 50–60% and a relatively short plasma half-life of 1.5 to 4.5 hours [12, 13]. Many patients experience gastrointestinal symptoms like abdominal discomfort, nausea, and diarrhoea, especially in the first weeks of treatment, which

limits wider use of the drug [14]. Side effects and the need for two or three daily doses at higher amounts can also make it harder for patients to stick to their treatment. A sustained-release (SR) version that keeps drug levels steady for 10 to 16 hours could allow Metformin HCl to be taken just once a day.

Candesartan Ciloxetil is chemically ( $\pm$ )-1-Hydroxyethyl 2-ethoxy-1-[p-(o-1H-tetrazol-5ylphenyl) benzyl]-7-benzimidazolecarboxylate, cyclohexyl carbonate (ester), is an orally administered antihypertensive agent characterized by poor water solubility. This property leads to variable bioavailability and bioequivalence. Candesartan is a highly potent, long-acting, and selective angiotensin II type 1 (AT1) receptor blocker. It is administered as the inactive pro-drug Candesartan Ciloxetil, which is rapidly and completely converted to Candesartan during gastrointestinal absorption. The recommended oral dosage of Candesartan Ciloxetil is 4 to 32 mg per day, administered once or twice daily.

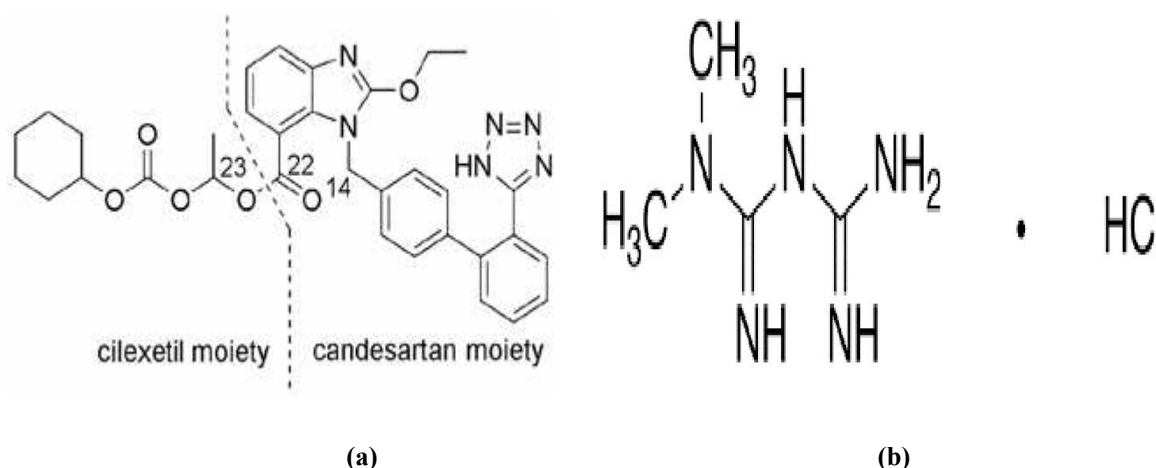


**Figure 1 Chemical structure of (a) Candesartan Ciloxetil (b) Metformin hydrochloride**

Literature survey states that Metformin Hydrochloride is official in IP, USP and BP and Candesartan Ciloxetil is official in USP and BP and JP [1, 6-7] however, combination of Metformin Hydrochloride and Candesartan Ciloxetil is not official in any Pharmacopoeia. Various analytical methods like Spectrophotometry [8-12], Spectrofluorimetry [13, 14], HPLC [15-25] and HPTLC [26-29] methods were reported in literature for the determination of Metformin Hydrochloride and Candesartan Ciloxetil either alone or with other drugs in different dosage forms. However, no analytical methods are reported for combination of Metformin Hydrochloride and Candesartan Ciloxetil.

Hence, this is the first study to report the development and validation of an HPTLC method — following ICH Q2(R1) guidelines — for the simultaneous estimation of Metformin Hydrochloride and Candesartan Ciloxetil. Because multiple chromatographic factors can influence the response simultaneously, a multivariate experimental design approach was adopted rather than varying one factor at a time. Among available design types, CCD is widely regarded as particularly well suited to modelling and optimization tasks of this kind, and it was accordingly used here to optimize the HPTLC separation conditions. Its appeal lies in the flexibility it offers, along with its capacity to reveal both individual factor effects and interactions between them — information that a simpler design would not capture [34]. Viewing the simplicity, frugality, less time-consuming and few processing parameters, the objective of this research work was to develop a simple, rapid, precise and meticulous HPTLC method using Design of Experiment (DOE) approach for quantitative analysis of Metformin Hydrochloride and Candesartan Ciloxetil and method validation in line with ICH guidelines

## **2. Materials and Methods**

### **2.1 Materials**

Analytically pure Metformin Hydrochloride (Alembic pharmaceuticals Ltd., Vadodara, India) and Candesartan Cilexetil (Alembic pharmaceuticals Ltd., Vadodara, India) were obtained as gift samples respectively. All solvents and chemicals used were of analytical grade, purchased from Merck Specialities Pvt. Ltd., India.

### **2.2 Instrumentation**

Hamilton microlitre syringe (Linomat syringe 659.0014, Hamilton-Bonaduz Schweiz, Camag, Switzerland), pre-coated silica gel aluminium plate 60 F<sub>254</sub>, (10 × 10 cm, 100 µm thickness; E. Merck, Darmstadt, Germany), Linomat 5 sample applicator (Camag, Switzerland), Twin trough chamber (20 × 10 cm; Camag, Switzerland), UV chamber (Camag, Switzerland), TLC scanner 4 (Camag, Switzerland) and operated by winCATS version 1.4.6 software (Camag, Switzerland) and electronic balance (AUW 220, Shimadzu Corp., Japan) were used in the study.

### **2.3 Preparation of standard solutions**

10 mg of standard Metformin Hydrochloride and Candesartan Cilexetil were accurately weighed, transferred into two separate 10 ml volumetric flasks, dissolved in methanol and the volumes were made up to the mark with methanol, to obtain solution containing 1000 µg/ml. Aliquots of the stock solutions were appropriately diluted using methanol to yield working standards of 1000 µg/ml of Metformin Hydrochloride and 100 µg/ml of Candesartan Cilexetil.

### **2.4 Chromatographic procedure**

Working standards of varying concentrations were applied as 6 mm bands onto silica gel 60F254 pre-coated aluminium plates, using a micro syringe fitted to a Camag Linomat 5 applicator. Development was carried out by the linear ascending technique in a twin-trough glass chamber, with a mobile phase consisting of methanol, carbon tetrachloride, ammonium acetate (1% in methanol and n-butanol), and ammonia combined at a 1.32:4.6:4.6:0.1 ratio. Prior to development, the chamber was allowed to saturate for 27.81 minutes at room temperature (25 ± 2 °C), and the plates were run over a track length of 7.76 cm. Once development was complete, plates were dried with an air dryer before scanning. Densitometric analysis was performed on a Camag TLC Scanner 4 controlled by winCATS software, operating in reflectance-absorbance mode at 288 nm. Scanning parameters included a slit width of 6.00 × 0.30 mm (micro), a scan speed of 20 mm/s, and a data resolution of 100 µm/step, with a deuterium lamp providing continuous UV radiation across 200-400 nm. Drug concentrations were calculated from the intensity of diffusely reflected light, with quantification based on standard linear regression of peak areas

### **2.5 Software aided method optimization**

To examine how key factors — including main, interaction, and quadratic effects — influence the retardation factor (R<sub>f</sub>) of both drugs, and to optimize the mobile phase composition accordingly, a central composite design (CCD) was employed. CCD occupies a central place within response surface methodology, as it allows quadratic response surfaces to be explored and second-order polynomial models to be constructed without the need for a full three-level factorial experiment [34, 35].

Selection of the critical factors and their optimization ranges drew on both preliminary univariate experiments and prior chromatographic experience. Mobile phase composition itself was defined in terms of the methanol volume relative to the total mobile phase volume. Fifteen experiments, including five center points, were conducted by varying three factors: methanol content in the mobile phase (A), chamber saturation time (B), and distance travelled (C). The R<sub>f</sub> values of Metformin Hydrochloride and Candesartan Cilexetil were measured as responses for both drugs, as shown in Table 1. The nominal values for factors A, B, and C were 1.32 ml, 27.81 min, and 7.76 cm, respectively. Methanol content (A) was varied between 0.16- and 1.84-ml. Chamber saturation time (B) ranged from 26 to 36 min, and distance travelled (C) ranged from 7.6 to 8.6 cm. The coded value of  $\alpha$  was set at 1.41. Design Expert (Version 9.0.0.1, Stat-Ease Inc., Minneapolis, MN, USA) trial version statistical software was used for Data Analysis. The significance of the factors was determined using Bartlett's statistical test for the Analysis of Variance (ANOVA) model. All experiments were randomized to minimize bias from uncontrolled variables. Replicates (n=5) of the center points were included to estimate experimental error.

## **2.6. Method validation**

The HPTLC method was validated for linearity, sensitivity, precision, accuracy, specificity limit of detection (LOD), limit of quantification (LOQ), specificity, robustness, in accordance with ICH Q2 (R1) guideline [36].

### **2.6.1 Linearity**

Varying volumes (1–6  $\mu$ l) of the standard solutions were spotted onto the HPTLC plate to achieve concentration ranges of 120–280 ng/band for Candesartan Cilexetil and 2000–6000 ng/band for Metformin Hydrochloride, with each measurement performed in five replicates. Peak areas obtained were plotted against the corresponding drug concentrations and assessed by ordinary linear regression analysis. Homoscedasticity of variance along each drug's regression line was confirmed using Bartlett's test [37].

### **2.6.2 Sensitivity**

The limit of detection (LOD) and limit of quantification (LOQ) for both drugs were established from the standard deviation of the response and the slope of the calibration curve, following ICH guidelines. These were calculated as:

$$\text{LOD} = 3.3 \times \sigma/S$$

$$\text{LOQ} = 10 \times \sigma/S$$

where  $\sigma$  represents the standard deviation of the y-intercepts of the regression lines, and S is the slope of the calibration curve.

### **2.6.3 Precision**

Method precision was evaluated through both intra-day and inter-day studies. For intra-day precision, three concentrations of each drug — 200, 240, and 280 ng/band for Candesartan Cilexetil (CAN), and 2000, 3000, and 4000 ng/band for Metformin Hydrochloride (MET) — were analyzed in triplicate within a single day. Peak areas were recorded and expressed as percent relative standard deviation (% RSD). Inter-day precision was assessed similarly, using the same concentrations for both drugs analyzed in triplicate across three separate days.

### **2.6.4 Accuracy**

Accuracy of the method was assessed via recovery studies conducted at three levels — 80%, 100%, and 120% of the target concentration. For MET, three different amounts of MET standard (3600, 4000, and 4400 ng/band) were added to the dosage form (2500 ng/band) using the standard addition method. Similarly, for CAN, three different

amounts of CAN standard (200, 240, and 280 ng/band) were added to the dosage form (160 ng/band) using the standard addition method.

#### **2.6.5 Specificity**

Specificity of the method was established by comparing tablet formulation samples against standard drug samples. The identity of Candesartan Cilexetil and Metformin Hydrochloride in the formulation samples was confirmed by comparing R<sub>f</sub> values and overlaying peak purity spectra with those of the reference standards. Peak purity for both compounds was evaluated by comparing spectra at three points along the peak: the start (S), the apex (M), and the end (E).

#### **2.6.6 Robustness**

Robustness of the method was evaluated by introducing small, deliberate variations to key parameters — including the methanol volume in the mobile phase, chamber saturation time, solvent migration distance, and detection wavelength — and assessing their effect on the results. For each parameter, the percentage relative standard deviation (% RSD) of both R<sub>f</sub> values and peak areas was calculated to quantify this effect.

#### **2.7 Analysis of marketed formulation**

The content of Candesartan Cilexetil and Metformin Hydrochloride in the MUPS formulation was determined as follows. Twenty capsules were accurately weighed, and their mean weight was calculated. The powdered contents were then transferred to a glass mortar, and a quantity equivalent to 16 mg of Candesartan Cilexetil and 2500 mg of Metformin Hydrochloride was weighed out and placed in a 10 mL volumetric flask. Five millilitres of methanol were added, and the volume was made up with methanol; the mixture was sonicated for 15 minutes and then filtered through Whatman No. 42 filter paper pre-wetted with methanol. This solution was further diluted to produce a sample stock solution containing 160 µg/mL of Candesartan Cilexetil and 2500 µg/mL of Metformin Hydrochloride. One microlitre of this solution — equivalent to 160 ng/band of Candesartan Cilexetil and 2500 ng/band of Metformin Hydrochloride — was applied to the HPTLC plate, followed by development and scanning. All analyses were performed in triplicate.

### **3. Results and discussion**

#### **3.1 Selection of wavelength**

To determine the optimal detection wavelength, densitometric scanning of the developed HPTLC plate was performed across the UV-Visible range of 200–700 nm, and the resulting overlain spectra were recorded using a CAMAG TLC Scanner 4. Both Candesartan Cilexetil and Metformin Hydrochloride showed strong absorbance at 248 nm, and this wavelength was accordingly selected for detection (Figure 2).

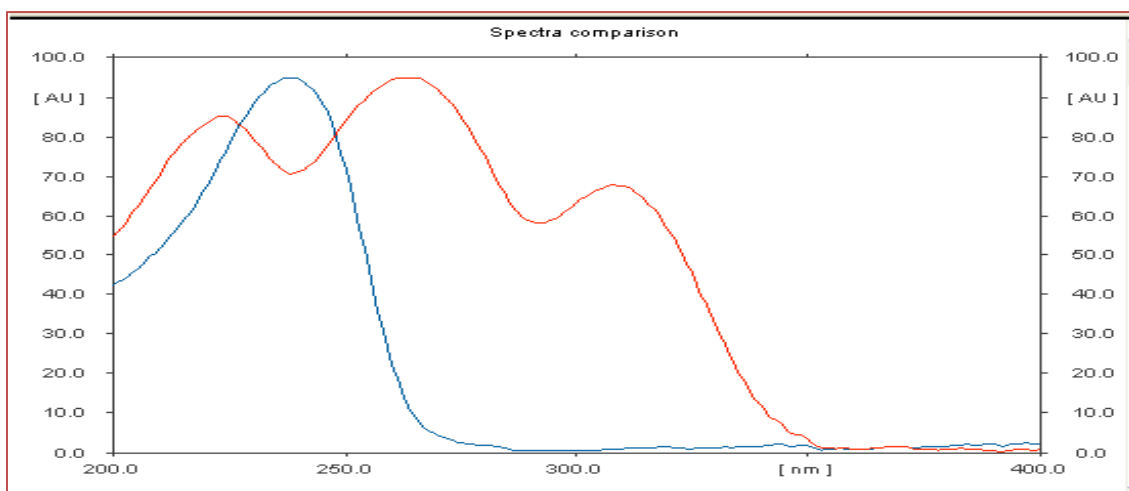


**Figure 2: Overlay UV spectra of Metformin Hydrochloride (1000 ng/band and Candesartan Cilexetil (100 ng/ band) at 248 nm**

### 3.2 Optimization of chromatographic conditions

Chromatographic conditions were optimized to develop a HPTLC method for the simultaneous detection of Metformin Hydrochloride and Candesartan Cilexetil in both bulk substances and pharmaceutical dosage forms.

#### 3.2.1 Preliminary study

Multiple mobile phase combinations were evaluated to optimize the chromatographic peaks of Metformin Hydrochloride and Candesartan Cilexetil. Literature review indicates that HPTLC methods have been reported for these compounds individually or in combination with other drugs, using mobile phases containing methanol, ammonia, chloroform, toluene, glacial acetic acid, n-butanol, ammonium acetate, acetonitrile, dichloromethane, ethanol, formic acid, triethylamine, and water. Accordingly, these solvents were tested in various combinations and proportions during the preliminary study.

Chamber saturation time and solvent migration distance are critical parameters for HPTLC chromatographic separation. In this study, a chamber saturation time of 27.81 minutes was used. Solvent migration distances exceeding 77.6 mm resulted in analyte band diffusion.

Additional chromatographic conditions were optimized to achieve well-defined, compact bands for Candesartan Cilexetil and Metformin Hydrochloride, with acceptable  $R_f$  values (less than 0.8) for both drugs, using the CCD approach.

#### 3.2.2 Optimization of chromatographic conditions using CCD

CCD was chosen for its flexibility in optimizing HPTLC separation conditions, offering a comprehensive view of both the main and interaction effects among the experimental factors. A three-factor, rotatable Central Composite Design was implemented, consisting of 15 experimental runs, five of which were center points. Methanol content in the mobile phase (A), chamber saturation time (B), and distance travelled (C) served as the independent variables; these, together with the corresponding responses across all trial runs, are summarized in Table 1. Model fitting showed that a linear model best described the  $R_f$  value of Candesartan Cilexetil, while a quadratic model provided the best fit for Metformin Hydrochloride — determined by the lowest PRESS value and an adjusted  $r^2$  approaching 1 in each case.

**Table 1 CCD model for optimization of mobile phase study**

| Run | Methanol content in the total mobile phase (ml) | Saturation time (min) | Developing distance (mm) | R <sub>f</sub> value |      |
|-----|-------------------------------------------------|-----------------------|--------------------------|----------------------|------|
|     |                                                 |                       |                          | CAN                  | MET  |
| 1   | 0.5                                             | 35.00                 | 75.00                    | 0.38                 | 0.21 |
| 2   | 0.5                                             | 25.00                 | 75.00                    | 0.43                 | 0.28 |
| 3   | 1                                               | 30.00                 | 80.00                    | 0.67                 | 0.25 |
| 4   | 1.5                                             | 35.00                 | 75.00                    | 0.8                  | 0.33 |
| 5   | 1.5                                             | 35.00                 | 85.00                    | 0.94                 | 0.33 |
| 6   | 1.5                                             | 25.00                 | 75.00                    | 0.74                 | 0.29 |
| 7   | 0.5                                             | 25.00                 | 85.00                    | 0.62                 | 0.25 |
| 8   | 1.5                                             | 25.00                 | 85.00                    | 0.83                 | 0.42 |
| 9   | 0.16                                            | 30.00                 | 80.00                    | 0.12                 | 0.49 |
| 10  | 1.0                                             | 30.00                 | 80.00                    | 0.67                 | 0.25 |
| 11  | 1.0                                             | 21.59                 | 80.00                    | 0.66                 | 0.39 |
| 12  | 1.0                                             | 30.00                 | 88.41                    | 0.74                 | 0.29 |
| 13  | 1.0                                             | 30.00                 | 71.69                    | 0.62                 | 0.22 |
| 14  | 1.0                                             | 30.00                 | 80.00                    | 0.67                 | 0.25 |
| 15  | 1.0                                             | 30.00                 | 80.00                    | 0.67                 | 0.25 |
| 16  | 1.0                                             | 30.00                 | 80.00                    | 0.67                 | 0.25 |
| 17  | 0.5                                             | 35.00                 | 85.00                    | 0.6                  | 0.36 |
| 18  | 1.0                                             | 30.00                 | 80.00                    | 0.67                 | 0.25 |
| 19  | 1.84                                            | 30.00                 | 80.00                    | 0.93                 | 0.63 |
| 20  | 1.0                                             | 38.41                 | 80.00                    | 0.67                 | 0.34 |

**n = 3 replicates, MET = Metformin Hydrochloride, CAN = Candesartan Cilexetil**

Model was also validated by analysis of variance (ANOVA) using Design Expert software and the results are as presented in Table 2. Effects were considered statistically significant at  $P < 0.05$ . Adequate Precision, which measures signal-to-noise ratio, is expected to exceed 4; the values obtained for both drugs confirmed an adequate signal [39]. Model reproducibility, assessed via the coefficient of variation (% CV), together with high adjusted  $R^2$  values, confirmed a strong correlation between the experimental data and the fitted models, with adjusted  $R^2$  values meeting the accepted threshold of  $\geq 0.80$  [40, 41] — indicating a good fit between the experimental data and the polynomial equations. The resulting equations, expressed in terms of actual factors, are presented in Table 2, where a positive coefficient reflects a factor that favors optimization and a negative coefficient reflects an inverse relationship between that factor and the response.

**Table 2 Predicted response models and statistical parameters obtained from ANOVA for CCD**

| Response (R <sub>f</sub> ) | Type of model | Polynomial equation model for Y | Model P-value | %CV | Adequate precision |
|----------------------------|---------------|---------------------------------|---------------|-----|--------------------|
|----------------------------|---------------|---------------------------------|---------------|-----|--------------------|

|           |           |                                                                                                                                                                                                                                      |      |                         |                 |        |
|-----------|-----------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------|-------------------------|-----------------|--------|
| MET       | Quadratic | Y <sub>1</sub> =Rf value of metformin HCl =<br>+0.25+0.037 * A-8.364E-003 *<br>B+0.032 * C-8.760E-003 * A *<br>B+1.260E-003 * A * C+3.760E-<br>003 * B * C+0.091 * A <sup>2</sup> +0.022 *<br>B <sup>2</sup> -0.023 * C <sup>2</sup> |      | 0.0066<br>(Significant) | 18.78           | 10.557 |
| Std. Dev. | r Squared | Adj. r-Squared                                                                                                                                                                                                                       | Mean | PRESS                   | Pred. r-Squared |        |
| 0.059     | 0.8331    | 0.6829                                                                                                                                                                                                                               | 0.3  | 0.28                    | 0.3415          |        |
| CAN       | Quadratic | Y <sub>2</sub> =Rf value of candesartan =+0.67<br>+0.19 * A+0.022 * B+0.066 *<br>C+0.030 * A * B -0.022 * A * C<br>+0.010 * B * C-0.036 * A <sup>2</sup> -6.073E-<br>003 * B <sup>2</sup> +0.013 * C <sup>2</sup>                    |      | 0.0001<br>(significant) | 10.24           | 14.85  |
| Std. Dev. | r-Squared | Adj. r-Squared                                                                                                                                                                                                                       | Mean | PRESS                   | Pred. r-Squared |        |
| 0.066     | 0.9328    | 0.8723                                                                                                                                                                                                                               | 0.65 | 0.33                    | 0.4905          |        |

Factors are in coded levels, MET = Metformin hydrochloride, CAN = Candesartan Cilexetil

CV for the proposed models is presented in Table 2, along with the regression equation generated for the final selected response. Only statistically significant coefficients ( $p < 0.05$ ) are included in the equations. A positive coefficient indicates an effect that favors optimization, whereas a negative coefficient signifies an inverse relationship between the factor and the response. "Adequate Precision" measures the signal-to-noise ratio; a ratio greater than 4 is considered desirable. The precision values in Table 2 demonstrate that the signal is adequate.

• **Perturbation Plot** The predicted models are illustrated using perturbation plots to facilitate interpretation of the results (Figure 3a and 3b). These graphs demonstrate how the response variable changes as each factor shifts from its defined reference value, while all other factors remain constant. A steeper slope or pronounced curvature indicates greater sensitivity of the response to a particular factor. The figures indicate that methanol content (factors A, B, and C) exerted the most significant influence on the  $R_f$  values of Metformin hydrochloride and Candesartan Cilexetil, respectively.

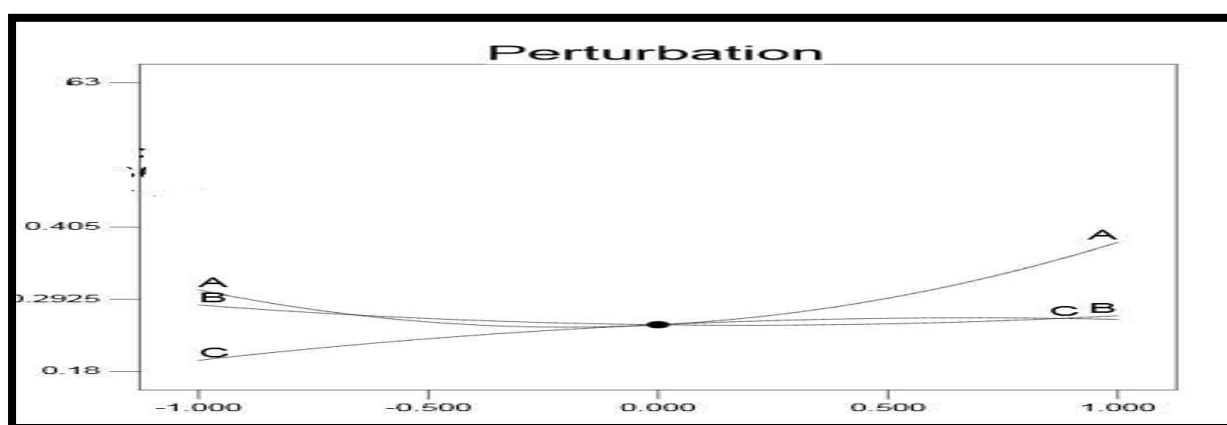


Figure 3a Perturbation graph showing the effect of each factor A, B, and C on  $R_f$  of MET

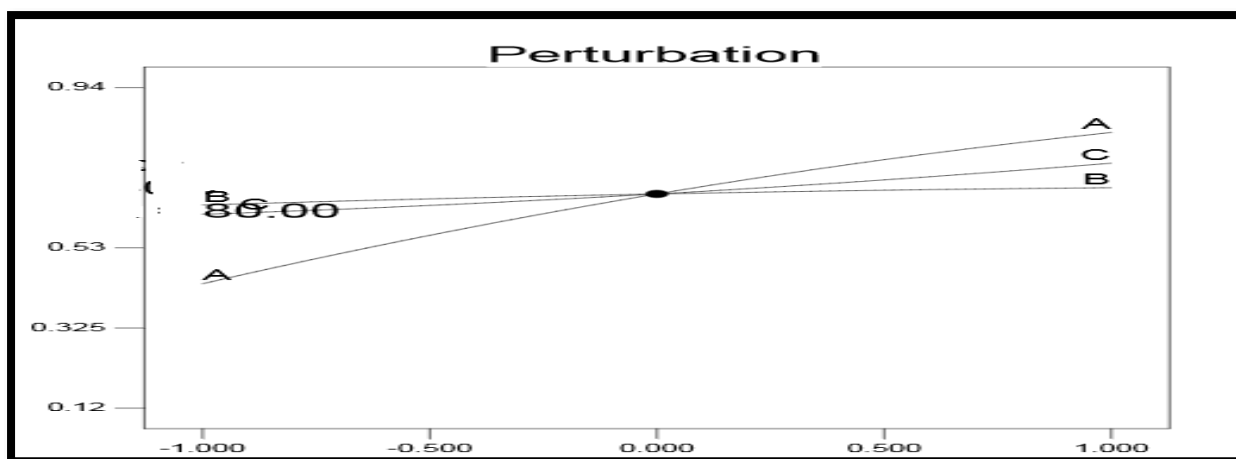


**Figure 3b** Perturbation graph showing the effect of each factor A, B, and C on  $R_f$  of CAN

### Contour Plots

The main results of this study are presented in contour plots which represent the relationship between the dependent and independent variables (Figure 4).

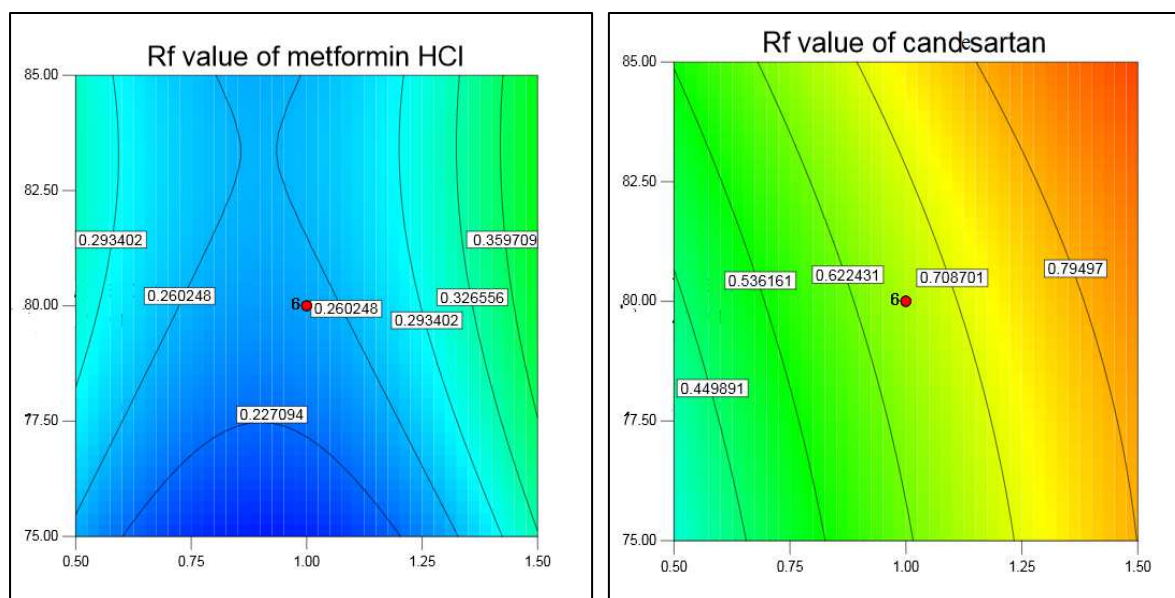


**Figure 4** contour plots showing the effect of each factor A, B, and C on  $R_f$  of MET and CAN respectively

The three-dimensional response surface plots, presented in Figure 5a and Figure 5b, illustrate the results of the response surface methodology (RSM) analysis. In these plots, the response model is graphed against two factors while the third factor is maintained at a constant, specified level.

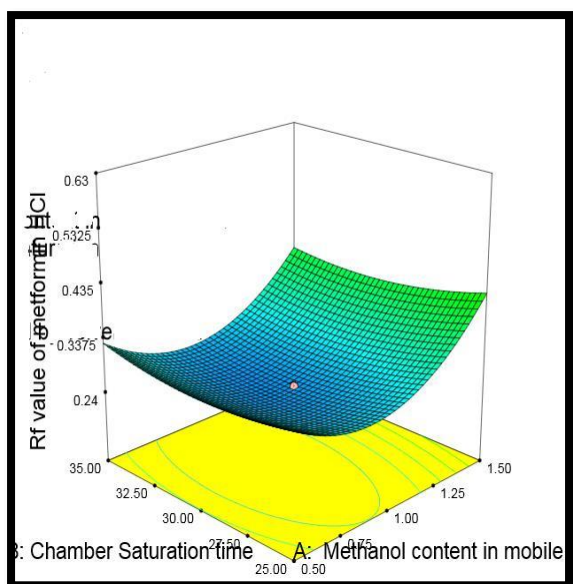


**Figure 5a 3D GRAPH (MET)**

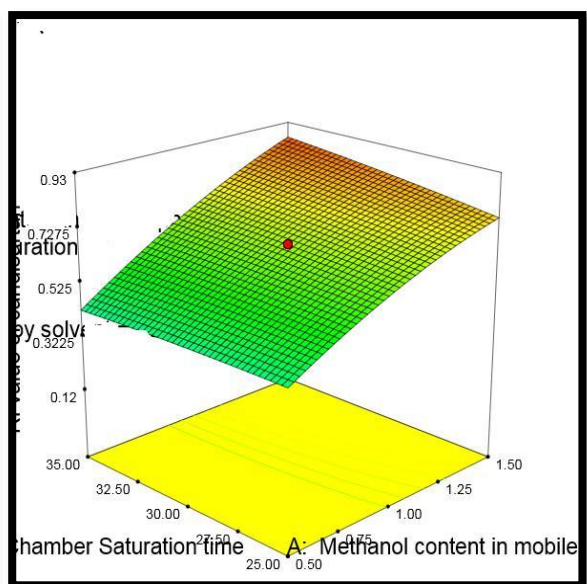


**Figure 5 3D GRAPH (CAN)**

Three-dimensional plots illustrate the relationship between chamber saturation time, methanol content, and the Rf values of Metformin hydrochloride and Candesartan Cilexetil. Following analysis of the experimental data using ANOVA and derivation of the final equation, the optimal levels for each factor and response were determined using the Numerical Optimization function in Design Expert software version 7.0.0.

**Table 3 Criteria for optimization of individual responses and factors with targeted values.**

| Name                       | Goal     | Lower Limit | Upper Limit |
|----------------------------|----------|-------------|-------------|
| A- Methanol content        | In range | 0.16        | 1.84        |
| B- Chamber saturation time | In range | 25          | 35          |
| C-Developing distance      | In range | 75          | 85          |
| Rf of MET                  | In range | 0.3         | 0.4         |
| Rf of CAN                  | In range | 0.5         | 0.75        |

The optimal separation conditions were determined using Derringer's desirability function [35]. During numerical optimization, target values were first defined for each factor and response. Of the 20 optimization solutions the software generated, three conditions with desirability values approaching 1 were selected for further evaluation. Figure 6 presents the response surface corresponding to the maximum value of Derringer's desirability function.

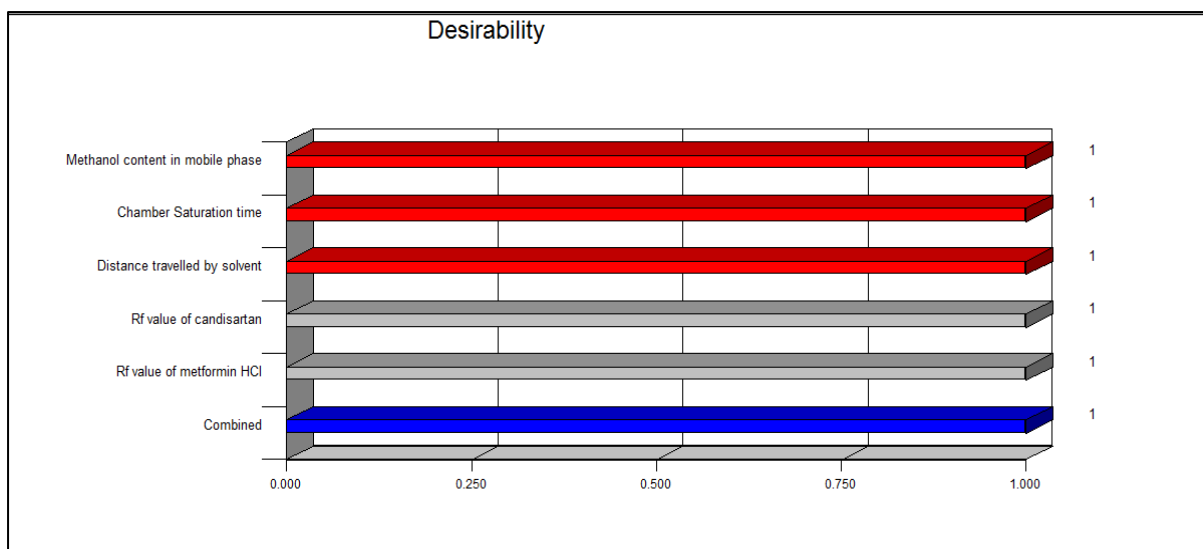


**Figure 6 Desirability plot**

Table 4 compares experimental and predicted responses for both drugs, along with predicted optimums 1, 2, and 3, to evaluate the model's predictability.

**Table 4 Comparison of experimental and predictive value of different experimental runs under optimum conditions**

| Optimum conditions | Response              | Responses (predicted) | Responses (observed) | Predicted error % |
|--------------------|-----------------------|-----------------------|----------------------|-------------------|
| <b>1</b>           | R <sub>f</sub> of MET | 0.31                  | 0.30                 | 0.98              |
|                    | R <sub>f</sub> of CAN | 0.73                  | 0.71                 | 0.98              |
| <b>2</b>           | R <sub>f</sub> of MET | 0.31                  | 0.30                 | 1.02              |
|                    | R <sub>f</sub> of CAN | 0.74                  | 0.71                 | 1.00              |
| <b>3</b>           | R <sub>f</sub> of MET | 0.32                  | 0.30                 | 0.99              |
|                    | R <sub>f</sub> of CAN | 0.74                  | 0.71                 | 0.99              |

Table 4, which presents the minimum percentage predicted error, indicates that a set of coordinates yielding a high desirability value ( $D = 1$ ) is achieved under Condition 1. Therefore, Condition 1 is proposed as the optimum experimental condition for the simultaneous analysis of Metformin hydrochloride and Candesartan Cilexetil.

Based on Table 4 and the % predicted error, the coordinates that yield a high desirability value ( $D = 1$ ) under optimum Condition 1 are recommended for the simultaneous analysis of Metformin Hydrochloride and Candesartan Cilexetil. The corresponding R<sub>f</sub> values are 0.71 for Candesartan Cilexetil (100 ng/band) and 0.30 for Metformin Hydrochloride (1000 ng/band), as illustrated in Figure 7.

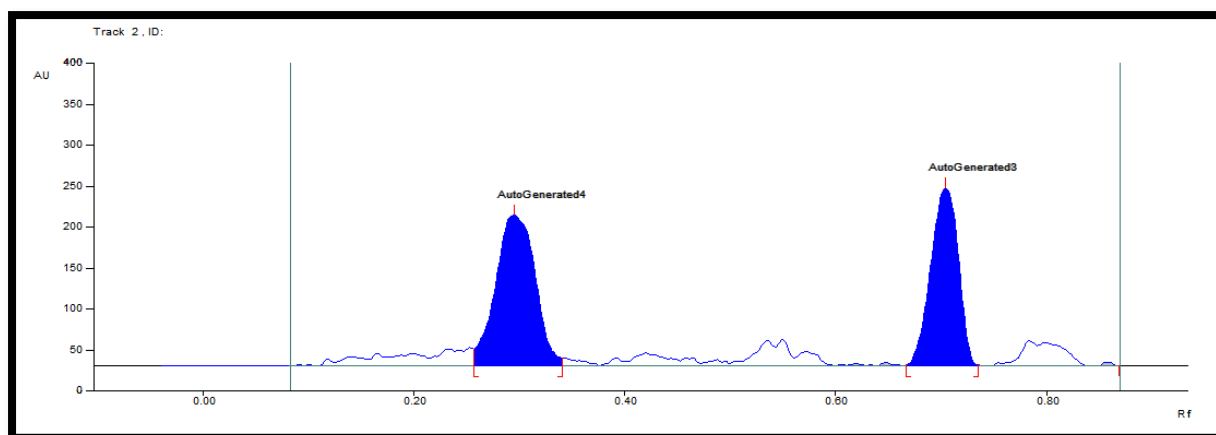


**Figure 7 HPTLC Densitogram under optimized conditions.**

### **3.3 Method validation**

#### **3.3.1 Linearity**

##### **Calibration curve**

Stock solutions of Metformin hydrochloride and Candesartan Cilexetil were prepared in methanol to achieve concentrations of 100–1000 µg/ml, respectively. Various volumes of these solutions were applied, and the linear relationship between peak area and drug concentration was evaluated over a range expressed in ng/band. Six replicate measurements were performed for each concentration: 2000–6000 ng/band for Metformin hydrochloride and 120–280 ng/band for Candesartan Cilexetil (Table 5). Calibration plots were generated by plotting the main band area against drug concentration and analyzed using ordinary regression analysis. Homoscedasticity of variance in peak area response was assessed using Bartlett's test.

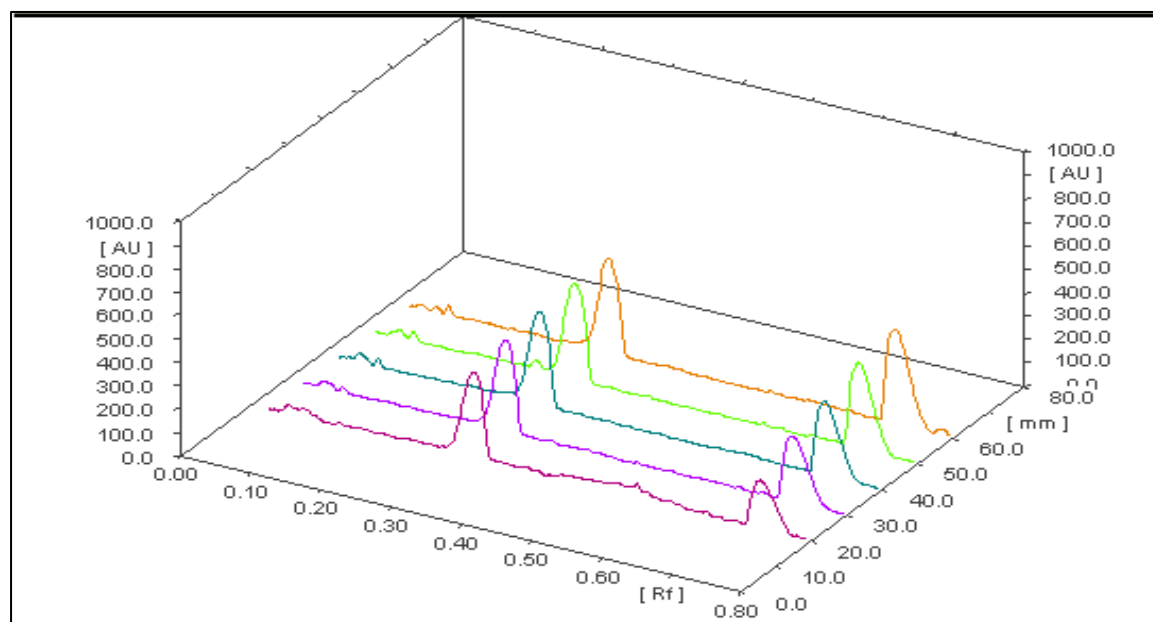


**Figure 8 HPTLC chromatogram (3D view) for linearity of MET and CAN at 248 nm**

**Table 5 HPTLC parameters for Metformin Hydrochloride and Candesartan Cilexetil**

| Parameters                                   | MET                    | CAN                   |
|----------------------------------------------|------------------------|-----------------------|
| Calibration range <sup>a</sup> (ng/spot) n=5 | 1000-5000              | 120-280               |
| Regression equation                          | $y = 0.4216x + 8940.2$ | $y = 37.74x + 1275.2$ |
| Correlation coefficient (r)                  | 0.9971                 | 0.9912                |
| Standard deviation of slope                  | 0.0093                 | 0.1018                |
| Confidence limit of slope                    | 0.4005-0.4425          | 35.8511-39.6249       |
| Standard deviation of intercept              | 85.0248                | 36.2538               |
| Confidence limit of intercept                | 8493.13-9387.15        | 1211.47-1339          |
| Limit of detection (ng/ band)                | 665.5801               | 3.1702                |
| Limit of quantification (ng/ band)           | 2016.9095              | 9.6067                |
| Bartlett's test <sup>b</sup> ( $\chi^2$ )    | 0.01444                | 0.0002138             |

<sup>a</sup> mean,  $\pm$  SD, n=5 replicates, <sup>b</sup> Calculated value  $\chi^2$  less than critical value  $\chi^2$  (0.05, 4) = 9.488, <sup>c</sup> Confidence interval at 95% confidence level and four degree of freedom

### 3.3.2 Precision

To evaluate intra-day precision, the experiment was repeated three times within a single day, and the average percentage relative standard deviation (% RSD) was calculated. Inter-day precision was similarly assessed by repeating the procedure across three separate days, with average % RSD values determined for the peak areas of both Candesartan Cilexetil and Metformin Hydrochloride. The resulting % RSD values, all below 2%, confirmed the method's precision for both intra-day and inter-day conditions (Table 5).

### 3.3.3 Accuracy

When the proposed method was applied to recovery studies at three concentration levels (80%, 100%, and 120%) using standard spiking, percentage recoveries for both Candesartan Cilexetil and Metformin Hydrochloride fell within the acceptable range of  $100 \pm 2\%$  [38].

### 3.3.4 Specificity

Application of the developed method to the formulation resulted in two peaks: an R<sub>f</sub> value of 0.30 for Metformin Hydrochloride and an R<sub>f</sub> value of 0.71 for Candesartan Cilexetil. These R<sub>f</sub> values were consistent with those observed for both standards, as confirmed by densitogram comparison. The peak purity of Candesartan Cilexetil and Metformin Hydrochloride was also assessed.

Evaluation of the formulations by comparing spectra at the peak start, apex, and end positions, as shown in Figure 9a and 9b, demonstrated strong correlation coefficients. For Metformin Hydrochloride, r(S,M) and r(M,E) were 0.9989 and 0.9981, respectively, whereas for Candesartan Cilexetil, the values were 0.9970 and 0.9613, respectively. These results indicate that the method is specific in the presence of various excipients.

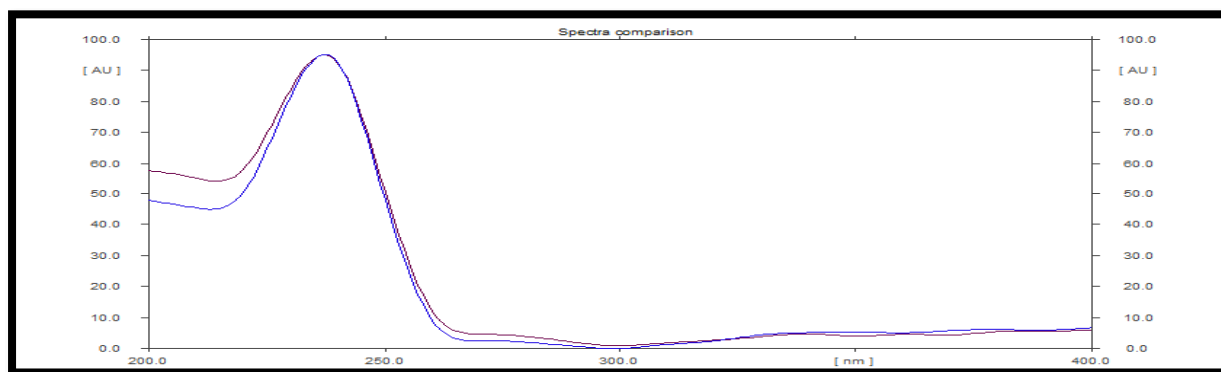


**Figure 9a** overlain spectra of Metformin Hydrochloride with reference spectra

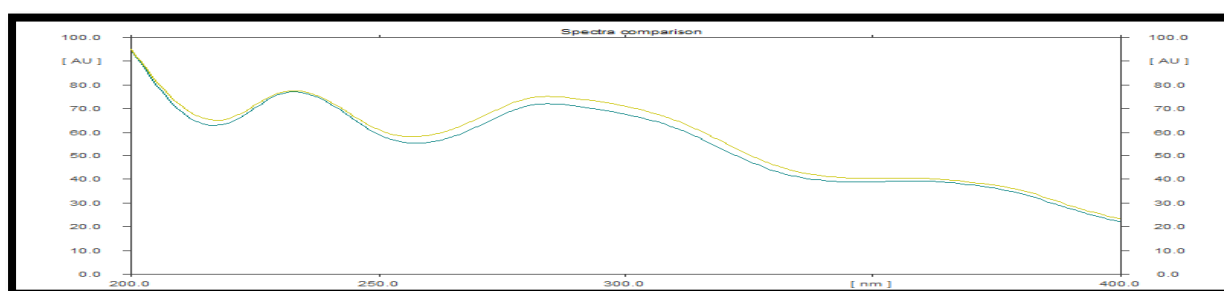


**Figure 9b** overlain spectra of Candesartan Cilexetil with reference spectra

### 3.3.6 Robustness

The robustness of the analytical procedure is defined as its ability to remain unaffected by small, deliberate variations in method parameters (International Conference on Harmonization, ICH Q2 (R1), 2005; Fabre 1996). Several factors, including methanol content in the total mobile phase ( $1.32 \pm 0.05$  ml), developing distance ( $77 \pm 5$  cm), saturation time ( $27.81 \pm 2$  min), spotting time (0 to 80 min), wavelength variation ( $248 \pm 2$  nm), and mobile phase volume ( $10 \pm 2$  ml), were selected based on their criticality as observed during trial runs and method development. The examined ranges represented minor deviations from the established method settings. The primary responses evaluated were the  $R_f$  value and peak area of different drugs. The impact of these variations on both  $R_f$  and peak area was assessed by calculating the percentage relative standard deviation (%RSD) for each parameter. Results were reported as % relative standard deviation.

**Table 6** Robustness study of developed HPTLC method.

| Parameters                              | SD <sup>a</sup> | (%) RSD | SD <sup>a</sup> | (%) RSD |
|-----------------------------------------|-----------------|---------|-----------------|---------|
| Methanol Content (1.27, 1.37)           | 0.0070          | 2.3183  | 0.0212          | 3.0522  |
| Saturation time (27.81 $\pm$ 2 min)     | 0.0070          | 2.3183  | 0.0070          | 1.0174  |
| Development distance (7.7 $\pm$ 0.5 cm) | 0.00            | 0.00    | 0.0212          | 3.0522  |

|                                                           |        |        |        |        |
|-----------------------------------------------------------|--------|--------|--------|--------|
| <b>Wavelength change (248±2nm)</b>                        | 0.0070 | 2.3183 | 0.0141 | 1.9918 |
| <b>Time from spotting to Chromatography (0 to 80 min)</b> | 0.0050 | 1.7094 | 0.0081 | 1.1833 |
| <b>Mobile phase volume (10±2mL)</b>                       | 0.0070 | 2.3183 | 0.0070 | 1.0174 |

### 3.4 Applicability of the proposed HPTLC method for determination of MET and CAN in newer MUPS dosage form.

| <b>Drugs</b>                         | <b>Amount of drug present(mg)</b> | <b>% Amount of drug found <sup>a</sup></b> | <b>SD</b> | <b>% RSD</b> |
|--------------------------------------|-----------------------------------|--------------------------------------------|-----------|--------------|
| <b>Metformin HCl (250 mg)</b>        | 257.4                             | 102.96                                     | 1.9519    | 0.7642       |
| <b>Candesartan Cilexetil (16 mg)</b> | 162.033                           | 101.27                                     | 1.1924    | 0.7411       |

<sup>a</sup> n =3 replicates, % RSD= Relative standard deviation

The MUPS dosage form containing 250.00 mg of MET and 16 mg of CAN was analysed, and the recovered amounts were expressed as percentages of the label claims. The percentage amounts for all drugs ranged from 95% to 105%. The %RSD was less than 2.

### Conclusion

The study successfully established a novel, validated HPTLC method for simultaneous quantification of Metformin Hydrochloride and Candesartan Cilexetil in MUPS formulations. Application of CCD allowed systematic optimization of chromatographic variables, yielding robust and reproducible results. The method demonstrated excellent linearity, precision, accuracy, and robustness, with R<sub>f</sub> values of 0.71 (Candesartan Cilexetil) and 0.30 (Metformin Hydrochloride) under optimized conditions. The low %RSD values and compliance with ICH Q2 (R1) validation criteria confirm its reliability for quality control and routine pharmaceutical analysis. This approach not only ensures accurate quantification but also highlights the utility of experimental design strategies in method development for combination drug formulations. Hence, the method was found to be adequate for quality control analysis of drugs in combined MUPS dosage form.

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