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# Development of a Novel Stability Indicating HPLC Method for the Simultaneous estimation of Cefepime and Enmetazobactam in Dosage Form Using Response Surface Methodology

**H. Neelofar[*1], Dr. D. Ranganayakulu[2]**

[*1]Assistant Professor Research Scholar, Sri Padmavathi school of Pharmacy, Tiruchanoor, JNTUA
[2]Professor & Principal, Sri Padmavathi School of Pharmacy, Tiruchanoor, JNTUA

**Corresponding author**
MS.H. Neelofar,
Assistant Professor, research scholar
Sri Padmavathi school of Pharmacy, Tiruchanoor,
JNTUA.

**Abstract:**
The current study highlights the systematic quality by design assisted creation of an efficient analytical technique for the estimation of Cefepime and Enmetazobactam in EXBLIFEP intravenous (IV) infusions form. Response surface methodology in the design of experiments was used for identifying key material attributes and critical process parameters that influence the designated critical analytical attributes. Separation was achieved on the Sunfire C18 column (250x 4.6 mm, 5µm). The effects of acetonitrile content (v/v), flow rate, and column temperature on the retention times of the two drugs and their resolution and the number of theoretical plates were investigated and optimized. The optimum chromatographic conditions within the design space were found to be an isocratic mobile phase consisting of water and acetonitrile (50: 50, v/v) with a flow rate of 1mL/min and a run time of 6 min. The retention times of Cefepime and Enmetazobactam were found to be 2.36-min and 2.98-min. Different validation parameters were established, and the approach was validated in agreement with the ICH and FDA requirements. Data analysis using statistical methods has revealed that the method is reliable, accurate, and robust.

**Keywords:** design of experiments, response surface methodology, central composite design, critical analytical attributes and Design-Expert 10.0.2 software.

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## INTRODUCTION:

Compared to a traditional or formal approach, a quality by design (QbD) approach to method creation emphasizes risk management more, which results in a more robust and long-lasting method [1, 2]. Understanding how dependent variables and the effects they have on responses through a suitable analysis is a crucial component of QbD. In this work, a risk-based high performance liquid chromatography (HPLC) approach for estimating the amounts of Cefepime and Enmetazobactam in tablets is developed and validated. The optimization of traditional HPLC techniques involves identifying one component at a time while holding the other variables constant, which leads to several tests with inadequate comprehension of crucial parameters [3, 4]. QbD has developed into a powerful tool for creating chromatographic techniques in the present era. The advantages of this approach include a thorough understanding of the parameters influencing chromatographic separation by identifying the most important ones, as well as their impact on the selected responses, both positive and negative, and the multifaceted interactions among them [5, 6]. Additionally, QbD suggests the key fixes and promotes the deliberate modification of the variables to achieve the intended outcome. Early on in the development phase, the QbD approach advises assessing the quality of the analytical process [7–11].

Cefepime is a broad-spectrum cephalosporin antibiotic used to treat various bacterial infections, including pneumonia and infections of the skin and urinary tract. It works by killing the bacteria that cause the infection. Common side effects may include nausea, diarrhea, and rash. It is administered intravenously and is effective against bacteria such as Pseudomonas and Escherichia [12-15].

Analytical techniques f or estimating Cefepime and Enmetazobactam are few. It has never been done analytically previously to estimate Cefepime and Enmetazobactam in EXBLIFEP intravenous (IV) infusions simultaneously by the application of the principles of Quality by Design, and we have created a novel analytical technique for the first time. In this study, a unique stability indicating HPLC method is developed and validated utilizing quality by design concepts for the simultaneous quantification of Cefepime and Enmetazobactam in pharmaceutical formulations.

## EXPERIMENTAL

Cefepime and Enmetazobactam working standards were procured from Shree Icon Pharmaceutical Laboratories, Vijayawada. Commercially available fixed-dose combination tablets containing both drugs were used for analysis. HPLC-grade acetonitrile, Hydrogen peroxide, Hydrochloric Acid (Analytical Reagent), and water were purchased from Merck (India). Orthophosphoric acid (OPA, AR grade) was used for buffer preparation. All other chemicals used were of analytical grade. Cefepime and Enmetazobactam combination EXBLIFEP intravenous (IV) infusions purchased from the local medical store.

### Standard Preparation:

Accurately weighed and transferred 40mg of Cefepime and 10 mg of Enmetazobactam working Standards into a 50ml clean dry volumetric flask and 3/4th volume of diluent was added and sonicated for 5 minutes and the final volume was made up with diluents. (800µg/ml of Cefepime and 200µg/ml of

Enmetazobactam) 1ml from each stock solution was pipetted out and taken into a 10ml volumetric flask and made up the volume with diluent. (80µg/ml of Cefepime and 20µg/ml of Enmetazobactam)

**Preparation of Sample stock solutions:** A vail containing solution equivalent to 2000 mg of Cefepime and 500mg of Enmetazobactam was transferred into a 500 ml volumetric flask and about 10 ml of dilutent was added and shaken for 20 minutes by mechanical means or manually and further sonicated for 5 min, further the volume was made up with diluent and filtered by HPLC filters (1600µg/ml of Cefepime and 400µg/ml Enmetazobactam)

**Preparation of Sample working solutions (100% solution):**0.5ml of filtered sample stock solution was transferred to 10ml volumetric flask and made up with diluent. (80µg/ml of Cefepime and 20µg/ml of Enmetazobactam). Label Claim: 2000 mg of Cefexime, 500mg of Enmetazobactam (The reconstituted EXBLIFEP solution will have a resultant concentration of 0.2 grams/mL (Cefepime 0.16 grams/mL and Enmetazobactam 0.04 grams/mL).

**Selection of appropriate detection wavelength**

Cefepime and Enmetazobactam concentrations were scanned in the 200–400 nm range with the wavelength maxima of 280 nm chosen as the detecting wavelength.

**Chromatographic specifications**

A Waters Acquity HPLC system was utilized, fitted with a quaternary solvent manager, a sample manager, a TUV detector controlled by Empower 2 software, a cooling autosampler, and a column oven facilitating temperature control of the analytical column. A Sunfire C18 (250x 4.6 mm x 5µm) was used for this study. The temperature of 30°C and flow rate of 1 mL/min were used for all investigations. The standard and sample injection volumes were both 20 µL. Prior to injection, every standard and sample solution was filtered using 0.2 µm filter tips. Column effluents were viewed with a photo diode array (PDA) operating at 280 nm.

**Table 1. Independent variables (factor) and responses investigated in CCD design**

| Independent variable(factor) | Level of variable | | | Response | Target |
|---|---|---|---|---|---|
| | low (–1) | medium (0) | high (+1) | | |
| Flow rate of the mobile phase, ( mL/min) | 0.838 | 1.0000 | 1.17 | Retention of Cefepime $RT_1$; retention of Enmetazobactam $RT_2$; Resolution RS; Resolution NTP1; number of Theoretical plates NTP2; number of Theoretical plates | Minimizing the retention time of both drugs while maximizing the resolution and no of theoretical plates |
| Acetonitrile content in mobile phase, % (v/v) | 51.59 | 60.00 | 68.41 | | |
| Temperature, °C | 24.95 | 30.00 | 35.05 | | |

**Choosing a High-Quality Target Product Profile.**

The number of theoretical plates, resolution, and retention time (Rt) of Cefepime and Enmetazobactam were determined to be the quality target product profile (QTPP) for the suggested HPLC method.

**Identification of the Critical Quality Attributes:**

The QTPP is directly impacted by the critical quality attributes (CQAs), which are method variables. To maintain the QTPP allowable response range, three crucial procedure parameters had to be maintained: temperature, the mobile phase flow rate, and the amount of acetonitrile (v/v) in the mobile phase.

**Optimization Design:** The chromatographic conditions of the suggested method were refined through the use of an experimental design based on response surface methodology (RSM). After 20 tests, the central composite design (CCD) was chosen to optimize the three components. The independent variables and their levels were selected based on previous screening and existing knowledge. The dependent variables (responses) were retention times, the number of theoretical plates, and the resolution of the two medicines. The design matrix and the acquired data were subjected to multiple regression analysis, and the relationship between the independent variables and the data was established using the second-order polynomial function that resulted [24]. The variables, their ranges, and responses with matching objectives are listed in Table 1.

**Statistical interpretation.** Using Design-Expert 10.0.2, the experimental design and statistical analysis were completed (free trial version). The importance of each model, terms, and their interactions were assessed using analysis of variance (ANOVA). A model was considered statistically significant if its *p*-value was less than 0.05. *F*-values were also checked for each diagnostic tools like the residual vs. predicted plot and normal probability plot of residuals, suitability of the model was evaluated.

**Validation of analytical methods**. In terms of specificity, solution stability, linearity range, accuracy, precision, system applicability, and forced degradation tests, the novel method was verified in accordance with ICH, USP, and FDA criteria.

**Specificity.** To ensure that no contaminants, degradation products, or excipients affected the drug peaks in the chromatogram, the method's specificity was examined. In addition to ocular assessment, peak purity indices were used to assess the drugs' specificity.

**Stability of the solution**. To find out if the drug combination would stay stable in the diluting solvent, test solutions were kept in tightly sealed vials and refrigerated at 5°C for 24 hours at room temperature. The solutions at 0 and 24 hours were analyzed using the established approach. The area does not appear to have changed. The relative standard deviation (RSD) of the drug's stability in the diluting solvent was found to be less than 2.0%.

**Range of linearity**. Peak area was plotted against several concentrations (20–150% of nominal) to create a calibration curve, which was then used to evaluate the method's linearity and operating range. The linear equation, regression coefficient ($r^2$), slope, and intercept were all calculated.

**Precision**. The information comes from at least nine analyses at three concentrations. The intra-day (repeatability) and inter-day (intermediate precision) levels of precision evaluation are the two most often utilized. In the current study, the three concentration levels of intraday precision were evaluated using three replicates at each concentration level. Two HPLC systems were used in two different days to evaluate intermediate precision. The relative standard deviation's overall fluctuation was calculated.

**System Suitability**. It is common practice to employ system appropriateness to make sure that a method is suitable for a certain analysis. Among the parameters that were validated in the current investigation were theoretical plate count, tailing factor, area resolution and repeatability, limit of detection, etc.

**Forced degradation.** The objective of these research is to intentionally deteriorate a sample. The purpose of these studies is to evaluate an analytical technique's sensitivity. The crucial information on the drug breakdown pathway that forced degradation research provides helps with both the rational formulation of the treatment and the development of multiple dose forms. Drug compounds or products deteriorate by 10–30% when exposed to water, oxidizing and reducing chemicals, bases, acids, and ultraviolet (UV) light. The procedure is then used to the degrade samples to determine whether the drug molecule and associated substance(s) interfere in any way.

## RESULTS AND DISCUSSION

Method Development. *Initial Screening.* To determine the important elements and their levels and effects, a preliminary screening and literature search were carried out. According to screening experiments, Cefepime and Enmetazobactam are separated by a 40% methanol and 60% water mixture and the column used was Agilent C18 (4.6 x 250mm, 5µm); nevertheless, the Enmetazobactam peak is not eluted and Cefepime peak is not symmetrical and tailing is observed. In contrast, the mobile phase in the second trial was a 0.01 N $KH_2PO_4$ (50%) acetonitrile (50%) mixture; all chromatographic conditions were kept constant, resulting in peaks that were asymmetrical with tailing and merged. In the third trial, mobile phase, which was composed of 0.01N Ammonium acetate (60%): Acetonitrile (40%) was examined. In this trail also Cefepime and Enmetazobactam peaks shape is not symmetrical, so further trail is carried out. In the fourth trial, mobile phase, which was composed of 0.01 N $KH_2PO_4$ (60%)− acetonitrile (40%) was examined by changing the column to Sunfire C18 (4.6 x 250mm, 5µm).In this trail also Cefepime and Enmetazobactam peaks shape is not symmetrical and base line noise was observed, so further trail is carried out. In the fifth and final trial, mobile phase, which was composed of Water (50%)− Acetonitrile (50%) was examined by the mobile phase in this final trail Cefepime and Enmetazobactam peak shapes are symmetrical with good resolution and no base line noise is observed.

**Statistical analysis and experiments**. Nine specifications derived from the design of trials with six repetitions at the domain's center were incorporated in the entire factorial design, which encompassed 20 experimental situations. Each of these situations resulted in a chromatogram.

**Table 2.** Randomized run order, factor combinations, and corresponding responses

| | | Factor 1 | Factor 2 | Factor 3 | Response 1 | Response 2 | Response 3 | Response 4 | Response 5 |
|---|---|---|---|---|---|---|---|---|---|
| Std | Run | A:FR | B:MP | C:Temp | RT1 | RT2 | RS | NTP1 | NTP2 |
| | | ml/min | % | 0 C | min | Num | num | Num | num |
| 1 | 19 | 0.9 | 55 | 27 | 2.772 | 3.375 | 4 | 5046 | 6971 |
| 2 | 16 | 1.1 | 55 | 27 | 2.224 | 2.747 | 3.7 | 4327 | 5657 |
| 3 | 12 | 0.9 | 65 | 27 | 2.736 | 3.624 | 4.9 | 5667 | 6954 |
| 4 | 8 | 1.1 | 65 | 27 | 2.324 | 3.011 | 4.3 | 4338 | 5567 |
| 5 | 18 | 0.9 | 55 | 33 | 2.45 | 3.022 | 3.7 | 4478 | 6214 |
| 6 | 10 | 1.1 | 55 | 33 | 2.037 | 2.476 | 3.4 | 3908 | 4907 |
| 7 | 15 | 0.9 | 65 | 33 | 2.445 | 3.325 | 4.7 | 4813 | 5907 |
| 8 | 5 | 1.1 | 65 | 33 | 2.135 | 2.674 | 4.2 | 3270 | 5025 |
| 9 | 4 | 0.831821 | 60 | 30 | 2.748 | 3.527 | 4.5 | 5347 | 7064 |
| 10 | 17 | 1.16818 | 60 | 30 | 2.034 | 2.571 | 3.7 | 3536 | 5010 |
| 11 | 2 | 1 | 51.591 | 30 | 2.368 | 2.834 | 3.5 | 4461 | 5779 |
| 12 | 9 | 1 | 68.409 | 30 | 2.401 | 3.252 | 4.8 | 4563 | 5827 |
| 13 | 13 | 1 | 60 | 24.9546 | 2.61 | 3.313 | 4.3 | 5075 | 6501 |
| 14 | 11 | 1 | 60 | 35.0454 | 2.167 | 2.734 | 3.9 | 3791 | 5148 |
| 15 | 6 | 1 | 60 | 30 | 2.368 | 2.990 | 4.1 | 4425 | 6123 |
| 16 | 3 | 1 | 60 | 30 | 2.369 | 2.990 | 4.1 | 4474 | 6114 |
| 17 | 1 | 1 | 60 | 30 | 2.370 | 2.991 | 4.1 | 4514 | 6086 |
| 18 | 20 | 1 | 60 | 30 | 2.372 | 2.995 | 4.1 | 4602 | 5975 |
| 19 | 7 | 1 | 60 | 30 | 2.376 | 3.000 | 4.2 | 4463 | 5992 |
| 20 | 14 | 1 | 60 | 30 | 2.382 | 3.025 | 4.2 | 4460 | 5978 |

The table 2 shows the responses that go with them. Cefepime's retention time varied from 2.03 to 2.77 minutes, whereas Enmetazobactam retention time varied from 2.47 to 3.37 minutes. As a result, these data can be used and analyzed to create the ideal environment for achieving the current study's goals. The significance of 2FI experimental models was examined using ANOVA; the results are shown in Tables 3 and 4. Fisher's ratio was used to assess each model's significance. The model appears to be significant based on the model F-values for responses 1 (RT1 of 408.99), 2 (RT2 of 402.73), 3 (RS of 144.12), 4 (NTP1 of 142.39), and 5 (NTP2 of 142.39). It was discovered that all three models had p-values below 0.0001, indicating their relevance. Every other term was also significant based on the associated F- and p-values. The quality of the resulting polynomial regressions was assessed using the determination

coefficient ($R^2$), adjusted determination coefficient (adj. $R^2$), and anticipated determination coefficient (pred. $R^2$). The $R^2$ values were found to be extremely near to 1 in each case, suggesting that the regression curve fit the data with an accuracy of more than 99%. There was a good degree of agreement (difference less than 0.2) between the modified $R^2$ values and the projected $R^2$ values. It is possible to estimate sufficient precision based on the signal-to-noise ratio, which should be higher than 4. The current investigation found high accuracy values, which suggests a sufficient signal. The model is summarized in Table 5. Each model's coded polynomial equations were as follows (Eqs. (1)– (5)):

RT1 of Cefepime = +2.37-0.2112 A +0.0156 B -0.1270 C -0.0299 AB +0.0296 AC +0.0036 BC +0.0067 $A^2$+0.0044 $B^2$ +0.0058 $C^2$ (1)

RT2 of Enmetazobactam = +3.00 -0.2962 A+0.1257 B -0.1636 C -0.0113 AB +0.0055AC-0.0015BC+ 0.0159$A^2$+0.0138 $B^2$ +0.0069 $C^2$ (2)

RS = +4.12-0.2230 A +0.4017 B -0.1152C -0.0625 AB +0.0125 AC +0.0375 BC (3)

NTP1 = +4477.90-527.70A +36.65 B -371.13C -197.88AB -8.13 AC -116.88BC (4)

NTP2 = +6043.97-611.00A -15.76 B -393.32C +44.00AB ++64.00AC -10.25BC +1.84 $A^2$ -80.89 $B^2$- 73.29$C^2$ (5)

Where A represents the flow rate, B the percentage of acetonitrile, and C the temperature of the column. While AB, AC, and BC show their interaction impact, A, B, and C show the main effect terms. To ascertain whether the model was sufficient, a visual examination of the residual vs. predicted plot and the normal probability plot was conducted. A satisfactory fit to the data was shown by the residuals' normal distribution along the straight line with minimal scatter. The equation in terms of coded factors can be used to anticipate the response for particular levels of each factor. The residual plots showed a random distribution of residuals between +4 and 4 with no trend, indicating the lack of any systematic bias or outliers.

**Function of Derringer desirability**. Fig. 1 shows the specification for utilizing the Derringer desirability function to optimize each and every response. This methodology is based on the desirability value for each response.

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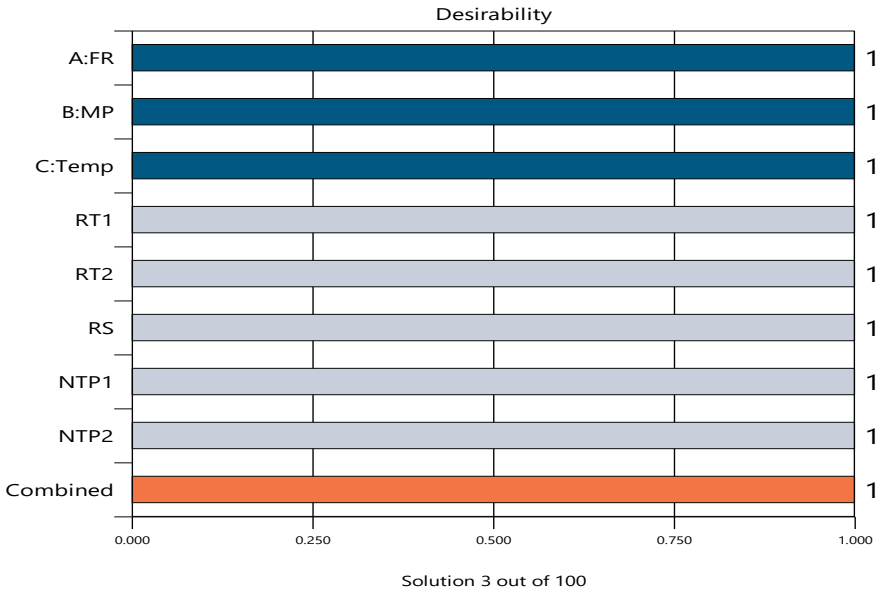


**Fig 1: Desirability function graph**

On the desirability function scale, which goes from zero to one, a completely wanted response requires a value around one; zero is considered the most undesired response. Based on their worth, the most attractive trials were selected. Therefore, the first trial with desirability one (i = 1) was selected for method optimization. The results are shown in Table 3.

**Table 4. Optimized trials suggested by software based on desirability value**

| Trail No. | Flow rate, mL/min | Organic phase content, % | Temperature, °C | $RT_1$, min | $RT_2$, min | $RS_1$ | NTP1 | NTP2 | Desirability |
|---|---|---|---|---|---|---|---|---|---|
| 3 | 1.000 | 60.000 | 30.000 | 2.373 | 2.999 | 4.120 | 4477.900 | 6043.970 | 1.000 |

**Selected Design area and ideal conditions for separation**. Figs. 2–6 illustrate the standardized impacts of independent factors on the responses and their interplay with each other using two-dimensional (2D) overlay contour plots and three-dimensional (3D) response surface graphs. Using the data for the experimental settings and accompanying responses, an overlay contour plot was constructed in order to determine the design space and the optimal mobile phase composition. In this case, the objective was to decrease the retention time of both medications while also increasing resolution Various specifications within the design space were analyzed for desirability before the most advantageous ones were selected. The entire target was achieved with a desirability value of 1.00 using a mobile phase containing roughly 40% (v/v) acetonitrile and a buffer concentration of about 10 mM. Finally, at a column temperature of 30°C and a flow rate of 1.0mL/min, acetonitrile blend of 60.0% (v/v) and 0.1% ortho-phosphoric acid buffer was determined to be the best isocratic mobile phase. Furthermore, the method operable design

region (MODR) and ideal chromatographic conditions were delineated by the graphical optimization as shown in Fig. 7.

**Table 4.** ANOVA for response surface 2FI and quadratic models (degrees of freedom, *F*-value)

| Source | Degrees of freedom | | | | | *F*-value | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | RT of CP | RT of EB | RS | NTP1 | NTP2 | RT of CP | RT of EB | RS | NTP1 | NTP2 |
| Model | 9 | 9 | 9 | 9 | 9 | 412.99 | 423.73 | 154.12 | 147.39 | 141.38 |
| A | 1 | 1 | 1 | 1 | 1 | 2812.63 | 2263.18 | 567.36 | 804.18 | 908.18 |
| B | 1 | 1 | 1 | 1 | 1 | 501.10 | 504.23 | 1138.86 | 32.09 | 32.09 |
| C | 1 | 1 | 1 | 1 | 1 | 899.76 | 680.83 | 30.78 | 175.60 | 175.60 |
| AB | 1 | 1 | 1 | 1 | 1 | 0.1210 | 2.33 | 0.3621 | 0.3826 | 0.3826 |
| AC | 1 | 1 | 1 | 1 | 1 | 34.57 | 20.15 | 0.3621 | 12.01 | 12.01 |
| BC | 1 | 1 | 1 | 1 | 1 | 0.0000 | 0.0032 | 3.26 | 0.1781 | 0.1767 |
| A2 | 1 | 1 | 1 | 1 | 1 | 28.59 | 23.54 | 27.44 | 10.61 | 10.61 |
| B2 | 1 | 1 | 1 | 1 | 1 | 27.73 | 35.35 | 0.4584 | 81.02 | 81.02 |
| C2 | 1 | 1 | 1 | 1 | 1 | 2.11 | 0.8532 | 3.28 | 75.81 | 65.82 |

Table 4. ANOVA for response surface 2FI and quadratic models (*p*-value)

| **Source** | | | ***p*-value, prob > *F*** | | |
|---|---|---|---|---|---|
| | **RT of CP** | **RT of EB** | **RS** | **NTP1** | **NTP2** |
| Model | < 0.0001 | < 0.0001 | < 0.0001 | < 0.0001 | < 0.0001 |
| A | < 0.0001 | < 0.0001 | < 0.0001 | < 0.0001 | < 0.0001 |
| B | < 0.0001 | < 0.0001 | < 0.0001 | 0.0002 | 0.0002 |
| C | < 0.0001 | < 0.0001 | 0.0002 | < 0.0001 | < 0.0001 |
| AB | 0.6412 | 0.1677 | 0.5607 | 0.5500 | 0.5500 |
| AC | 0.0006 | 0.0012 | 0.5607 | 0.0061 | 0.0061 |
| BC | 1.0000 | 0.9515 | 0.1012 | 0.5818 | 0.6818 |
| $A^2$ | 0.0004 | 0.0006 | 0.0005 | 0.0045 | 0.0067 |
| $B^2$ | 0.0018 | 0.0001 | 0.5183 | < 0.0001 | < 0.0001 |
| $C^2$ | 0.2768 | 0.3619 | 0.2001 | < 0.0001 | < 0.0001 |

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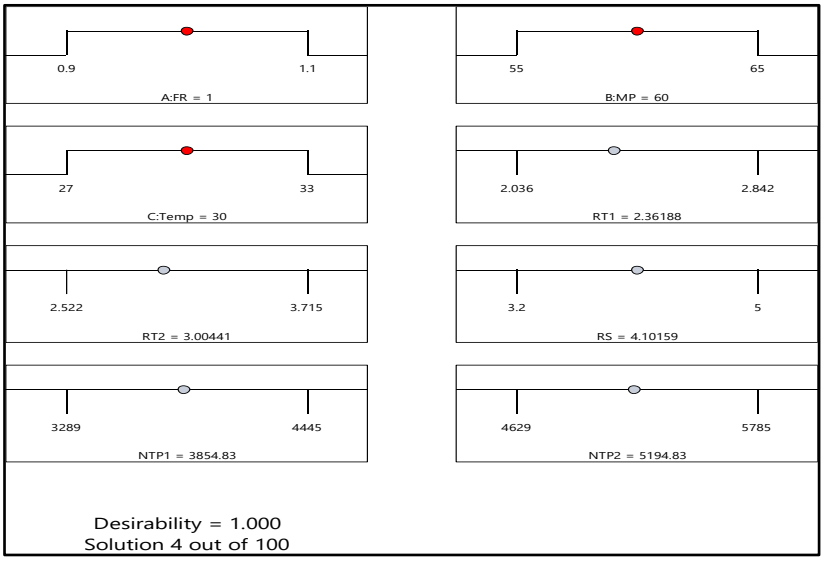
**Table 5. Regression model summary**

| Model | $R^2$ | Adj. $R^2$ | Pred. $R^2$ | Adeq. precision | Std. dev. | CV% |
|---|---|---|---|---|---|---|
| Model for RT1 | 0.9973 | 0.9949 | 0.9846 | 69.3035 | 0.0165 | 0.6910 |
| Model for RT2 | 0.9972 | 0.9948 | 0.9822 | 74.3667 | 0.0227 | 0.7432 |
| Model for RS | 0.9923 | 0.9855 | 0.9688 | 43.4387 | 0.0588 | 1.46 |
| Model for NTP1 | 0.9923 | 0.9853 | 0.9667 | 44.8695 | 36.01 | 0.9564 |
| Model for NTP2 | 0.9923 | 0.9853 | 0.9667 | 44.8695 | 36.01 | 0.9564 |

Fig. 1. Ramps (a) and bar graph (b) of Derringer's desirability function representing the optimized experimental conditions, the individual and combined desirability values.



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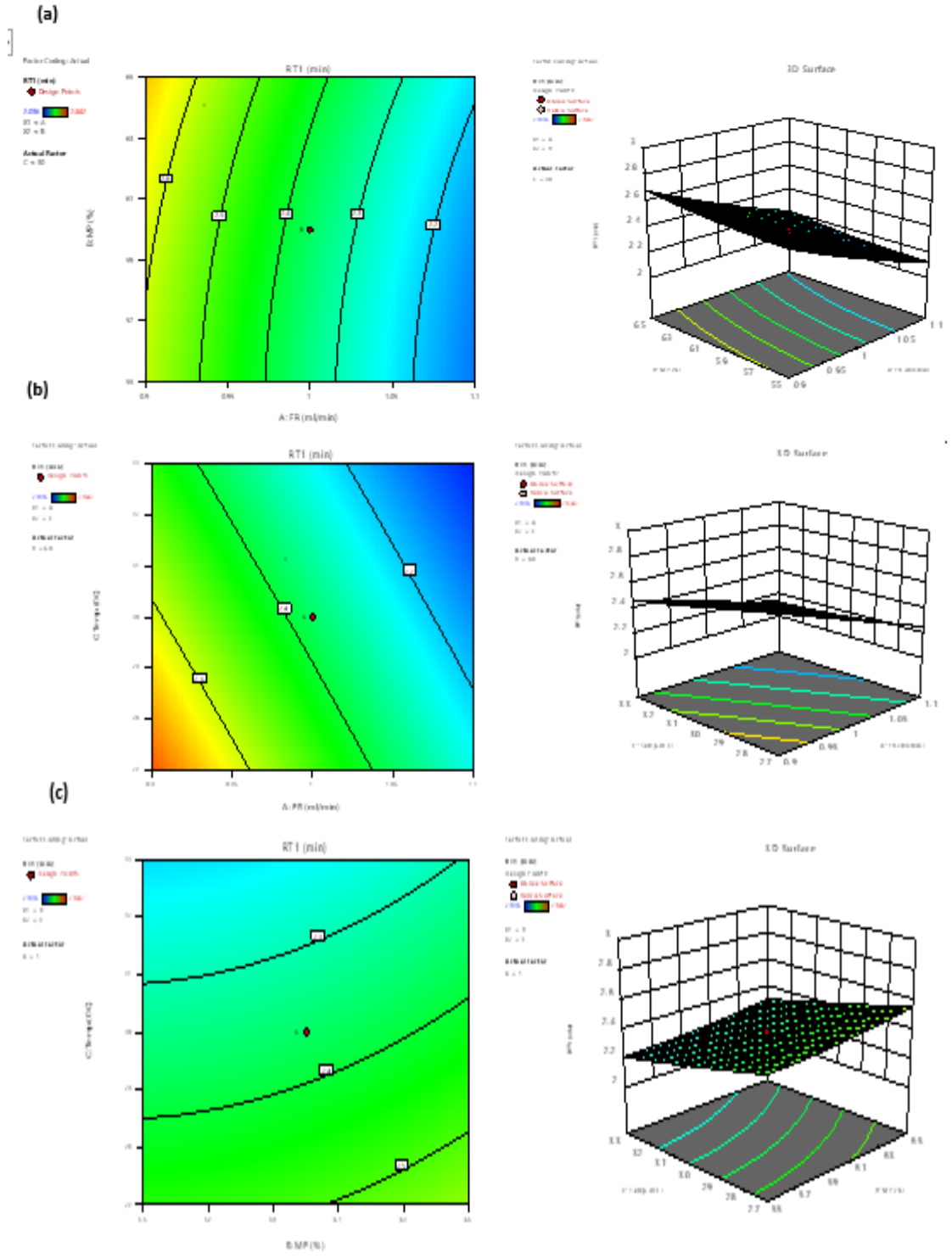


**Fig. 2.** 2D-contours and 3D-response surface plots showing the influence of CMPs: (a) flow rate (A) and organic phase content (%) (B); b) organic phase content (%) (B) and oven temperature (C); (c) flow rate (A) and oven temperature (C) on retention time (RT1) as the Critical Analytical Attribute(CAA).

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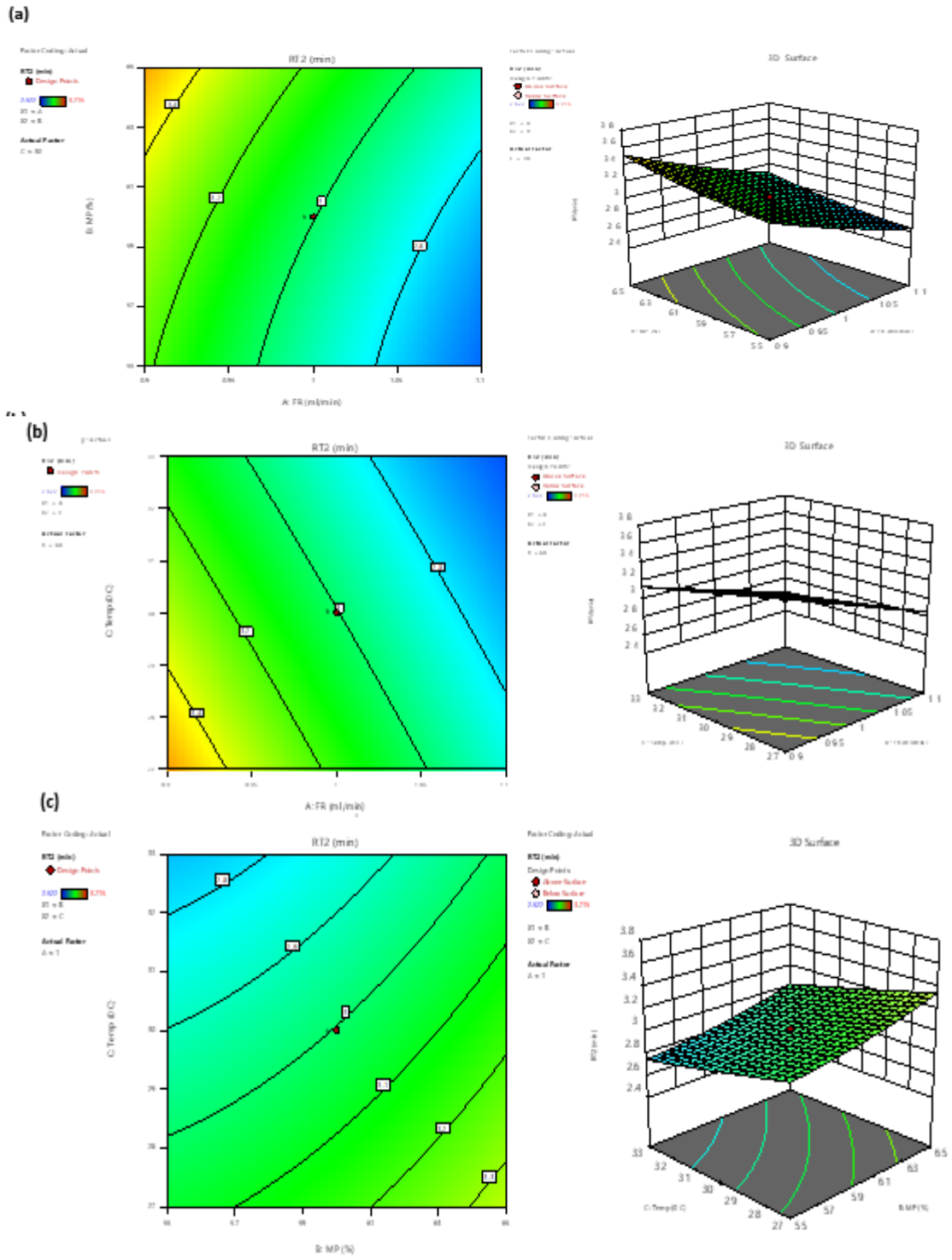


**Fig. 3.** 2D-contours and 3D-response surface plots showing the influence of CMPs: (a) flow rate (A) and organic phase content (%) (B); b) organic phase content (%) (B) and oven temperature (C); (c) flow rate (A) and oven temperature (C) on retention time (RT2) as the Critical Analytical Attribute(CAA).

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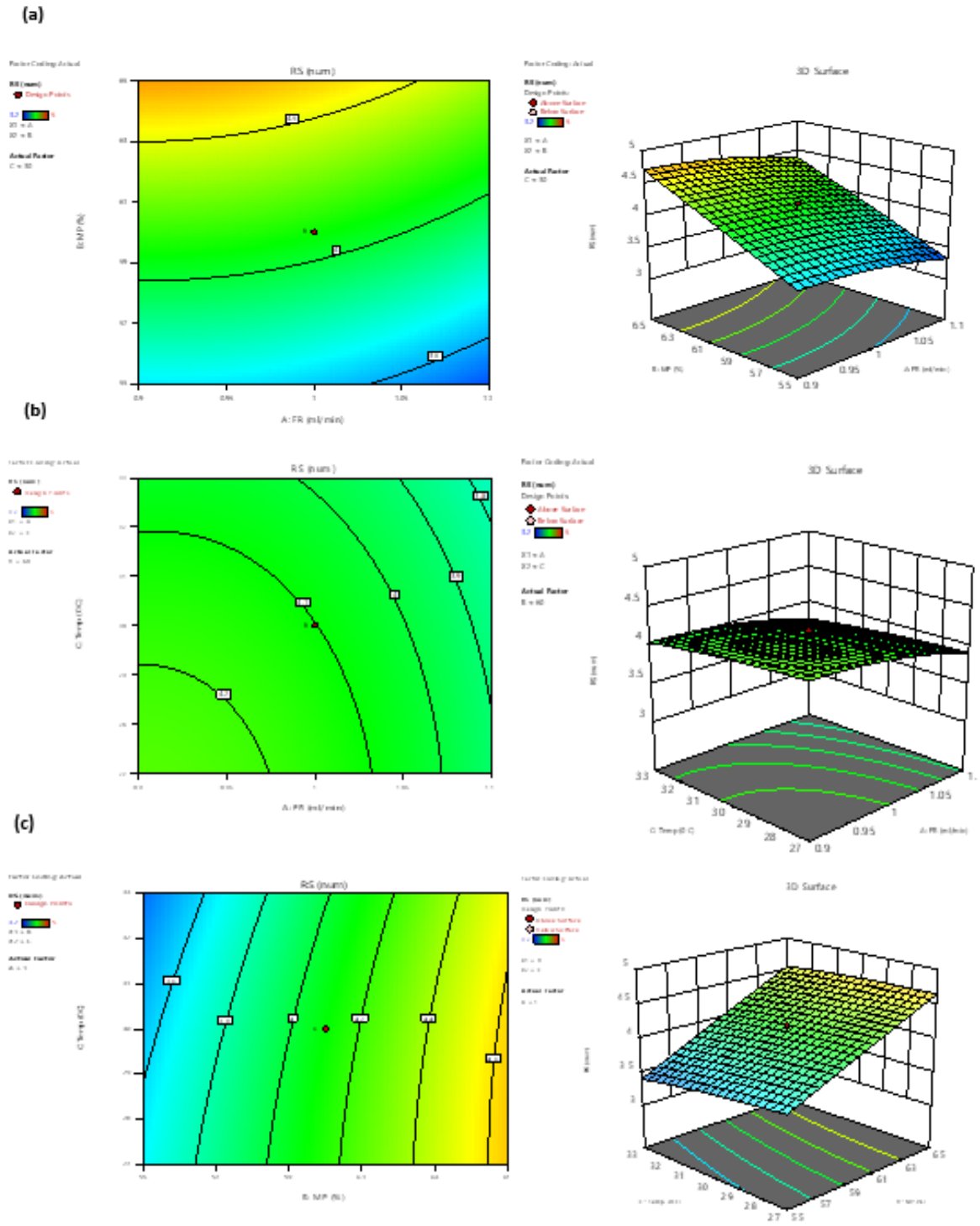


**Fig.4.** 2D-contours and 3D-response surface plots showing the influence of CMPs: (a) flow rate (A) and organic phase content (%) (B); b) organic phase content (%) (B) and oven temperature (C); (c) flow rate (A) and oven temperature (C) on resolution (RS) as the Critical Analytical Attribute(CAA).

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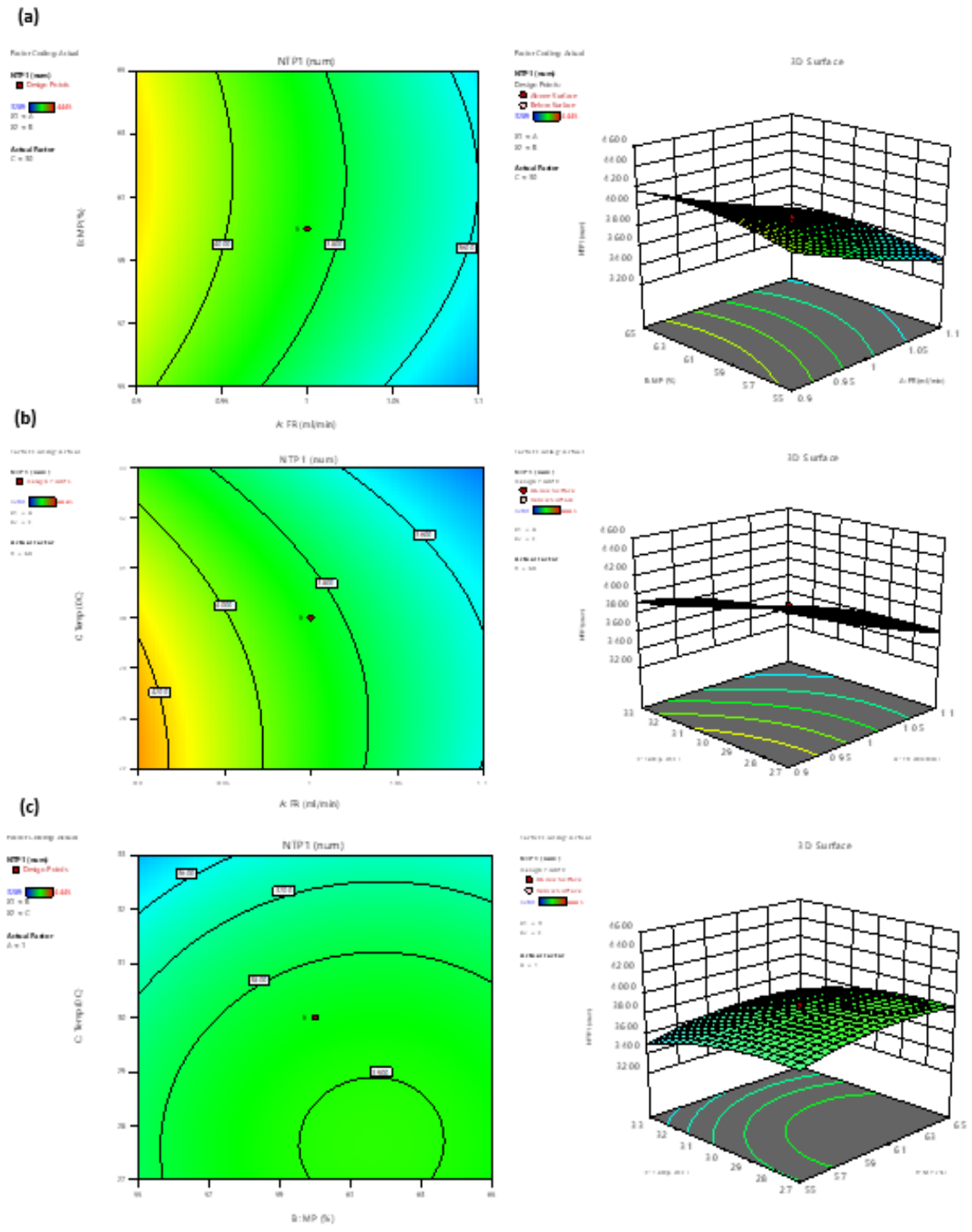


**Fig.5.** 2D-contours and 3D-response surface plots showing the influence of CMPs: (a) flow rate (A) and organic phase content (%) (B); b) organic phase content (%) (B) and oven temperature (C); (c) flow rate (A) and oven temperature (C) on No of Theoretical Plates (NTP1) as the Critical Analytical Attribute(CAA).

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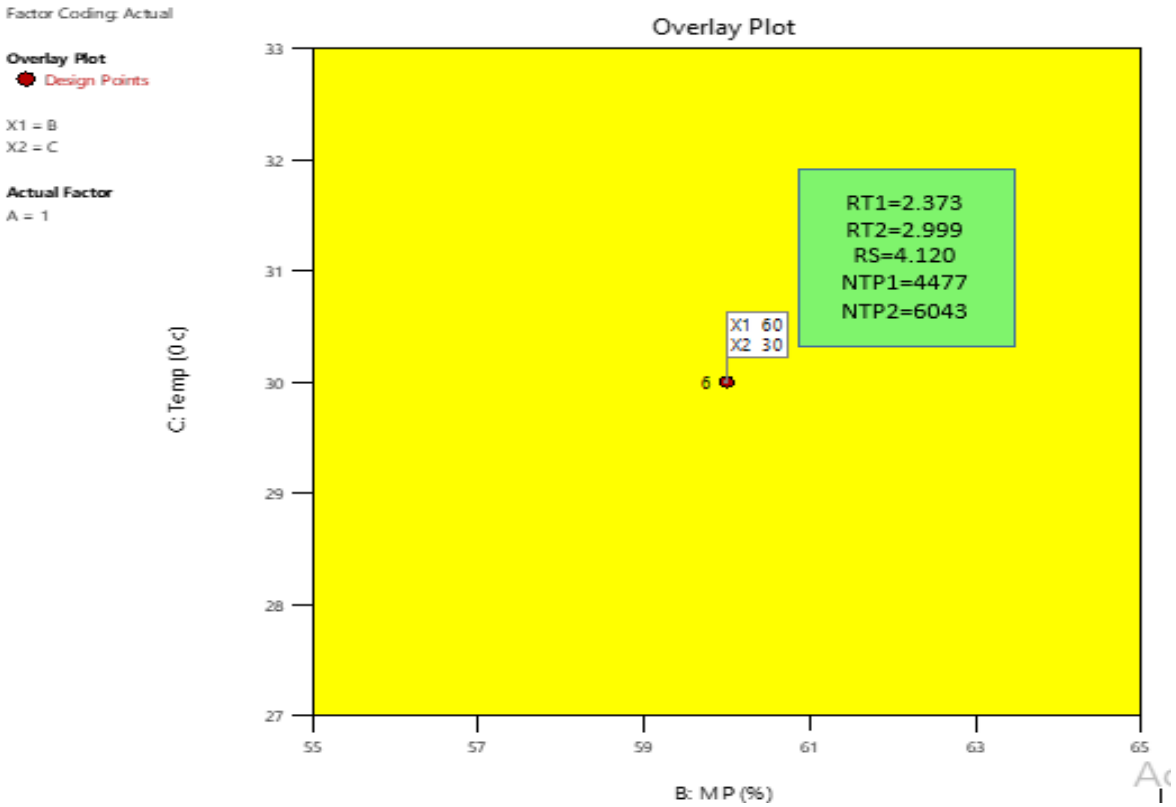


**Fig. 7.** Illustration of the overlay plot in graphical form displaying the ideal design space or technique operational design region.

**Method validation.** *Specificity.* No interference from any other peak of impurities or excipients was discovered after visual examination of the standard chromatogram, assay sample chromatogram, and forced degradation sample chromatogram. Additionally, peak purity indices in each case were reviewed and were found to be greater than 0.9998, demonstrating the method specificity for the drug molecules. The chromatograms of the standard mixture and sample mixture are shown in Figs. 8b, 8c. The chromatogram of a blank obtained by a PDA detector is shown in Fig. 8a.

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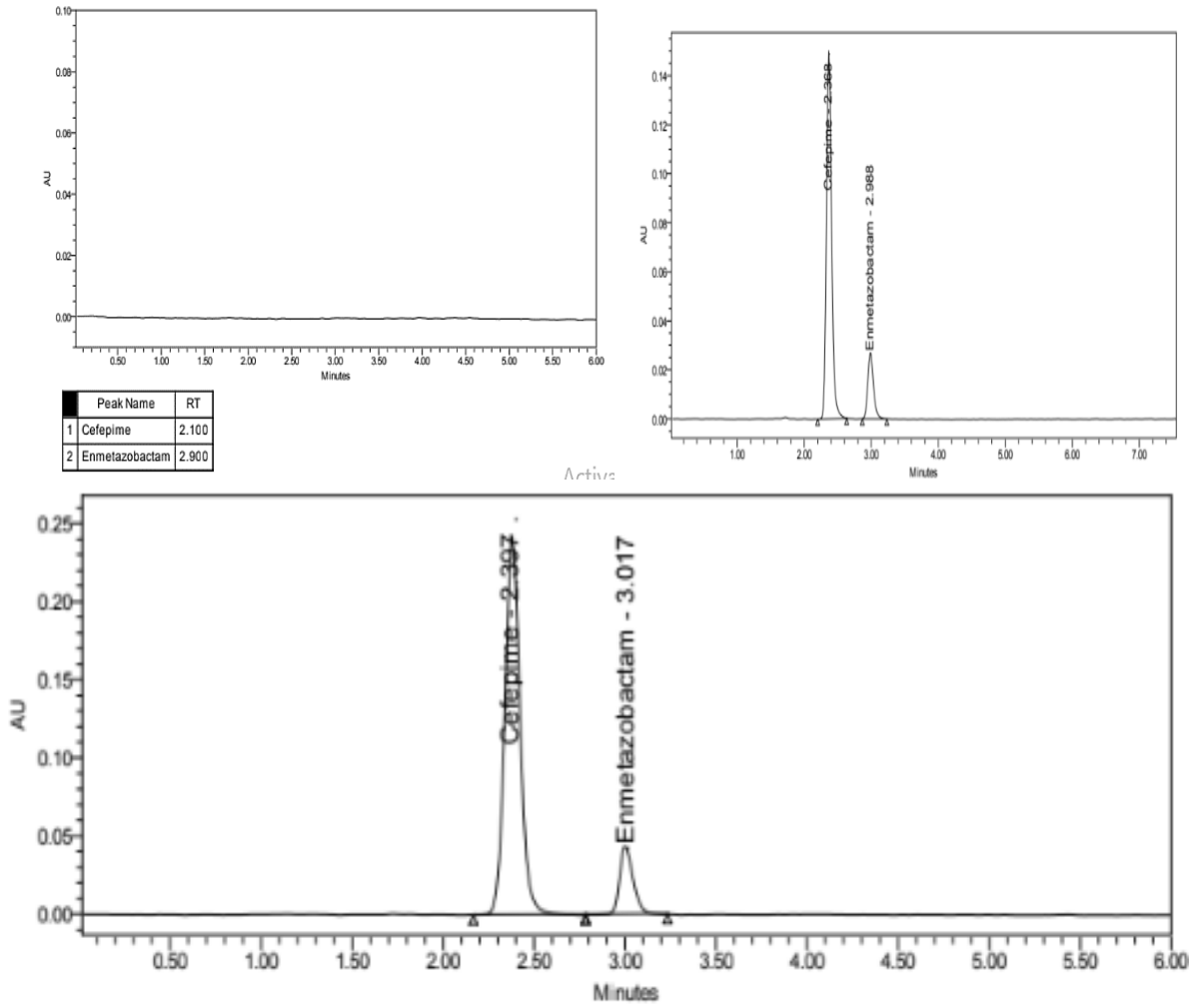


**Fig. 8.** Chromatograms of (a) blank, (b) standard Cefepime and Enmetazobactam, and (c) sample.

Solution stability. Vials with working standards at the nominal concentration were placed at room temperature and in the refrigerator, and tests were performed at 0 and 24 h to determine stability in this solvent. The peak areas were then contrasted with those at 0 h. No appreciable alterations in the area were seen. The drugs appeared to be stable in the diluting solvent as the RSD was discovered to be less than 2.0%.

Linearity, working range, and accuracy. The nominal standard concentration in the current validation procedure was 80 µg/mL of Cefepime 20 µg/mL of Enmetazobactam. Plotting the peak areas against the corresponding concentration, we prepared standard solutions at 25, 50, 75, 100, 125, and 150% of the nominal concentration. Both drugs had determination coefficients ($r^2$) above 0.999, indicating a satisfactory fit of the data by the regression line. The linearity range was found to be 20 – 120 µg/mL for Cefepime and the linearity range for Enmetazobactum was found to be 5 – 30 µg/mL for Cefepime The regression equations were as follows (Eqs. (6) and (7)): Cefepime $y = 5435.1x + 1324.3$ ($r^2 > 0.999$), (6)

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Enmetazobactam: $y = 5433.1x + 1378.9$ ($r^2 > 0.999$) (7)

Table 7. Linearity data for Cefepime and Enmetazobactam

| Cefepime | | Enmetazobactam | |
|---|---|---|---|
| concentration, µg/mL | average area ratio | concentration, µg/mL | average area ratio |
| 20 | 205864 | 5 | 41620 |
| 40 | 416908 | 10 | 82635 |
| 60 | 629297 | 15 | 123796 |
| 80 | 838819 | 20 | 165159 |
| 100 | 1046446 | 25 | 206620 |
| 120 | 1239504 | 30 | 245304 |

Table 8. Accuracy data for Cefepime and Enmetazobactam

| % of nominal | Amount spiked, µg/mL | | Amount recovered, µg/mL | | Recovery, µg/mL | |
|---|---|---|---|---|---|---|
| | Cefepime | Enmetazobactam | Cefepime | Enmetazobactam | Cefepime | Enmetazobactam |
| 50% | 45 | 2.5 | 44.6 | 2.51 | 99.0 | 98.5 |
| | 45 | 2.5 | 45.3 | 2.50 | 100.7 | 100.3 |
| | 45 | 2.5 | 45.2 | 2.48 | 100.4 | 98.9 |
| 100% | 90 | 5 | 90.0 | 4.98 | 100.1 | 98.9 |
| | 90 | 5 | 89.3 | 4.97 | 99.2 | 99.8 |
| | 90 | 5 | 89.9 | 4.99 | 99.8 | 99.7 |
| 150% | 135 | 7.5 | 135.0 | 7.49 | 100.0 | 99.3 |
| | 135 | 7.5 | 133.7 | 7.44 | 99.1 | 98.7 |
| | 135 | 7.5 | 135.8 | 7.47 | 100.6 | 99.3 |
| Mean recovery, % | | | | | 99.9 | 99.3 |

Table 9. Summary of precision

| **Injection** | **Cefepime** | | **Enmetazobactam** | |
|---|---|---|---|---|
| | **method precision** | **inter-day precision** | **method precision** | **inter-day precision** |
| 1 | 835379 | 834430 | 165243 | 164660 |
| 2 | 835218 | 828898 | 165457 | 165497 |
| 3 | 837099 | 836105 | 164454 | 164290 |
| 4 | 832880 | 833000 | 165430 | 166368 |

| | | | | |
|---|---|---|---|---|
| 5 | 851104 | 832916 | 165064 | 163851 |
| 6 | 838010 | 825909 | 165764 | 163600 |
| Mean | 838282 | 831876 | 165235 | 164711 |
| SD | 6524.9 | 3774.3 | 448.6 | 1050.4 |
| RSD, % | 0.8 | 0.5 | 0.3 | 0.6 |

The developed analytical method for the estimation of Cefepime and Enmetazobactam showed good system suitability results. The percentage RSD of peak area was 0.8% for Cefepime and 0.3% for Enmetazobactam, which is within the acceptable limit of ≤ 2%, indicating good precision of the method. The RSD of retention time was 1.2% for Cefepime and 1.03% for Enmetazobactam, also within the acceptable limit, showing good repeatability. The tailing factor values were 1.40 for Cefepime and 1.422 for Enmetazobactam, which are within the USP limit of ≤ 2, indicating proper peak shape. The resolution between the two drugs was 5.5, which is greater than the required value of 2.0, showing good separation between the peaks. The limit of detection (LOD) was 0.21 µg/mL for Cefepime and 0.02 µg/mL for Enmetazobactam, while the limit of quantification (LOQ) was 0.65 µg/mL and 0.06 µg/mL, respectively. These results indicate that the method is sensitive, precise, and suitable for routine analysis of the two drugs.

The robustness of the developed method for Cefepime and Enmetazobactam was evaluated by making small deliberate changes in chromatographic conditions such as flow rate, mobile phase composition, and column temperature. The results showed that slight variations in these parameters did not significantly affect the peak area or system performance.When the flow rate was decreased by 10%, the peak areas obtained were 846471 for Cefepime and 166525 for Enmetazobactam, with RSD values of 0.4% and 0.2%, respectively. Similarly, when the flow rate was increased by 10%, the peak areas were 832454 and 164375, with RSD values of 0.5% and 0.2%. For mobile phase composition variation (–10%), the peak areas were 835098 for Cefepime and 164222 for Enmetazobactam, with RSD values of 0.8% and 0.3%. When the mobile phase was increased by 10%, the peak areas were 846044 and 166680, with RSD values of 0.3% and 0.2%.

The accuracy of the method for the simultaneous estimation of the drugs was demonstrated by the percent recoveries which were well within the limit (100 ± 2%). Tables 7, 8 present the findings. Precision. In the current study, triplicate injections of three different solutions with known quantities of added drugs (50, 100, and 200% of the nominal concentration) were conducted. Regression equations were used to calculate percent recoveries (concentrations). Table 9 displays the findings of the precision study. The method is precise within the required recovery range, as shown by the low RSD of the total variation.

## CONCLUSIONS

The Quality by Design (QbD) approach was applied to develop an HPLC analytical method based on the analytical target product profile. Critical method parameters such as mobile phase composition, flow rate, and column temperature were studied at different levels to optimize the chromatographic conditions. A

central composite design using Design Expert software was employed to evaluate the interaction between these variables and to establish the final design space. The developed method was successfully validated and showed acceptable linearity, precision, accuracy, and robustness for the analysis of Cefepime and Enmetazobactam. Therefore, the proposed method is reliable and suitable for routine quality control analysis of these drugs in pharmaceutical formulations.

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