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EXPERIMENTAL DESIGN-BASED OPTIMIZATION APPROACHES IN UPLC METHOD DEVELOPMENT: SIMULTANEOUS QUANTITATIVE ESTIMATION OF SULFADIAZINE AND TRIMETHOPRIM IN A COMBINED VETERINARY FORMULATION

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ABSTRACT. This study reports a comparative application of three different experimental designs to develop ultra-performance liquid chromatography (UPLC) methods for simultaneous quantification of sulfadiazine and trimethoprim in veterinary tablets. The effects of column temperature, buffer ratio, and flow rate on chromatographic response were systematically evaluated by response surface methodology using full factorial design (FFD), Box–Behnken design (BBD), and central composite design (CCD). Full quadratic models were constructed and evaluated for each design, using statistical parameters and studying the response surface plots. Then, optimal conditions providing efficient peak resolution in shortest runtime were calculated, and experimentally confirmed. FFD and BBD produced nearly identical optimal conditions, while CCD yielded conditions with shorter run time and sufficient resolution. The optimal conditions were determined from two experimental design methods: the first set from FFD and BBD (collectively referred to as FFD/BBD-UPLC), and the second set from CCD (referred to as CCD-UPLC). Both sets of conditions underwent system suitability tests and were validated for linearity, limits of detection and quantification, precision, accuracy, and specificity. Recoveries for sulfadiazine and trimethoprim were within 99.3-100.5% with relative standard deviations less than 1.8%. The developed methods were successfully applied to commercial veterinary tablet analysis, with statistically comparable assay results in accordance with the label claims for both drugs.

Keywords: *UPLC, optimization, experimental design, sulfadiazine, trimethoprim, pharmaceutical analysis*

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INTRODUCTION

Veterinary drug formulations, especially those intended for food-producing animals, play a critical role in ensuring both animal health and food safety. These formulations must demonstrate the intended therapeutic efficacy and comply with regulatory requirements to minimize the risk of drug residues in animal-derived food products and to help control the development of antimicrobial resistance [1]. Fixed-dose combinations, such as sulfadiazine (SDZ) and trimethoprim (TMP), are commonly used in veterinary medicine due to their synergistic antibacterial effects. SDZ and TMP inhibit sequential steps in bacterial folate synthesis, resulting in selective and enhanced and selective bactericidal activity, reduced resistance development, and broad antimicrobial coverage [2].

Literature survey reveals that the simultaneous determination of SDZ and TMP in pharmaceutical preparations has been carried out by chemometrics-assisted spectrophotometric methods [3-5] thin-layer chromatography [6], capillary zone electrophoresis [7, 8] and HPLC [9, 10]. The development of fast, feasible, accurate, and reproducible analytical methods that serve as alternatives to existing ones have always been important for pharmaceutical industry, for both human and veterinary drugs. Ultra-performance liquid chromatography (UPLC) offers a modern, high-efficiency alternative to HPLC with reduced run times and improved resolution, making it highly suitable for simultaneous analysis of combination drugs. However, the full benefits of UPLC can only be realized when chromatographic conditions are carefully optimized.

Optimization in analytical chemistry, particularly in chromatographic method development, is critical to achieve selectivity, sensitivity, feasibility, and efficiency. Traditional one-variable-at-a-time optimization is labor-intensive and often fail to detect interactions between variables. Response surface methodology (RSM) has emerged as a rational, statistically robust strategy for optimization in many fields including pharmaceuticals, engineering, and food sciences. RSM is based on fitting a polynomial equation to experimental data using mathematical and statistical techniques. It enables simultaneous evaluation of multiple experimental factors, the construction of predictive models, and the determination of optimal factor levels to achieve the best system performance [11, 12].

In response surface methodology, it is necessary to select an experimental design that defines which experiments will be conducted. Experimental designs for first-order models are typically used for screening purposes or when curvature is not expected in the response surface [13]. However, in order to model the interaction of factors, curvatures, and critical

response points such as maxima or minima, experimental designs with at least three levels are required. Such designs are able to fit full quadratic models which allow to predict the optimal levels of experimental factors that yield the best response [11, 14]. The choice of experimental design directly influences the number of required experiments, the quality of the fitted model, and the accuracy of the predicted optimum. Among the most commonly employed RSM designs are full factorial design (FFD), Box–Behnken design (BBD), and central composite design (CCD). FFD is a cubic design which includes all possible combinations of factor levels. It enables detailed exploration of all factor interactions but becomes resource-intensive with increasing number of factors. BBD is more economical, requiring fewer runs and avoiding extreme factor levels, making it preferable when the corner points in the design space may be operationally difficult. CCD includes axial points, which may introduce challenges under extreme experimental conditions but offers superior predictive capabilities and greater flexibility in exploring the design space [15, 16].

While each of these designs has theoretical and statistical advantages, comparative studies evaluating their practical outcomes in chromatographic optimization remain limited. Such comparisons are valuable for guiding method developers in selecting the most appropriate design based on analytical performance, resource demands, and optimization success.

This study aimed to compare FFD, BBD, and CCD designs for optimizing a UPLC method for the simultaneous quantification of SDZ and TMP in veterinary tablet formulations. As it is important have an efficient resolution in a short analysis time in pharmaceutical chromatographic analyses, a chromatographic response function that ensures sufficient resolution between peaks, narrow peak width and short analysis time was used as response. Using this response as the optimization criterion, each design was assessed for its ability to produce efficient, fast, and high-resolution chromatographic conditions. The optimal conditions obtained from each design were validated and compared in terms of analytical performance and practical suitability for the quantitative determination of SDZ and TMP in veterinary tablet samples.

RESULTS AND DISCUSSION

Response surface methodology offers a powerful alternative to conventional one-variable-at-a-time optimization in various fields including science, technology, and industry. It involves several critical decisions that collectively influence the quality and the performance of a predictive model that would be used for a defined purpose. The key steps include (1) selecting

relevant factors and their levels, ensuring they cover the experimental space of interest; (2) identifying measurable responses that align with the optimization objectives; (3) choosing an appropriate experimental design and carrying out the experiments; (4) employing a suitable fitting technique (e.g., linear regression, polynomial models, or machine learning algorithms) to model the system's behavior; and (5) applying an optimization strategy (such as grid-search, derivative functions, or gradient-based methods). Each step impacts the model's accuracy, interpretability, generalizability, and predictive performance, which overall affect the outcome as the optimal conditions and the optimal response [11].

The focus of this work was the third step, selecting the optimal design for response surface methodology. Quadratic models, which are needed to find an optimum response of a system, can be estimated using full factorial designs with three or more levels for each factor, but these designs require a high number of runs. More efficient designs, such as CCD and BBD, are generally preferred due to the reduced number of runs. The choice of design depends on desired precision, the nature of the design space, and experimental constraints such as the number of experiments and available resources, often the most critical consideration. BBD is preferred if fewer runs are needed and avoiding extreme points is desirable [17, 18]. On the other hand, CCD is the most widely used design because it has better predictive power and can be a good choice after a factorial screening design when converting the screening phase into an RSM by adding central and axial points into the design [19, 20]. In this work, we compared the most common RSM designs to evaluate their practical performance to choose the best set of experimental conditions for optimal response. Throughout the all RSM procedure a chromatographic response function developed by Rakić et al. [21] was used as the dependent variable. This function abbreviated as CRF is calculated by measuring various parameters such as retention times, peak widths, height of the peaks, height of the valleys (in case of overlapping peaks), and elution time. It typically decreases with narrow peak widths, shorter analysis time, and sufficient resolution between the peaks. Therefore, the optimal chromatographic settings are characterized by the lowest CRF values. Previous works indicated CRF was a suitable response for UPLC method development, achieving efficient chromatographic separation in a short run time [22-25].

Development of Chromatographic Method by Experimental Design

In this work, some preliminary experiments were conducted instead of a factorial screening step because most UPLC factors affecting the chromatographic response were predictable from our experience with UPLC

analyses. Categorical factors such as column type and mobile phase composition (organic solvent and buffer type) were determined prior to the experimental design process. A mixture of HCOOH-HCOONa buffer and methanol was found suitable on the C18 column to observe significant shifts in the retention times of both drugs with the variations in the ratio of methanol and buffer. Potential factors, namely, concentration, pH and ratio of the buffer in mobile phase, column temperature, and flow rate were tested to observe if they have an apparent effect on the retention times. A change in the concentration of the buffer (from 20 mM to 100 mM) did not affect the elution times, and 20 mM was chosen to reduce the possibility of salt precipitation. The pH of the buffer did not change the retention times of the drugs; however, pH 4.4 had a significantly lower solvent peak. Hence, the pH of the buffer was excluded as a factor from the design, and 20 mM pH 4.4 sodium formate buffer was used during the remaining experiments. Column temperature, buffer ratio, and flow rate were selected as factors x_1, x_2, and x_3, respectively, as they showed observable changes in chromatographic behavior.

A common design space was needed to compare FFD, BBD, and CCD so we aimed to use standardized factor levels (−1, 0, +1) across all three designs. The levels of factors for FFD, BBD, and CCD were systematically determined based on preliminary screening work and critical instrumental constraints such as UPLC backpressure and run time, ensuring the experimental design covered the widest possible chromatographic design space. The objective was to define a range where the chromatographic behavior of the peaks spanned from maximum separation with the constraint of a maximum 7-minute run time to minimal separation, which is near-complete overlapping of the peaks. Specifically, a low column temperature (30 º C), high buffer ratio (78.0 %) and low flow rate (0.16 mL/min) was defined as the maximum separation conditions. The maximum overlap conditions were defined as high column temperature (50 º C), low buffer ratio (52.0 %) and high flow rate (0.28 mL/min). These values were considered as -1 and +1 factor levels, and central point of the values were defined as 0 level, as listed in Table 1. The value of the axial point was calculated using the formula $\alpha = 2^{k/4} = 2^{3/4} \approx 1.682$ to obtain a rotatable CCD. Table 1 depicts the factor levels used in all three designs.

Design matrices of FFD, BBD and CCD were generated according to the factor levels in Table 1, and they are provided in supplementary material. The number of experiments were calculated using the formulas 3^k for FFD, $2k \times (k-1) + 1 + n_0$ for BBD and $2^k + (2k+1) + n_0$ for CCD, where k is the number of factors and n_0 is the number of replicate in the center point (0, 0, 0), which was 3 and 5 for BBD and CCD, respectively. In the end, three different design matrices containing 27, 15, and 20 experimental runs were generated for FFD, BBD, and CCD, respectively.

Table 1. Factors and their corresponding levels used in FFD, BBD, and CCD

	x_1	x_2	x_3
Level	Column temp (ºC)	Buffer (%)	Flow rate (mL/min)
-α	23.2	43.1	0.12
-1	30.0	52.0	0.16
0	40.0	65.0	0.22
+1	50.0	78.0	0.28
+ α	56.8	86.9	0.32

All experimental runs in three different design matrices were performed by injecting a standard solution of 20 µg/mL SDZ and 20 µg/mL TMP, by detection at 245 nm. Figure 1 illustrates the chromatograms from 27 runs required by the FFD. Figure 2 and 3, depict the chromatograms recorded during the application of BBD and CCD, respectively. Data from these chromatograms were used to calculate the CRF response for each run. Table S1, Table S2, and Table S3 in the supplementary material list the experimentally measured responses at each run.

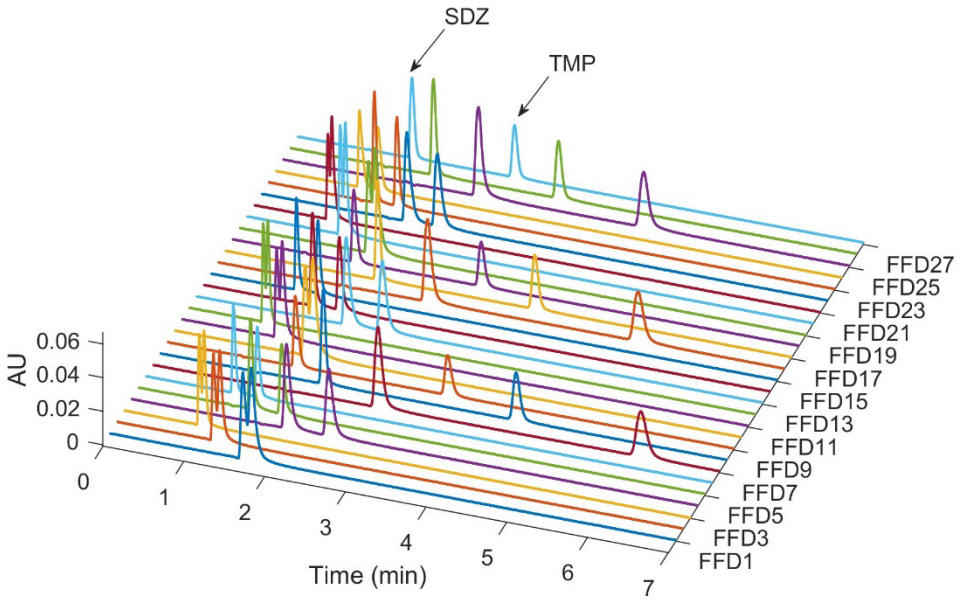

Figure 1. Illustration of 27 chromatograms obtained from FFD design matrix

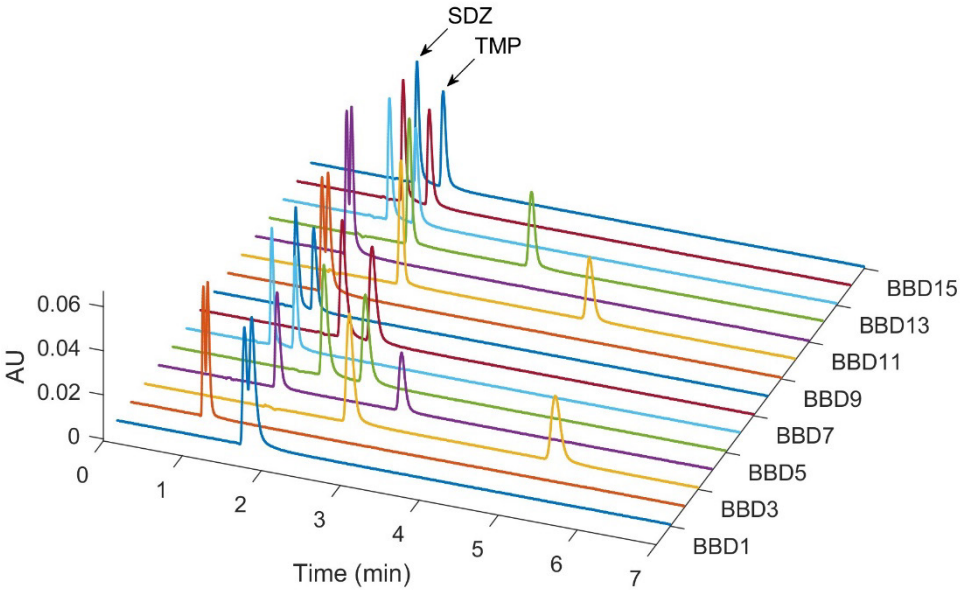

Figure 2. Illustration of 15 chromatograms obtained from BBD design matrix

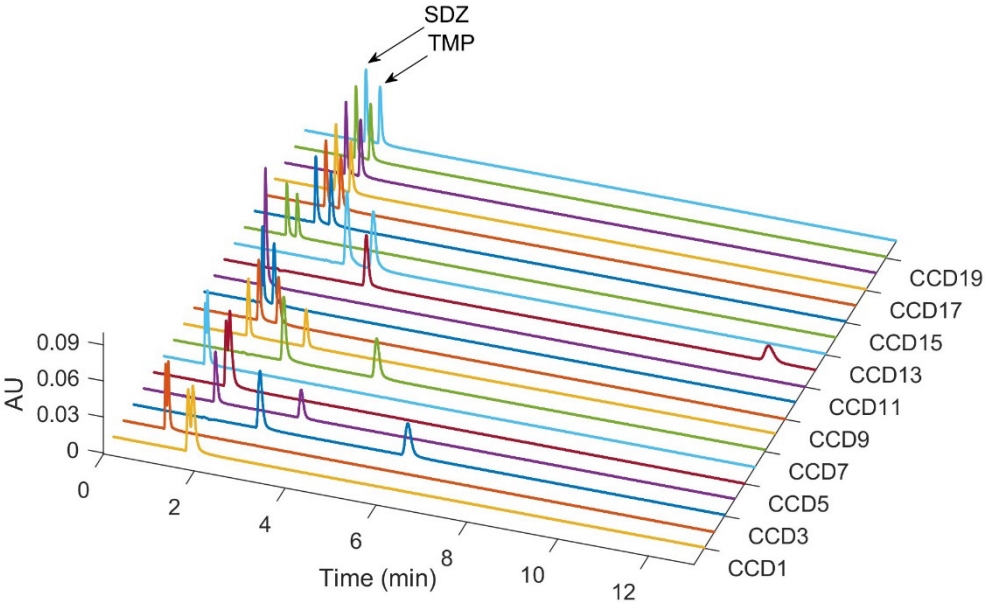

Figure 3. Illustration of 20 chromatograms obtained from CCD design matrix

For each design, a full quadratic model containing three variables (x_1, x_2, x_3), their squared terms and the binary interactions was constructed to the experimental data using least squares regression. A representative model is given as

$$\hat{y} = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{33} x_3^2 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3$$

where x_1, x_2, and x_3 represent the variables, β_0 is the intercept, β_1, β_2, and β_3 are the linear terms, β_{11}, β_{22}, and β_{33} are the quadratic terms, β_{12}, β_{13}, and β_{23} are the interaction terms.

Table 2 presents the estimated coefficients (β), t-test statistics, and p-values for three quadratic regression models corresponding to FFD, BBD, and CCD, highlighting the statistical significance of linear, quadratic, and interaction effects ($p<0.05$).

Table 2. Estimated coefficients, t-test statistics, and p-values for three quadratic regression models obtained from FFD, BBD and CCD

	FFD		BBD		CCD	
term	**estimate**	**p-value**	**estimate**	**p-value**	**estimate**	**p-value**
β_0	2.394	1.94×10^{-38}	2.457	2.10×10^{-13}	2.183	2.29×10^{-29}
β_1	0.028	1.74×10^{-11}	0.028	8.83×10^{-05}	0.098	1.18×10^{-17}
β_2	-0.753	1.39×10^{-35}	-0.694	1.01×10^{-11}	-1.705	4.48×10^{-30}
β_3	-0.230	7.73×10^{-27}	-0.244	1.88×10^{-09}	-0.402	8.41×10^{-24}
β_{11}	-0.013	7.44×10^{-4}	-0.073	5.46×10^{-06}	0.058	1.54×10^{-15}
β_{22}	0.914	5.8×10^{-33}	0.829	2.87×10^{-11}	2.125	3.77×10^{-31}
β_{33}	0.161	3.73×10^{-20}	0.148	1.59×10^{-07}	0.148	1.36×10^{-19}
β_{12}	-0.094	9.05×10^{-19}	0.010	3.78×10^{-02}	-0.374	2.52×10^{-22}
β_{13}	0.006	1.12×10^{-2}	0.022	1.35×10^{-03}	0.003	6.55×10^{-3}
β_{23}	0.080	1.55×10^{-17}	0.144	1.47×10^{-07}	0.069	5.69×10^{-15}

The quadratic models detailed in Table 2 were used to plot the response surface and contour plots. These figures illustrate the interaction of each pair of selected factors, enabling the visualization of their combined effects on CRF in two dimensions within the design space. As there are three pairs in each model, three plots were generated for each design, namely FFD, BBD, and CCD, presented in Figure S1, Figure S2, and Figure S3,

respectively in supplementary material. The response surfaces of the same factor pair obtained by different designs have similar features, but the greater design space of CCD reveals more curvatures. One may utilize these plots to choose an acceptable region of factor levels that would result in a desirable response. However, it is also possible to predict the optimal levels of factors that would result in the best possible response by using the models. Indeed, in this work, the optimal levels of variables minimizing the CRF were computed using the models given in Table 2. Table 3 summarizes the optimal values for each factor, using the models obtained by FFD, BBD, and CCD. As can be seen in this table, the values obtained by FFD and BBD are very similar—or even identical. In order to compare the optimal conditions obtained by different designs, a mixture of SDZ and TMP was injected into the UPLC system with 10 replications using the conditions given in Table 3. A representative chromatogram from each condition is presented in Figure 4. When these chromatograms are examined, there is almost no difference between FFD and BBD in terms of resolution, peak width and analysis time. However, the optimal conditions determined using CCD resulted in a shorter analysis time with a slightly lower but still sufficient resolution.

Table 3. The optimal chromatographic conditions obtained by FFD, BBD, and CCD

	Factor	FFD	BBD	CCD
x_1	Column temperature (°C)	38.5	39.3	44.9
x_2	Buffer (%)	69.9	69.7	70.5
x_3	Flow rate (mL/min)	0.26	0.26	0.30

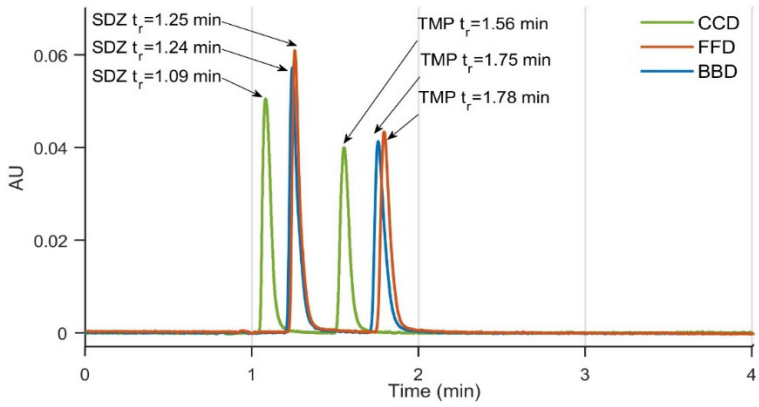

Figure 4. Chromatograms obtained with optimal conditions calculated from FFD, BBD and CCD.

In order to perform a more rational comparison of optimal conditions obtained by different designs, CRF values calculated from ten chromatograms were compared statistically. Statistical comparison of CRF values obtained from FFD design versus BBD and CCD design was performed using F-test and Student's t-test. As presented in supplementary material Table S4, the average CRF values obtained by FFD, BBD and CCD were calculated as 2.4142, 2.4282 and 2.0314, respectively.

The difference between CRF values obtained by FFD and BBD was not statistically significant at a significance level of 0.05, which is also observed visually Figure 4. FFD and BBD cover the same design space, but unlike BBD, FFD includes the corner points. It is evident that BBD has more statistical efficiency compared to other designs, meaning it requires fewer experiments and has the same exploratory power. Hence, it was concluded that BBD might be a better choice than FFD for optimization purposes.

However, CRF values obtained by CCD were found to be statistically different from FFD (p=0.05). The smaller response obtained by CCD indicated that using CCD enabled reaching a smaller CRF value; hence, it outperformed FFD and BBD, possibly due to the greater design space. Indeed, the optimal flow rate predicted by CCD was 0.30 mL/min, which was out of the range of factor levels of FFD and BBD.

FFD provided a comprehensive evaluation of the system, but require a high number of experimental runs (27), making it the least efficient option for complex optimizations. BBD was the most statistically efficient design in the range of -1 and -1 design space. It required significantly fewer runs (15) than FFD while yielding a statistically similar optimal CRF value (2.4282 vs. 2.4142), demonstrating equal modeling power within the factor cube. However, BBD is limited by its inability to explore the corners of the design space. In contrast, the CCD, despite requiring more runs (20) than BBD, offered the superior optimal solution, by covering a greater design space using the axial points. This expanded range allowed the model to predict an optimal flow rate (0.30 mL/min) outside the -1 and +1 limits, which resulted in a statistically lower CRF value (2.0314). Consequently, the CCD was the most effective method for this specific optimization, as its broader domain allowed it to locate a more favorable true minimum for the response surface.

The implementation of experimental design-based optimization of chromatographic methods aligns strongly with the principles of green analytical chemistry [26]. This approach serves as an efficient tool to reduce the environmental impact of analytical method development by conserving energy, reducing solvent consumption, and decreasing the number of experimental trials. Statistical experimental designs such as FFD, BBD, and CCD help to find the real optimal conditions by maximizing the information gained with a

pre-defined number of experiments, as opposed to the classical one variable at a time approach [27]. Reducing the number of experimental trials results in a significant reduction in solvent and reagent consumption, aligning with green chemistry's principle of waste prevention, thereby reducing hazards to both the environment and human health. Furthermore, the final optimal conditions predicted by RSM, such as the shortest analysis time obtained by CCD design in this work, would minimize solvent and energy consumption during the routine application of the UPLC method.

We also aimed to compare the analytical performance of each optimal condition for the simultaneous quantification of SDZ and TMP in commercial samples. Since the chromatographic conditions obtained using FFD and BBD designs were not significantly different, the average of the optimal conditions derived from these two designs was employed for the quantitative analysis of SDZ and TMP. This chromatographic method, with a column temperature of 38.9 ºC, 69.8 % buffer, and a 0.26 mL/min flow rate, was called the FFD/BBD-UPLC method and resulted in retention times of 1.24 min and 1.77 min for SDZ and TMP, respectively. In contrast, the chromatographic conditions obtained from the CCD design differed from those of the other two designs. To enable comparison of analytical results, a second UPLC method using these CCD-derived conditions with a column temperature of 44.9 ºC, 70.5 % buffer and 0.30 mL/min flow rate was referred to as the CCD-UPLC method, with retention times of 1.07 and 1.54 for SDZ and TMP, respectively.

Application of Optimized Methods for the Quantitative Analysis

Two different UPLC methods were developed using different designs by RSM approach, aimed for implementation in the quantitative analysis of SDZ and TMP in veterinary tablet samples. For this aim, the calibration set of analytes were prepared as explained in Section "Preparation of Standard Solutions", and were injected into the UPLC system using two different methods, FFD/BBD-UPLC and CCD-UPLC. Calibration and prediction steps were performed at the detection wavelength of 267 nm and 230 nm for SDZ and TMP, respectively. The chromatograms of the calibration set recorded at 267 and 230 nm by the application of FFD/BBD-UPLC method are illustrated in Figure 5(I)a and Figure 5 (I)b, respectively. Figure 5 (II)a and Figure 5 (II)b depict the chromatograms of the calibration set detected at 267 and 230 nm, respectively.

The peak areas of SDZ and TMP were calculated from the UPLC chromatograms recorded at 267 nm for SDZ and 230 nm for TMP, for both UPLC methods (See Figure 5). The linear regression between the peak area

and concentration for each substance was modelled using the least squares approach. Detection wavelengths, linear ranges and statistical data of regression analysis were summarized in Table 4. As can be seen here, the statistical parameters of FFD/BBD-UPLC and CCD-UPLC methods were similar. The determination of SDZ and TMP in both validation and commercial tablet samples was performed using the linear regression equations shown in Table 4.

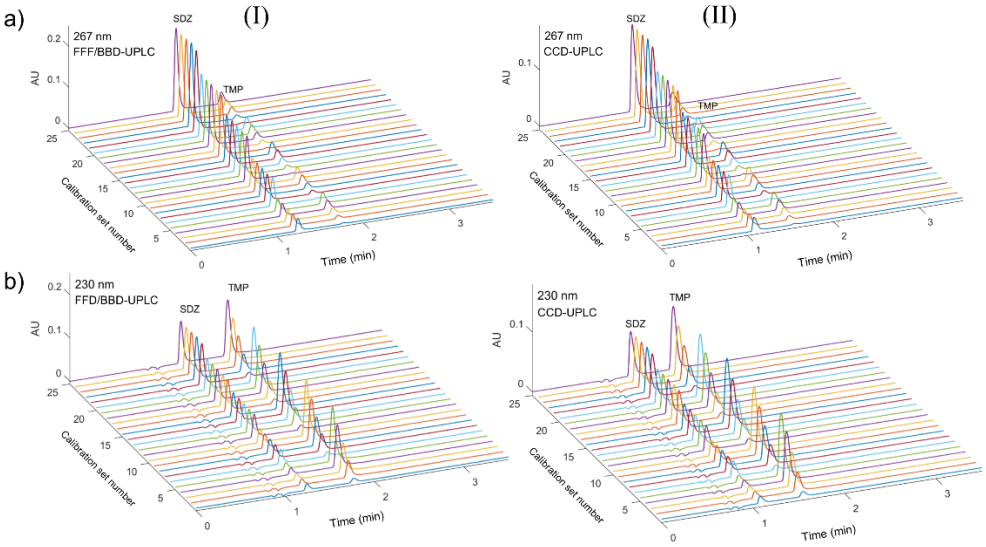

Figure 5. Chromatograms of the calibration set recorded at **a)** 267 nm for the determination of SDZ and **b)** 230 nm for the determination of TMP, obtained by the application of **(I)** FFD/BBD-UPLC and **(II)** CCD-UPLC methods

Table 4. Regression analysis results of SDZ and TMP during the application of FFD/BBD-UPLC and CCD-UPLC methods

	FFD/BBD-UPLC		**CCD-UPLC**	
	SDZ	**TMP**	**SDZ**	**TMP**
Detection wavelength (nm)	267	230	267	230
Linear range (µg/mL)	6-38	2-26	6-38	2-26
Slope	2.847	3.111	2.495	2.748
Intercept	-1.151	0.473	-1.016	0.269
Correlation coefficient	0.9992	0.9997	0.9991	0.9998
Standard error of slope	0.024	0.016	0.022	0.012
Standard error of intercept	0.604	0.259	0.539	0.204
Standard error of correlation coefficient	1.382	0.672	1.233	0.528
Limit of detection (µg/mL)	0.70	0.28	0.71	0.24
Limit of quantitation (µg/mL)	2.12	0.83	2.16	0.74

Validation of the optimized methods

Prior to analytical validation experiments, system suitability tests were carried out to evaluate the suitability and effectiveness of the optimized chromatographic methods. A standard solution containing 20 µg/mL SDZ and 20 µg/mL TMP was injected 10 times into UPLC and system suitability parameters were calculated using the Waters Empower2 software. The average values of these parameters, reported in Table 5, were found to be suitable for continuing the validation procedures.

Table 4. Regression analysis results of SDZ and TMP during the application of FFD/BBD-UPLC and CCD-UPLC methods

	FFD/BBD-UPLC		**CCD-UPLC**		**Recommended**
	SDZ	**TMP**	**SDZ**	**TMP**	
Retention time (min)	1.24	1.77	1.07	1.54	
Capacity factor (k')	1.07	1.95	1.15	2.08	k' > 1
Resolution (R)	-	6.13	-	4.67	R > 2
Tailing factor (T)	1.58	1.45	1.64	1.46	T < 2
Theoretical plate number (N)	4140	5598	2480	4111	N > 2000
RSD of retention time	0.11	0.10	0.19	0.13	RSD < 1
RSD of peak area	0.89	1.15	0.97	1.17	RSD < 1

RSD: relative standard deviation

For both FFD/BBD-UPLC and CCD-UPLC methods, linearity and high correlation coefficients were reported in the working concentration ranges of SDZ and TMP. Limit of detection and limit of quantification values were calculated using the standard deviation of the intercept and slope values of calibration equations, given in Table 4. As can be seen in Table 4, the values for both drugs obtained from the FFD/BBD-UPLC and CCD-UPLC methods were very similar, all compatible with the working ranges and serving the intended purpose of the methods.

The accuracy and precision of the optimized UPLC methods were evaluated by recovery studies of binary synthetic mixtures. A test set of 11 binary mixtures was analyzed by FFD/BBD-UPLC and CCD-UPLC methods, and their analysis results were reported in Table S5 in supplementary material. The mean and standard deviation values of SDZ recoveries were reported to be 100.16% with 1.31 using FFD/BBD-UPLC method and 100.54% with 1.06 using CCD-UPLC method. On the other hand, mean

recovery with standard deviation values of TMP were reported to be 99.30% with 1.79 and 100.36% with 1.74 using FFD/BBD-UPLC and CCD-UPLC methods, respectively.

Another approach to assess accuracy and precision was the application of FFD/BBD-UPLC and CCD-UPLC methods to the analysis of inter-day and intra-day samples. Standard mixture solutions at three concentration levels (80%, 100%, and 120% of test level) were analyzed three times on the same day and on three consecutive days. The analysis results, along with recovery, standard deviation, relative standard deviation and relative standard error values are listed in Table S6 in supplementary material. These results indicated that the optimized UPLC methods gave accurate and precise results with good reproducibility.

Specificity/selectivity of the optimized methods was evaluated by the standard addition technique. The standard addition samples, which were prepared in triplicate as described in section “Preparation of Standard Solutions”, were analyzed by FFD/BBD-UPLC and CCD-UPLC methods. The added recover and relative standard deviation were calculated as shown in Table 6.

Table 6. Analysis results of standard addition samples by the application of FFD/BBD-UPLC and CCD-UPLC techniques

		Added		Found		Recovery (%)		RSD	
		SDZ	TMP	SDZ	TMP	SDZ	TMP	SDZ	TMP
FFD/ BBD-UPLC	Tablet +	10	5	10.09	4.94	101.0	98.8	0.19	0.19
	Tablet +	15	12	14.98	12.06	99.9	100.5	0.06	0.20
	Tablet +	20	19	20.25	19.21	101.2	101.1	0.05	0.18
CCD-UPLC	Tablet +	10	5	10.15	4.99	101.5	99.7	0.30	0.16
	Tablet +	15	12	14.81	11.93	98.7	99.4	0.12	0.03
	Tablet +	20	19	20.27	19.46	101.3	102.4	0.10	0.16

RSD: Relative standard deviation

Overall, both UPLC methods, optimized using distinct experimental designs, demonstrated comparable and acceptable analytical performance across all validation parameters.

Analysis of commercial tablet samples by the optimized methods

Following the validation procedures, FFD/BBD-UPLC and CCD-UPLC methods were applied for the quantitative estimation of SDZ and TMP in commercial tablet samples. The tablet solutions mentioned in " 2.4. Preparation of Commercial Sample Set" were injected into UPLC instruments under the optimized conditions of FFD/BBD-UPLC and CCD-UPLC methods. The stacked chromatograms of the commercial sample set obtained by the FFD/BBD-UPLC method are shown in Figure 6(I)a (267 nm) and Figure 6(I)b (230 nm). Figure 6(II)a and Figure 6(II)b depict the chromatograms of the commercial samples set obtained by the application of CCD-UPLC method for determination of SDZ at 267 nm and for TMP at 230 nm.

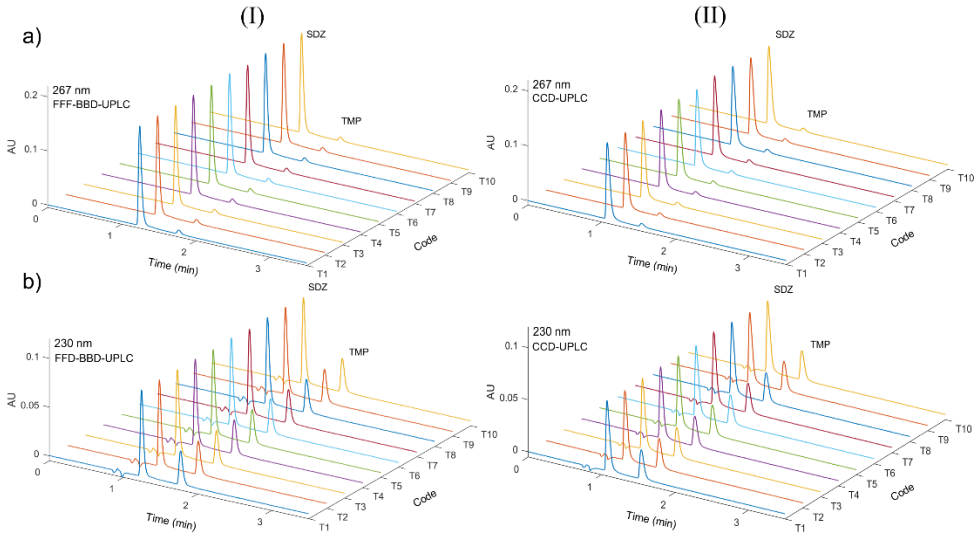

Figure 6. Chromatograms of the commercial sample set recorded at **a)** 267 nm for the assay of SDZ and **b)** 230 nm for the assay of TMP, obtained by the application of **(I)** FFD/BBD-UPLC and **(II)** CCD-UPLC methods

The peak areas in these chromatograms and calibration equations given in Table 4 were used to calculate the concentration of SDZ and TMP in the commercial sample set. Corresponding amounts of SDZ and TMP per tablet were computed as listed in Table 7. The average assay results were 997.60 mg/tablet and 1002.38 mg/tablet for SDZ, and 197.46 mg/tablet and 197.76 mg/tablet for TMP, both in accordance with the manufacturer's declaration. Relative standard deviation and relative error values were

smaller than 2% for both drugs using the optimized methods. The assay results from two different methods were statistically compared using F-test and Student's t-test, each applied at a significance level of 0.05, and were found to be comparable. Both UPLC methods successfully achieved accurate and reliable determination of SDZ and TMP in tablets for veterinary use.

Table 7. Assay results of commercial tablet samples obtained by FFD/BBD-UPLC and CCD-UPLC.

Code	FFD/BBD-UPLC		CCD-UPLC	
	SDZ (mg/tablet)	**TMP (mg/tablet)**	**SDZ (mg/tablet)**	**TMP (mg/tablet)**
T1	993.1	198.0	1000.7	201.1
T2	1013.1	196.3	986.5	193.5
T3	988.6	192.5	998.9	195.0
T4	988.1	200.4	1012.5	201.1
T5	992.9	200.2	1002.6	201.7
T6	986.5	201.5	991.4	193.3
T7	1010.4	192.4	1018.5	194.7
T8	1002.8	196.0	1015.9	201.2
T9	994.3	200.4	998.5	201.5
T10	1006.2	196.8	998.4	194.5
Mean	997.6	197.5	1002.4	197.8
SD	9.74	3.27	10.34	3.80
RSD	0.98	1.65	1.03	1.92
SE	3.08	1.03	3.27	1.20
RE	-0.24	-1.27	0.24	-1.12
CI	991.6-1003.6	195.4-199.5	996.0-1008.8	195.4-200.1
F-test	1.13	1.35	F-tabulated: 3.18	
t-test	1.11	0.25	t-tabulated: 2.26	

Label claim: SDZ:1000 mg/tablet, TMP: 200 mg/tablet
SD: Standard deviation
RSD: Relative standard deviation
SE: Standard error
RE: Relative error
CI: confidence interval (p=0.05)

CONCLUSIONS

This study explores the selection of experimental design matrices (FFD, BBD, and CCD) during response surface methodology to optimize UPLC conditions for the simultaneous quantification of sulfadiazine and trimethoprim in commercial tablets for veterinary use. The comparative performance of FFD, BBD, and CCD for UPLC optimization was studied with standardized factor levels. All designs provided successful models with significant effects on the response, and optimal conditions provided satisfactory outcomes for efficient and fast chromatographic analysis. CCD performed better factor exploration, possibly due to broader design space with the inclusion of axial points, resulting in chromatographic conditions with shorter analysis time while maintaining desirable resolution between the peaks. Application of FFD and BBD gave rise to very similar, if not the same, optimal conditions, confirming the efficiency of BBD for UPLC optimization. These results indicate CCD may be preferred for UPLC optimization when the resources permit, while BBD is a viable alternative for constrained settings. As three different designs resulted in two different optimal chromatographic conditions, each were validated and applied to quantitative analysis of SDZ and TMP in tablets. They showed good analytical performance during the validation studies, and achieved assay results that were statistically comparable and consistent with the labeled content. The optimized UPLC methods were found suitable for quality control of SDZ and TMP in veterinary tablet formulations and may be extended to other drug combinations with similar challenges.

EXPERIMENTAL SECTION

Chemical and reagents

Sulfadiazine (99.35 %) and trimethoprim (99.55 %) standard materials were purchased from Alfa Aesar (USA). Gradient grade methanol was procured from Sigma-Aldrich, (USA), and ultrapure water was obtained from a Milli-Q water purification system (Millipore, USA). Formic acid (98%-100%, d=1.22g/mL) and NaOH (≥98%) were of ACS reagent grade and were supplied by Sigma-Aldrich (USA) and Riedel-de Haën (Germany), respectively. Biotrin Tablet, containing 1000 mg sulfadiazine and 200 mg trimethoprim per tablet, was produced by Ceva Animal Health Inc. and was procured from a veterinary clinic in Ankara, Türkiye.

Instrumentation, Chromatographic Conditions, and Software

Chromatographic studies were performed using an ACQUITY UPLC H-Class system (Waters, USA) equipped with a quaternary pump, a cooling autosampler, a temperature-controlled column compartment with pre-heater, and a photodiode array detector. All chromatographic work was carried out on a Waters UPLC BEH C_{18} 100 mm (130Å, 1.7 µm, 2.1 mm) column.

The temperature of the autosampler was maintained at 15 °C, the solvent of injected samples was a mixture of methanol and water (50:50, *v/v*). Detection of sulfadiazine and trimethoprim was performed at 267 nm and 230 nm, respectively, with an injection volume of 1 µL. Different ratios of methanol and sodium formate buffer were used as mobile phase during the experimental design and optimization steps. The remaining chromatographic parameters were determined using different experimental designs via RSM, as explained in the section Results and Discussion. All samples were filtered through nylon syringe filters (0.2 µm) before injection. The inorganic component of the mobile phase was filtered through a cellulose nitrate membrane of 0.2 µm.

Empower2 software (Waters, USA) was used to control the UPLC instrument and chromatographic data collection. The generation of the experimental design matrices, response surface modelling, optimization, as well as calibration models and quantification were performed by concurrent use of Microsoft Excel (Microsoft, USA) and Matlab (MathWorks, USA). Figures were plotted in Matlab (MathWorks, USA).

Preparation of Standard Solutions

A mixture of ultrapure water and gradient grade methanol (50:50, *v/v*) was prepared previously and was used as solvent for all the standard and sample solutions. Individual stock solutions of sulfadiazine and trimethoprim were prepared by dissolving 20 mg and 10 mg of standard material in a 100 mL solvent mixture by manual shaking, and ultrasonication for 5 minutes. Standard solutions for calibration and validation were prepared by mixing and diluting the appropriate amounts of stock solutions.

The calibration set was planned using a factorial design of 5^2 (5 levels with equal steps and 2 analytes) within the working range of 6-38 µg/mL for sulfadiazine and 2-26 µg/mL for trimethoprim.

Three sets of analytical validation samples were prepared in a similar manner, by mixing and diluting required volumes. The first set, called binary synthetic mixture was used to assess the accuracy and the precision of the methods. The binary synthetic mixture set consisted of 11 solutions with different concentration of drugs from the calibration set, as given in Table S5 in supplementary material.

The second validation set consisted of intra-day and inter-day samples, that were freshly prepared at three different concentration levels: 80% (20 µg/mL SDZ + 4 µg/mL TMP), 100% (25 µg/mL SDZ + 5 µg/mL TMP), and 120% (30 µg/mL SDZ + 6 µg/mL TMP) of the specified test concentration and analyzed both on the same day and on three consecutive days to assess repeatability and intermediate precision.

The last set of validation samples, named standard addition samples, was prepared in triplicates to evaluate the effect of excipients in the solid dosage form and to evaluate the selectivity of the method. Three concentration levels of standards (10, 15, and 20 µg/mL for SDZ and 5, 12, and 19 µg/mL for TMP) were spiked into a tablet solution at a fixed volume (theoretically containing 15 µg/mL SDZ + 3 µg/mL TMP, according to the label claim). An extra tablet solution, without adding the standards, was prepared to calculate the added recovery values based on the hypothetically added amount.

Preparation of Commercial Sample Set

Three Biotrin tablets for veterinary use were weighed on an analytical balance and finely powdered in a mortar. A quantity of powder equivalent to 1/10 of a tablet by mass was transferred and diluted to 100 mL. After mixing the contents of the volumetric flask in an ultrasonic bath for 15 minutes followed by 15 minutes of magnetic stirring, the solution was filtered through a 0.2 µm nylon membrane syringe filter. This filtrate was further diluted to ensure SDZ and TMP concentrations fell within their working ranges. This process was repeated 10 times to obtain the commercial sample set.

SUPPLEMENTARY MATERIAL

The supplementary material for this article has been uploaded to Zenodo and is available at: https://doi.org/10.5281/zenodo.17937817.

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