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Method Development for the Chromatographic analysis of a Two-Component Tablet Formulation Using Chemometric Optimization Technique

Faysal SELİMOĞLU[1], Betül SARITAŞ[2], Erdal DİNÇ[3*]

ABSTRACT

A novel chromatographic method, ultra-performance liquid chromatography (UPLC) was improved to determine sulfamethoxazole and trimethoprim in a two-component tablet formulation. In the implemented of the method, the chromatographic parameters were optimized by using the experimental design and optimization procedure. The central composite design and fitting model was applied to identify the suitable chromatographic conditions providing a desirable elution of sulfamethoxazole and trimethoprim in a chromatogram. In the central composite design, temperature, flow rate and buffer% were selected as the effective factors on the chromatographic resolution. The buffer system was the mixture of 0.1 M CH_3COOH and CH_3COONa (pH 4.75) in mobile phase system. In the optimization step, the chromatographic conditions were found to be 0.20 mL/min for flow rate, 38.0 °C for the column temperature and 66% for the acetate buffer system (v/v) in the mobile phase. Analysis of the investigated drugs was accomplished on a stationary phase based on Waters BEH C_{18} column (50 mm-2.1 mm, 1.7 mm i.d.). In the validation step, recovery study was performed by analyzing the synthetic binary mixture of sulfamethoxazole and trimethoprim. Recovery results were found as 99.12% for sulfamethoxazole and 99.44% for trimethoprim. Assay results showed that the optimized chromatographic technique was very suitable for the quantitation of sulfamethoxazole and trimethoprim in tablets.

Keywords: Chemometric optimization, ultra-performance liquid chromatography, Sulfamethoxazole, trimethoprim,

1. INTRODUCTION

Sulfamethoxazole (SMX) is a sulfonamide derivative, which is one of the well-known antibacterial substances [1]. SMX is slightly off-white in crystal form, and its chemical formula is $C_{10}H_{11}N_3O_3S$. It is poorly soluble in polar but highly soluble nonpolar solvents. SMX can commercially be synthesized by reacting acetyl sulfonyl chloride with 2-amino-4,6-dimethyl pyrimidine 3-amino-5-methylisoxazole in

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acetone and pyridine [2]. SMX provides a broad-spectrum with a strong antibacterial effect on both gram-negative and gram-positive bacterial growth. SMX is used in the treatment of urinary tract infection, respiratory tract infection, and intestinal infection [3-4]. SMX interferes with the synthesis of bacterial dihydrofolate, which is a vital reproduction cofactor for eukaryotic and prokaryotic cells [5-6].

The SMX and TMP combination in the same formulation provides an extensive antibacterial spectrum to medicate more bacterial infections. TMP derives from trimethoxybenzyl-pyrimidine. TMP is an odorless, white powder in solid crystalline form and insoluble in water but soluble in dimethyl sulfoxide. TMS's chemical formula is $C_{14}H_{18}N_4O_3$. The chemical synthesis of TMP is done by two stepped reactions of 3,4,5-Trimethoxybenzaldehyde, 3-ethoxypropionitrile, and guanidine in acidic media [9]. TMP has a broad-spectrum antibiotic agent. TMP has the primary enzyme property that catalyzes the bacterial folic acid synthesis in the mechanism of action.

Due to the extensive and increasing use of SMX and TMP in human and veterinary medicines, it is needed to develop new analytical method for the quality control and quantitative assessment of SMX-TMP products. In the literature, there are many methods are mentioned about the quantification of SMX and TMP in pharmaceutical formulations [10]. However, these methods are not related to chemometric optimization for improving an UPLC approach for the quantification of TMP and SMX mixtures.

Most of the methods are based on HPLC [11-12], HPTLC [13], UV-VIS absorption spectrophotometry [14]. Multivariate statistical techniques such as, H-point standard additions method HPSAM [15], partial least squares PLS regression [16].

In the analytical chemistry laboratory, the design and optimization techniques allow an opportunity to find suitable experimental conditions for developing a new liquid chromatographic method with a few numbers of experiments. In literature, it is observed that central composite design CCD is preferable than others (for example, full factorial design and Box-Behnken design) since it can provide good predictions throughout the experimental data of the variable process and their parameters [17-18].

In this study, a chemometric methodology was used to reach optimal conditions for developing a new chromatographic determination based on the UPLC technique for the simulations quantitative evaluation of SMX and TMP in commercial tablets. After the method validation, the quality control and routine analysis of the investigated drug compounds in commercial tablets were accomplished by the optimized and validated UPLC technique.

2. EXPERIMENTAL

2.1. Instrument and Software

Chromatographic recordings of the related drugs and their samples were obtained using a Waters® Acquity H-Class UPLC system equipped with cooling autosampler and an oven allowing control the chromatographic column's temperature, «photodiode array» detector (PDA), Chromatographic instrument was controlled with Empower 2 software (Waters, USA) Chromatographic separation of analytes was accomplished by using a Waters UPLC BEH C_{18} column (50 mm x 2.1mm i.d., 1.7 µm).
Calibration curve and mathematical calculations of the obtained data were made by using an m-file algorithm written in MATLAB software (MathWorks Inc.) and Microsoft Excel program.

2.2. Chromatographic Conditions of UPLC Analysis

In this study, chromatographic analyses were archived on a Waters UPLC BEH C18 (50 mm x 2.1 mmi.d., 1.7 µm) used as a stationary phase. The chromatographic elution of TMP and SMX was carried out by using a mobile phase consisting of a mixture of methanol and 0.1 M sodium acetate buffer (at pH 4.75) (34:66, v/v) containing 1.0 mL trimethylamine per liter. The flow rate of the mobile phase was 0.2 mL/min, and the sample injection volume was 1.5 µL. During the separation process, column

temperature was 38.0 C°, respectively. Quantification of analytes was performed by using a PDA detection at 270 nm and sulfadiazine was used as internal standard (IS).

2.3. Chemicals and Standard Solutions

Methanol was of HPLC grade (Merck, DE). Acetic acid and sodium acetate anhydrous, Reagent Plus®, ≥99.0%, Triethylamine ≥99.0% were obtained from Sigma-Aldrich (St. Louis, MO, U.S.). In the UPLC analysis of analytes, double distilled water was used during chromatographic experiments. Standards of SMX (99.7% purity) and TMP (99.6% purity) were kindly supplied from Santa Pharma Inc. (Istanbul, Turkey). A pharmaceutical tablet formulation (BACTRIM Forte Tablet produced by Deva Pharm. Ind., Istanbul, Turkey) containing 800 mg SMX and 160 mg TMP per tablet was bought from a local pharmacy. For the UPLC analysis of the drugs, the standard stock solutions for both SMX and TMP were individually obtained by dissolving of 10.0 mg of each analyte in 100 mL methanol. A series of the calibration solutions containing SMX and TMP in the working range of 1-36 µg/mL were prepared from the above stock solutions. For the validity of the proposed method, a set of 14 binary mixture containing SMX and TMP in different concentration levels. All solution of calibration, validation and commercial samples were prepared in methanol during this study.

2.4. Analysis Procedure of Tablet Samples

In applying the developed UPLC method to real tablet samples, ten tablets have been accurately weighed and pulverized in a mortar for sample preparation. A quantity of tablet powder equivalent to one tablet was dissolved in methanol in a 100 mL volumetric flask. The tablet solution was filtered through a 0.45 µm membrane filter. The sample solution was then diluted with methanol to the linear concentration range the resulting sample was injected into chromatographic instrument system. This analysis process of tablets was repeated ten times.

3. RESULTS and DISCUSSION

3.1. Chemomeetric Optimization Approach

Optimization of factors affecting the chromatographic elution of analytes is crucial to developing a new UPLC method. For this purpose, preliminary chromatographic studies were done to find factors affecting the resolution factor or chromatographic response. As a result of these studies, the column temperature (X_1), flow rate (X_2) and percentage (X_3) of buffer % (0.1 M acetate buffer at pH 4.75) in the mobile phase were selected as independent factor variables that affect the chromatographic response. The levels of these factors were demonstrated in this paper as (-1) low, as (0) medium, and as (+1) high (see Table 1).

Table 1
Selected factors, corresponding factor levels

Levels	Temperature X1	Flowrate X2	Buffer% X3
1.682	38.4	0.43	80.1
1	35.0	0.38	74.0
0	30.0	0.30	65.0
-1	25.0	0.22	56.0
-1.682	21.6	0.17	49.9

Low level (-1), medium level (0), high level (+1)

In the chemometric optimization procedure, the resolution factor (R) was used as a response function, which is given by the following expression:

$$R = 2\ \frac{(t_2 - t_1)}{(W1 + W1)} \qquad (1)$$

where W_1 and W_2 denote the chromatographic peak widths of peak$_1$ and peak$_2$, respectively; t_1 and t_2 represents the elution times of analytes in a chromatogram.

Considering the factors and their factor levels illustrated in Table 1, the central composite design involving three factors variables (the temperature X_1, flow rate X_2 and percentage X_3 of 0.1 M acetate buffer system (pH=5.75) in the mobile phase) was planned as presented in Table 2.

Considering chromatographic conditions of the central composite design, the UPLC chromatograms were plotted by using the chromatographic instrument. Then, the data of the chromatographic responses were computed by using Equation (1) and presented in Table 2. The following second-order polynomial equation was obtained by using the mathematical relationship between the independent factor variables (X_1, X_2, and X_3) with factor interactions and the chromatographic response (R) as follows:

$$y = 1.3138 - 0.2118x_1 - 0.1378x_2 + 0.3932x_3 + 0.1178x_1^2 + 0.0738x_2^2 - 0.2930x_3^2 + 0.0265x_1x_2 - 0.0801x_1x_3 - 0.0609x_2x_3 \quad (2)$$

In the calculation of Equation (2) by regression analysis, the values the predicted responses were obtained and listed in Table 2.

Table 2
Full factorial design and responses with calculated and predicted values

Experiment Number	Temp X_1	Flow X_2	Buffer % X_3	Responses	
				Calculated	Predicted
1	-1	-1	-1	2.1030	2.0683
2	-1	-1	1	2.1635	2.1902
3	-1	1	-1	0.8709	0.9295
4	-1	1	1	1.8991	1.8774
5	1	-1	-1	0.9841	0.9948
6	1	-1	1	1.3252	1.2556
7	1	1	-1	0.6220	0.5843
8	1	1	1	1.6474	1.6711
9	-1.682	0	0	2.5577	2.5353
10	1.682	0	0	1.4209	1.4590
11	0	-1.682	0	1.9373	1.9719
12	0	1.682	0	1.3825	1.3636
13	0	0	-1.682	0.1805	0.1770
14	0	0	1.682	1.1745	1.1935
15	0	0	0	1.5160	1.5102
16	0	0	0	1.5110	1.5102
17	0	0	0	1.5068	1.5102
18	0	0	0	1.5088	1.5102
19	0	0	0	1.5083	1.5102
20	0	0	0	1.5128	1.5102

Response = Chromatographic resolution (R)

In order to estimate the model, which correspond to Equation (2), the statistical t-test for the model coefficients were applied and then the results of the statistical test were indicated in Table 3. From this table, it was reported that the calculated t-test results and p-values at the 95% confidence level were greater than t-table and lower than p=0.05. These results showed that the model coefficients were significant to explain our quadratic model. In other words, the computed polynomial

regression model is enough to quantify the suitable chromatographic conditions allowing better peak separation and shortest runtime for the analysis of SMX and TMP.

Table 3
Statistical results for the *t*- tests with p-values for the coefficients of the polynomial regression model

	Coefficients	T Stat	P-value
Intercept	1.5102	90.57	6.59E-16
$\mathbf{X_1}$	-0.3199	28.92	5.69E-11
$\mathbf{X_2}$	-0.1808	16.34	1.53E-08
$\mathbf{X_3}$	0.3022	27.31	1.00E-10
$\mathbf{x_1^2}$	0.1721	15.98	1.90E-08
$\mathbf{X_2^2}$	0.0557	5.17	4.18E-04
$\mathbf{X_3^2}$	-0.2916	27.08	1.09E-10
$\mathbf{X_1*X_2}$	0.1821	12.60	1.85E-07
$\mathbf{X_1*X_3}$	0.0347	2.40	3.73E-02
$\mathbf{X_2*X_3}$	0.2065	14.29	5.58E-08

To demonstrate the factor interactions, three dimensional (3D) graphs and related contour plots were obtained by recording the chromatographic response versus the flow rate and temperature, the chromatographic response versus the acetate buffer (mixture of CH_3COOH+CH_3COONa mixture) and temperature, and the chromatographic response versus the acetate buffer (mixture of CH_3COOH and CH_3COONa) and low rate as shown in Figures 1a-c, respectively. The bends on 3D axis of the surface and contour recordings clearly exhibit significant interactions of the chosen factors

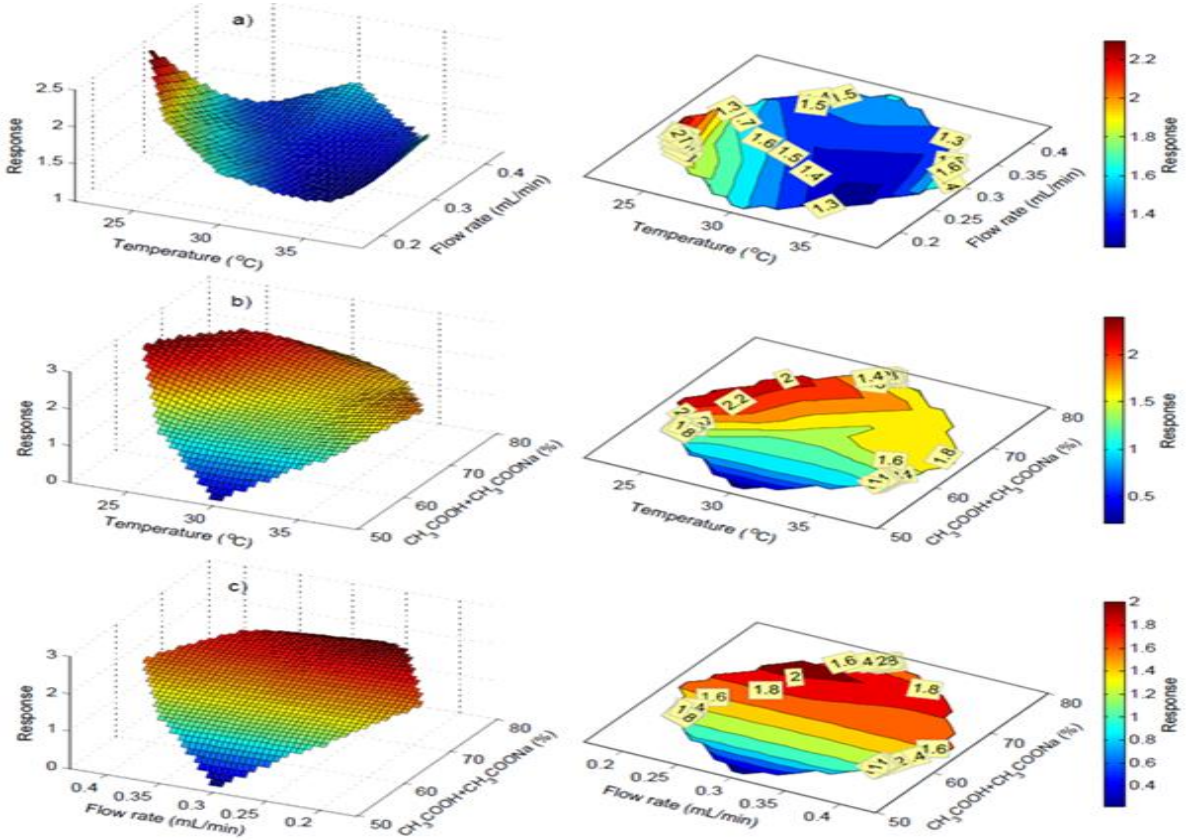

Figure 1 The plots of the surface and contour displays the relationships between flow rate-temperature and chromatographic response (a); flow rate-temperature and chromatographic response (b); flow rate and 0.1 M acetate buffer (at pH 4.75) (c).

Before the chemometric optimization of chromatographic conditions, Figure 2 shows a UPLC chromatogram with bad chromatographic elution of analytes and IS at the random experimental conditions. Such situation shows the necessity of chemometric optimization for chromatographic analysis of TMP and SMX in samples.

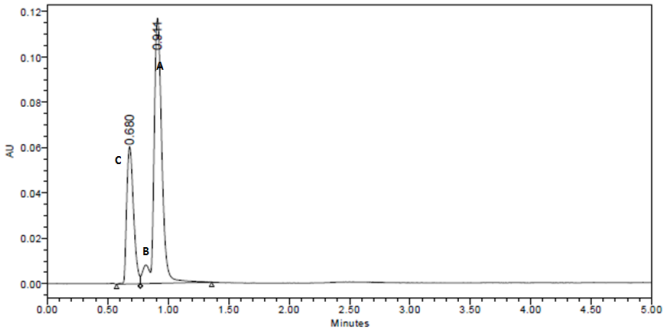

Figure 2 UPLC Chromatograms of a) 36 µg/mL SMX, b) 18 µg/mL TMP and c) 12 µg/ml IS were recorded before optimization with the detection at 270 nm.

To develop a new liquid chromatographic method, the suitable chromatographic experimental conditions for the temperature (X_1), the flow rate (X_2) and acetate buffer percentage (X_3) were computed from the mathematical solving of Equation (2) obtained by using central composite design. The calculated chromatographic parameters provided better elution of the analytes and short run time for For the preparation of calibration curves for TMP and SMX, the preparation of the calibration samples of analytes was done by mixing the standard stock solutions as depicted in the "Chemicals and Standard Solutions" section. Their chromatograms were recorded under optimized conditions, which determined as 38^{o}C for the column temperature, 0.2 mL/min for the flow rate of the mobile phase, 66% for the percentage for the acetate buffer at pH 4.74 in the mobile phase. Taking into account the percentage of acetate buffer defined by the optimization procedure, the mobile phase system containing a mixture of methanol and 0.1 M acetate buffer at pH 4.74 (34:66, v/v) was used.

The calibration curves were obtained by the linear regression of concentration on the chromatographic area ratio of analyte to IS. improving a new UPLC method that enables to determine SMX and TMP in their mixture. The above optimal UPLC conditions for the elution of SMX and TMP in their mixture were found to be 38.0°C for column temperature, 0.2 mL/min for flow rate, and 66 % for the percentage of 0.1M acetate buffer at pH 4.75 in mobile phase.

Under optimized UPLC conditions, the UPLC chromatogram of a calibration sample containing 36 µg/mL SMX and 18 µg/mL TMP in the presence of 12 µg/ml IS was recorded with the PDA detection at the wavelength of 270 nm. The recorded chromatogram was illustrated in Figure 3.

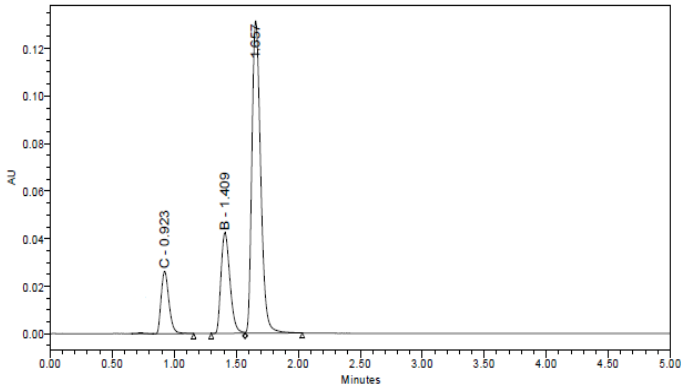

Figure 3 UPLC Chromatograms of the mixture of the standards: a) 36. µg/mL SMX, b) 18.0 µg/mL TMP and c)12.0 µg/ml IS were recorded after the central composite design and optimization procedure with the detection at 270 nm.

Regression analysis and its statistical results were given in Table 4. TMP and SMX were determined by using the calibration equations of the investigated drugs.

Table 4
Least square regression analysis and
Statistical results for the investigated drugs

Parameter	**SMX**	**TMP**
λ (nm)	270 nm	270nm
Range (µg/mL)	1.0-36.0	1.0-36.0
m	0.065117	0.021394

n	0.032512	-0.00857
r	0.99994	0.999771
SE(m)	0.00032	0.000205
SE(n)	0.002928	0.000443
SE(r)	0.009988	0.006382
LOD (µl/min)	0.36	0.16
LOQ (µl/min)	1.19	0.55

m=Slope of the regression function
n =Intercept of the linear regression function
r= Correlation coefficient of the regression function
SE (m)=Standard error of slope
SE(n)=Standard error of intercept
SE (r)= Standard error of correlation coefficients
LOD= Limit of detection
LOQ= Limit of quantitation.

3.2. Validity of the Method

The calibration curves for SMX and TMP were reported to be linear in the range of 1.0-36.0 µg/ml. High correlation coefficients were observed for the linear regression equations of TMP and SMX as shown in Table 4. A good separation of chromatographic peaks in a chromatogram with the resolution factor of 1.90 were reported as displayed in Figure 2. In the chromatogram indicated in Figure 2, the retention times for IS, TMP and SMX were reported as 0.900, 1.343 and 1.532, respectively.

The limit of detection (LOD) and the limit of quantitation (LOQ) in the dynamic concentration range were computed from the standard deviation of the intercept and slope values of calibration functions, and then their results were shown in Table 4. The developed UPLC method, which was applied in this study, supplies high chromatographic resolution with the shortest runtime for the analysis of the investigated drugs. Accuracy and precision of the newly developed UPLC method was tested by using recovery studies, including the analysis of the mixture of TMP and SMX in the presence of IS

The recovery results of the analysis were given in Table 5. As can be seen from Table 5, good accurate and precise recovery results with acceptable numerical values of the standard deviations and relative standard deviations were observed

Table 5
Recoveries of SMX and TMP in binary mixtures using the improved UPLC method

	Added (µg/ml)		**Found (µg/ml)**		**Recovery (%)**	
No.	**SMX**	**TMP**	**SMX**	**TMP**	**SMX**	**TMP**
1	1	6	0.97	6.07	96.6	101.1
2	6	6	5.68	6.02	94.6	100.3
3	12	6	12.13	6.00	101.1	100.0
4	18	6	17.78	5.62	98.8	93.7
5	24	6	24.66	5.91	102.7	98.5
6	30	6	29.66	5.80	98.9	96.7
7	36	6	35.97	5.81	99.9	96.8
8	30	1	29.81	1.05	99.4	105.0
9	30	6	30.18	5.84	100.6	97.3
10	30	12	29.68	11.74	98.9	97.8

11	30	18	29.34	17.75	97.8	98.6
12	30	24	29.23	24.32	97.4	101.3
13	30	30	30.76	30.19	102.5	100.6
14	30	36	29.52	37.59	98.4	104.4
				Average	99.12	99.44
				SD	2.21	3.04
				RSD	2.23	3.06

SD = Standard deviation; RSD = Relative standard deviation.

3.3. Analysis of Tablets

The optimized UPLC method was applied to the commercial tablets for the quantitative analysis of SMX and TMP amounts after the validation procedure. Under optimized chromatographic conditions, the chromatographic signals of the analytes in tablet formulation in the presence of IS were recorded as shown in Figure 4. Placing the chromatographic peak-area ratio of drug/IS to the calibration equations, SMX and TMP in tablets were computed. Tablet analysis procedure was repeated ten times.

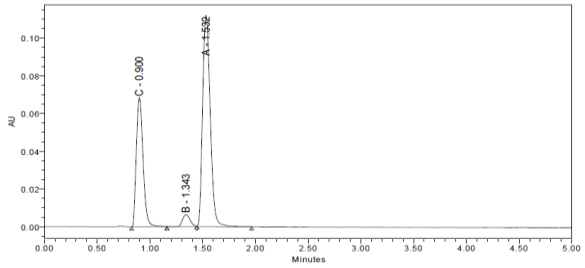

Figure 4 The UPLC Chromatograms of a) SMX b) TMP in a commercial tablet in presence of c)12 µg/ml IS with the detection at 270 nm.

The commercial tablets analysis results were listed in Table 6. These determination results were computed from the mean of a set of ten replicated experiments. The relative standard deviation values were calculated as 1.63% for SMX and 2.42 for TMP as illustrated in Table 6.

Table 6
Experimental determinations obtained by using the optimized chromatographic procedure for the analysis of the tablets.

	mg/tab	
No.	**SMX**	**TMP**
1	796.2	160.6
2	800.3	169.6
3	792.0	157.2
4	820.4	158.9
5	805.8	163.0
6	822.8	163.9
7	797.0	157.6
8	794.8	160.0
9	798.5	157.9
10	778.5	157.8
Mean	800.63	160.65
SD	13.09	3.89
RSD	1.63	2.42

SD = Standard deviation; RSD = Relative standard deviation.
Label claim is 800 mg SMX and 160 mg TMP per tablet

In conclusion, it was reported that the improved UPLC method is very appropriate for the simultaneous quantitative assessment of the related drugs in tablets.

5. CONCLUSIONS

In this paper, a chemometric methodology based on the central composite design and optimization procedure was used for finding optimal chromatographic condition for developing new UPLC method for the simultaneous quantitative assessments of SMX and TMP in binary mixtures and tablets.

This chemometric optimization approach provided the optimal chromatographic conditions giving enhanced chromatographic elution with short runtime and also economical, precise, accurate, and reliable analysis of TMP and SMX in tablets. Consequently, the improved RP-UPLC method is very suitable for the quality control and quantitative analysis of tablets containing TMP and SMX drugs.

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The Declaration of Conflict of Interest/ Common Interest
No conflict of interest or common interest has been declared by the authors.

The Declaration of Ethics Committee Approval
This study does not require ethics committee permission or any special permission.

Authors' Contribution
The authors contributed equally to the study.

The Declaration of Research and Publication Ethics

The authors of the paper declare that they comply with the scientific, ethical and quotation rules of SAUJS in all processes of the paper and that they do not make any falsification on the data collected. In addition, they declare that Sakarya University Journal of Science and its editorial board have no responsibility for any ethical violations that may be encountered, and that this study has not been evaluated in any academic publication environment other than Sakarya University Journal of Science.

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