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Optimization of chromatographic conditions via Box–Behnken design in RP-HPLC-PDA method development for the estimation of folic acid and methotrexate in bulk and tablets

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ABSTRACT

Simultaneous estimation of folic acid and methotrexate in bulk and tablet dosage form by RP-HPLC-PDA was conducted via Box–Behnken design application. Three-factor numerical values were finalized from the graphical and numerical optimization with built-in ANOVA in BBD. Sharp and symmetric peaks were observed at 4.138 and 6.929 min for folic acid and methotrexate, respectively. The mobile phase composition was methanol and 0.1% formic acid in water with a ratio of 31:69 and a flow rate of 1.1 ml/min. Both drugs were detected at a wavelength of 291 nm. The developed method was validated according to ICH guidelines. The results of the validation parameters were within acceptable limits. Stress stability studies have been performed under acidic, alkali, oxidation, neutral and photolytic conditions. Three different brand-marketed tablets were assessed with the developed method (MGXT, FOLTNAX and TRUXOFOL). In the tablet formulations, chromatogram percentages of folic acid and methotrexate were calculated at 99.13% and 99.50 in MGXT, 99.17% and 99.47 in FOLTNAX, and 99.91 and 100.05 in TRUXOFOL.

1. Introduction

Folic acid (FA) helps to lessen many of the side effects of methotrexate (MTX) while also protecting healthy cells in the body. It may reduce the likelihood of experiencing nausea, vomiting, or diarrhea [1]. MTX antimetabolites have been used for many years to treat rheumatoid arthritis, psoriasis, and neoplastic diseases [2]. Folic acid is a pteroyl monoglutamic acid. It consists of three moieties: a pteridine ring, *p*-aminobenzoate, and glutamic acid [3]. The glutamate substituent is joined to the pteridine ring by the *p*-aminobenzoate molecule. Therefore, a pteridine ring, *p*-aminobenzoic acid, and glutamic acid are present in MTX. MTX and FA have chemically similar structures, with the hydroxyl group in the pteridine ring of folic acid replacing the amine and the 10th nitrogen of *p*-aminobenzoic acid being different by adding a methyl group [4,5]. Chemical structures with the IUPAC name of both drugs are

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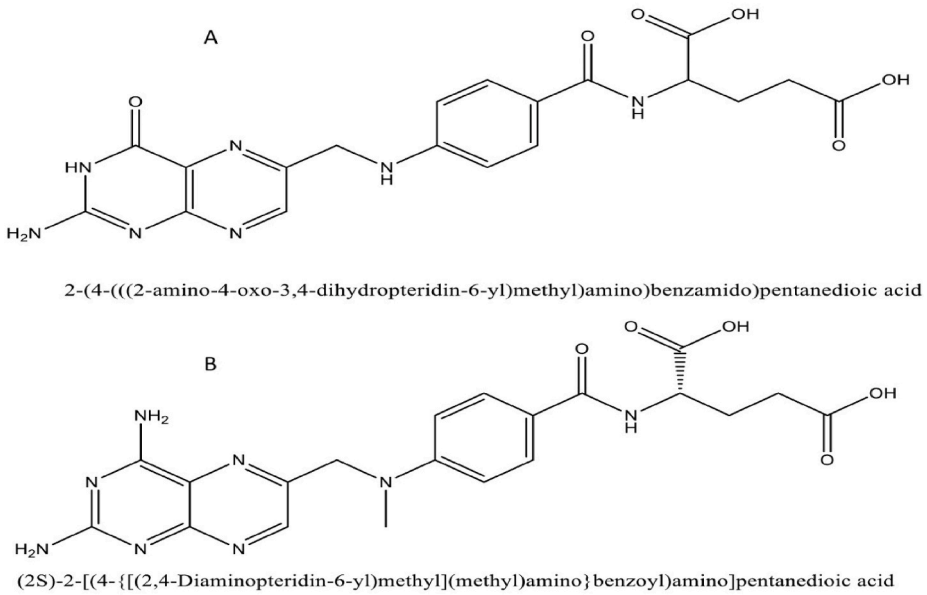

Fig. 1. Chemical structure of folic acid (A) and methotrexate (B).

presented in Fig. 1 (A-B).

Several researchers have performed FA and MTX quantitative analyses in the past. In addition, literature reviews have been performed to design the present research work.

MTX and metoclopramide have been reported simultaneously by the RP-HPLC method with a standard internal method. A Kromasil column was used. The column dimensions were 250 mm in length, 4.6 mm in diameter and 5 μm in particle size. The mobile phase composition and flow rates were CH_3OH and 0.05% CF_3COOH (36:64 v/v) and 1 mL/min, respectively. Elutions were detected at 290 nm at 40 °C oven temperature [6] High-performance liquid chromatography with a reversed-phase column and UV spectrophotometer was used to quantitatively analyze MTX and FA with QbD application. The mobile phase composition was CH_3OH: pH buffer (75:25 v/v) and pH buffer (pH 3.3 0.1% OPA with TEA). Elutions were detected at 249 nm at a 1.0 ml/min flow rate. The retention times of MTX and FA were 5.25 and 7.35 min, respectively [7]. The RP-HPLC analytical technique was used for the estimation of FA and MTX. The mobile phase composition was buffer (50 mM KH_2PO_4) and methanol in a ratio (60:40% v/v) at a 1 ml/min flow rate. Elutions were detected at 2.6 and 4.8 min for FA and MTX, respectively, at a detection wavelength of 270 nm [8]. The pharmacokinetic properties of active pharmaceutical ingredients (APIs) such as thiopurine and FA were assessed, and their quantities were measured before fixed-dose combination (FDC) development. The optimization, development, and validation by chromatographic method and its application for simultaneous estimation in commercial tablet formulations [9]. The amount of MTX was calculated using RP-HPLC with QbD application. CH_3CN and CH_3COONH_4 buffer (10 mM, pH 6) were the components of the mobile phase. MTX was detected at 257 nm in a 0.8 ml/min flow rate at 40 °C column oven temperature [10]. HPLC in the reversed-phase method was developed and validated to quantify tretinoin and MTX in pharmaceutical formulations and spiked plasma. CH_3CN: buffer (85:15, v/v) was used as the mobile phase. An RP C-18 column was used, and isocratic elution at a 1 ml/min flow rate elutes detected at 340 nm wavelength [11]. A straightforward, quick, and reliable HPLC analytical method was created with the QbD application. The findings demonstrated that the developed RP-HPLC method could be used with high resolution, accuracy, and precision to identify and quantify MTX in any dosage form [12]. HPLC-DAD and UV–vis spectrophotometer analytical techniques quantified MTX in different biodegradable microparticles. MTX was estimated in other dosage forms using mobile phase distilled H_2O: ACN in the 80:20 (v/v) ratio at pH 3 with HCOOH. Elute was detected at 211 nm wavelength [13]. Simultaneously, FA was estimated by the RP-HPLC method in the isocratic mode of mobile phase ACN-0.02 M KH_2PO_4 at pH 6 in a ratio of 80:20. FA was detected at 230 nm wavelength in mobile phase flow rate 0.9 ml/min [14]. The amount of FA in fortified salt was measured using a spectrophotometric technique that uses the folic acid-specific wavelength of 285 nm [15]. Analytical methods such as HPLC and HPTLC were employed to routinely analyze FA-based pharmaceutical formulations. These techniques bind FA with sodium nitroprusside, ammonia reagent, and colored species to create a dark, yellow-colored chromogen at 390 nm wavelength [16].

Since years ago, QbD tools have been used to estimate drugs in synthetic and herbal formulations. Analytical techniques such as spectrophotometry and chromatography were used to optimize the analytical method conditions by QbD. Screening of factors that influence the responses was selected by risk assessment studies. This tool's distinctiveness is its ability to save time, be precise, and concentrate on essential factors that affect responses [17–19].

Additionally, developing an analytical method requires thoroughly investigating various variables that could affect sensitivity and specificity. The Box–Behnken design (BBD) helps produce reliable scientific findings. The BBD strategy identifies the crucial characteristics in each process stage when developing analytical methods. The BBD tests of the chosen factors make high-order response

surfaces in the trials. This strategy is superior to the classical process of conducting trials. BBD tools can be used at various stages of developing an analytical method, including initial screening, monitoring chromatographic variables, optimizing chromatographic conditions, and testing the method's robustness [20–23].

Current analytical studies on both medications, whether in the same combination or other combinations, have produced successful results using various chromatographic settings and analytical techniques. Few researchers have also quantified the drug contents using QbD techniques. The UV-spectrophotometer detector in chromatographic analysis has some restrictions. Therefore, it was chosen for the current research to focus on new technology detectors such as PDAs. The capacity and efficacy of a typical UV–Vis spectrophotometer with a single channel detector were found to be constrained. This sparked interest in discovering new methods that may be used to replace UV spectrophotometers [24,25].

The present study was conducted to develop a novel method. The literature already available in the database was very helpful in designing the current research project. The development of RP-HPLC-PDA of the present analytical method used QbD tools, which made the method novel. Many researchers have reported methods using various analytical techniques, singly or simultaneously, in various dosage forms and bulk medications. Additionally, several researchers have reported simultaneous estimation of both drugs by QbD. However, to the best of our knowledge, there is no database input for RP-HPLC-PDA method development with BBD application for assessing FA and MTX in API and tablet dosage forms. The limitation of UV spectrophotometers is that they use single-channel focusing, which in research work is skipped and preferred to the advanced detector PDA (photodiode array), which works on direct focusing of dispersed light on the detector. This approach saves time. Using PDA detectors and BBD in this research work is novel and economical. Liquid chromatography uses detectors that are selected based on the chemistry of the analytes that the user is interested in. Most (U) HPLC detectors are light-absorbing devices with an emphasis on the ultraviolet (UV) and visible (Vis) spectra. The majority of organic analyte tests take place between 190 and 350 nm in the ultraviolet region. Typically, only a few user-selectable particular wavelengths can be measured simultaneously by HPLC/UV. The retention period of the particular analyte in the chromatography is used to confirm the findings.

The diode array detector (DAD), also known as the photo diode array (PDA), can measure the complete wavelength range in real time, which can have additional benefits. PDA may therefore be helpful in differentiating among chemicals with various absorbance spectra. Utilize various light wavelengths to identify and count the chemicals in a sample. Fewer chemicals are present in a sample because PDA detectors are more sensitive than UV detectors.

The research works that were previously reported do not use PDA detectors in the QbD approach. This method has reported a low detection limit, which will help to decide the doses in formulation development. Further research could help reduce the drug's toxicity by developing a formulation with a low dose.

2. Experimental work

2.1 Chemicals: Both APIs (FA and MTX) were obtained from Sigma Aldrich. HPLC grade methanol from S.D. Fine Chemical, Mumbai. Milli-Q water was used. Other chemicals, such as NaOH, HCl and formic acid, were used S.D. Fine chemical. From the local pharmacy, tablets were purchased (MGXT, FOLTNAX and TRUXOFOL).

2.1. Instrumentation

HPLC Agilent 1100 was used with a PDA detector. In the instrument, the software was installed (Chemstation 10.04). An RP-C18 column with 5 μm particle size dimensions (length 150 mm X diameter 4.6 mm) was used. Degassing of the solution before use in HPLC was performed in the Meltronics sonicator, and the pH of the solution was measured in an Elico pH meter.

2.2. Optimization of λmax

Twenty micrograms/ml concentrations of both APIs (FA & MTX) were examined over the ultraviolet range (200–400 nm) to determine *λmax*. The isosbestic point was observed at 291 nm, a λmax of the combination.

2.3. Optimization of chromatographic conditions by BBD

Before method development, the chromatographic conditions were optimized by BBD. Screening and selection of independent variables were performed. For this, the essential steps must be considered. The following are covered from the literature on QbD application in HPLC method development. Following the next steps, authentic literature search and initial risk assessment, analytical target profile (ATP) identifications, critical method attributes (CMA), risk assessment, and thane method optimization were performed [26,27]. Design Expert software, version 13.0.3.0, was used from Stat-Ease Inc., Minneapolis, USA. Several independent factors produce the sharp, symmetrical and resolved peak. In chromatographic analysis, BBD can be used to optimize the chromatographic procedure. The ANOVAs indicate the significant factors influencing the separation of analytes in simultaneous analysis. Here, BBD was used to finalize the chromatographic conditions for the simultaneous analysis of MTX and FA. The independent factors were screened among the factors influencing the dependent variables. With the help of the current literature, mobile phase composition, detection wavelength and flow rate were considered. The isosbestic point was determined in a UV spectrophotometer. The levels for the independent factors were (−1, 0, +1). Finally, the dependent variables (responses), retention time and peak area of the chromatogram for both drugs were considered. The ANOVA results and model graphs of seventeen trials help determine the final chromatographic

Table 1
Summary of Box–Behnken design.

	Factor 1	Factor 2	Factor 3	Response 1	Response 2	Response 3	Response 4
Run	A: MeOH conc. (%)	B: Flow rate (ml/min)	C: Wavelength (nm)	FA		MTX	
				RT	PA	RT2	PA 2
1	32	1.2	291	3.45	121.526	5.598	413.148
2	32	1	291	4.11	146.674	6.75	500.02
3	31	1.1	291	3.849	132.699	6.519	459.757
4	31	1.2	290	3.531	129.153	5.996	493.885
5	32	1.1	292	3.761	125.067	6.198	382.317
6	30	1.1	292	4.909	153.439	8.579	476.747
7	30	1	291	5.636	178.928	9.3747	620.254
8	31	1.1	291	4.731	161.747	7.971	557.46
9	31	1.1	291	4.821	167.632	7.985	552.49
10	31	1	292	5.105	164.786	8.639	510.044
11	30	1.1	290	4.862	172.545	8.471	662.312
12	31	1.1	291	4.739	152.901	8.015	471.938
13	30	1.2	291	4.528	151.248	7.931	527.009
14	32	1.1	290	4.642	171.721	7.604	668.377
15	31	1	290	5.195	191.365	8.776	728.087
16	31	1.2	292	5.195	140.742	7.385	435.886
17	31	1.1	291	5.235	143.741	7.381	436.885

Table 2
Calibration curve data.

Parameters	FA	MTX
Equation	y = 18.822x + 7.346	y = 8.6913x + 23.419
Regression	0.9999	0.9996
Range(μg/mL)	05–25	37.5–187.5
Avg. SD	0.62	1.5
Avg. %RSD	0.28	0.19
LOD μg/mL	0.11	0.33
LOQ μg/mL	0.57	1.72
RT(min)	4.132	6.935

conditions for method development. BBD trials are presented in Table 1.

2.4 Stock solutions: Standard stock solutions of both APIs were prepared. FA (50 mg) was dissolved in 50 ml of methanol (1 mg/ml = 1000 μg/ml), and the first standard stock solution was prepared. After that, a second standard stock solution with a strength of 100 μg/ml was prepared from the first standard stock solution. For a volume of 100 ml of the second standard stock solution, 10 ml from the first standard stock solution was mixed with 90 ml of diluents. The first standard stock solution of MTX (1 mg/ml = 1000 μg/ml) was prepared similarly. A second standard stock solution of 500 μg/ml MTX was prepared from the first standard stock solution. For a volume of 100 ml of the second standard stock solution, 50 ml of the first standard stock solution was mixed with 50 ml of diluents.

2.5 Preparation of calibration curve: The calibration curve at the λmax for both drugs was developed. Five dilutions were prepared across the 5–25 μg/ml range for FA and MTX 37.5–187.5 μg/ml and plotted between absorbance *vs.* concentration. Figure details of the calibration curve are presented in Table 2. The following equation calculates the limit of detection LOD (LOQ) and API quantification.

$$LOD = 3.3X\ Avg.SD/Slope\ LOQ = 10X\ Avg.SD/Slope$$

2.4. Method development

A new and novel isocratic method was developed with the suggested numerical value and graphical optimization from BBD trials. Agilent (1100) HPLC with PDA detector along with RP-C18 column. The dimensions of the column were 250 mm in length and 4.6 mm in diameter, and the stationary phase particle size was 5 μm. The column was maintained at 25 °C. The final solvent system was composed of methanol and 0.1% formic acid in water. The ratio was 31: 69. The mobile phase flow rate was 1.1 ml/min, and both drugs were detected at a wavelength of 291 nm. In 10 min, the run time and FA and MET retention times were found at 4.138 and 6.929 min, respectively. The injection volume was 10 μL.

2.5. Method validation

Validation of the new method was performed. According to ICH guidelines, experiments were conducted regarding system suitability, accuracy, precision, linearity, robustness, LOD, LOQ and solution stability [28,29].

(a) *System suitability test:* The HPLC instrument's functioning was evaluated per USP 24/NF 19. An experiment on system suitability was performed with repeatable outcomes. Six batches of middle-level quality control (MQC) standard solution concentrations of 15 μg/ml (FA) and 112.5 μg/ml (MTX) were used in the experiment. RSD values of retention time and plate number were considered for computing the percentage.
(b) *Linearity:* Three batches of each drug (FA & MTX) across the range were taken to check the linearity of the curve. Analyze the calibration curve regarding the correlation coefficient, slope, and intercept, which determine the linearity.
(c) *Precision and accuracy:* The precision of the RP-HPLC-PDA method was determined. Experiments were conducted with three quality control samples for interday and intraday precision. Three concentrations of quality control samples were considered. They are designated as low-level quality control (LQC), middle-level quality control (MQC) and high-level quality control (HQC) solutions. For FA, LQC (10 μg/ml), MQC (15 μg/ml) and HQC (20 μg/ml) and for MET, LQC (75.0 μg/ml), MQC (112.5 μg/ml) and HQC (150 μg/ml). Every experiment was reported with a % RSD value. The accuracy of the developed method was determined by performing the same experiments as the traditional method to calculate the percentage recovery of the drug. First, predetermined concentrations of both drugs, 5 μg/ml and 37.5 μg/ml for FA and MTX, respectively, were taken. Four concentration levels were prepared for the experiments: 0%, 50%, 100%, and 150%. The practical concentration was calculated from the calibration curve for each level. Then, the % recovery and % RSD values of each level were calculated.
(d) *Solution stability:* The stability of the solution was determined. MCQ-level quality control samples of drugs (15 μg/ml for FA and 112.5 μg/ml for MTX) were kept for two weeks at 25 °C and one month at 2–8 °C. The samples were analyzed (n = 6). The practical concentrations were determined with the calibration curve, and after that, % recovery and % RSD were reported.
(e) *Robustness:* The experiments were conducted with deliberate changes in the optimized chromatographic conditions. The observation was examined for each chromatographic condition. The mobile phase composition, flow rate, and wavelength influencing the retention time were considered in the experiments. The developed method was considered robust if the results were within acceptable limits.

3. Stress stability studies

Stress stability studies have been carried out. The ICH guidelines (Q1A, Q1B, and Q2B) for these studies were followed. These studies have been performed with intentional minor changes in the chemical and environmental conditions. Five conditions were selected: 0.1 N HCl, 0.1 N NaOH, 3% H_2O_2, neutral, and photolytic. In each state, experiments were conducted in three different time frames (1 h, 2 h, and 8 h) except in photolytic conditions, where experiments were performed after 24 h [30,31]. These studies try to determine whether the API is stable with degradants. Stability study data will help to finalize the shelf life of the formulations. The results of these studies indicate its stability.

(a) *Acid and alkali degradation studies:* Standard solutions of 200 μg/ml and 500 μg/ml API (FA and MTX) were prepared. One milliliter of each solution was mixed with 2 ml of 0.1 N HCl in a 10 ml volumetric flask. The solution was kept in the dark for 24 h at room temperature, and the same procedure was adopted for alkali stress degradation studies. These solutions were neutralized and brought to a volume of 10 ml with methanol. Required volumes of the solutions were taken for the injection.
(b) *Oxidative stress degradation studies:* 1 ml of each stock solution of both API (FA and MTX) mixed with 2 ml of 3% H_2O_2 in a 10 ml volumetric flask. For 24 h, it was kept in a dark place and covered with aluminum foil. The volume of the volumetric flask was made up of 10 ml with methanol. The required volume was used for injection.
(c) *Neutral condition of degradation:* 1 ml of each stock solution (FA and MTX) was placed in a 10 ml volumetric flask. First, 5 ml of distilled water was added to the volumetric flask, and then a 10 ml volume was made with diluents. Finally, the required volume from the volumetric flask was removed for the injection.
(d) *Photolytic degradation study:* 1 ml of each stock solution (FA and MTX) was placed in a 10 ml volumetric flask and kept under the longer wavelength for 24 h. The volume of 10 ml was made up of methanol just before the experiment. Then, the required volume was removed from the volumetric flask for injection.
(e) *Thermal degradation:* The powdered medication was exposed to 80 °C in an oven for 48 h to perform heat-induced degradation at higher temperatures for a shorter duration of time. The stock solutions for the study were then made from dried powder.

Assay of tablets: Three brands of both compositions (FA & MTX) were analyzed using the RP-HPLC-PDA method. Twenty tablets of each brand were weighed accurately and crushed separately [32,33]. Calculated amounts of powders were weighed from each brand with equivalent amounts of FA and MTX to give concentrations of 200 μg/mL and 500 μg/mL, respectively. It was dissolved in diluents, sonicated for 15 min, and then filtered at 0.45 μm. The same procedure was followed for all three brands. From all three stock solutions, a concentration of level MQC was prepared. Six samples of each brand were analyzed, and the quantity of drugs was calculated with the help of the standard calibration curve.

4. Results and discussion

4.1. Optimization of the chromatographic conditions by BBD

Three levels for each factor were applied. The numerical values for independent factors were methanol (30%, 31%, and 32%), flow rate (1 ml/min, 1.1 ml/min, and 1.2 ml/min) and detection wavelength (290 nm, 291 nm and 292 nm). The inbuilt ANOVA model

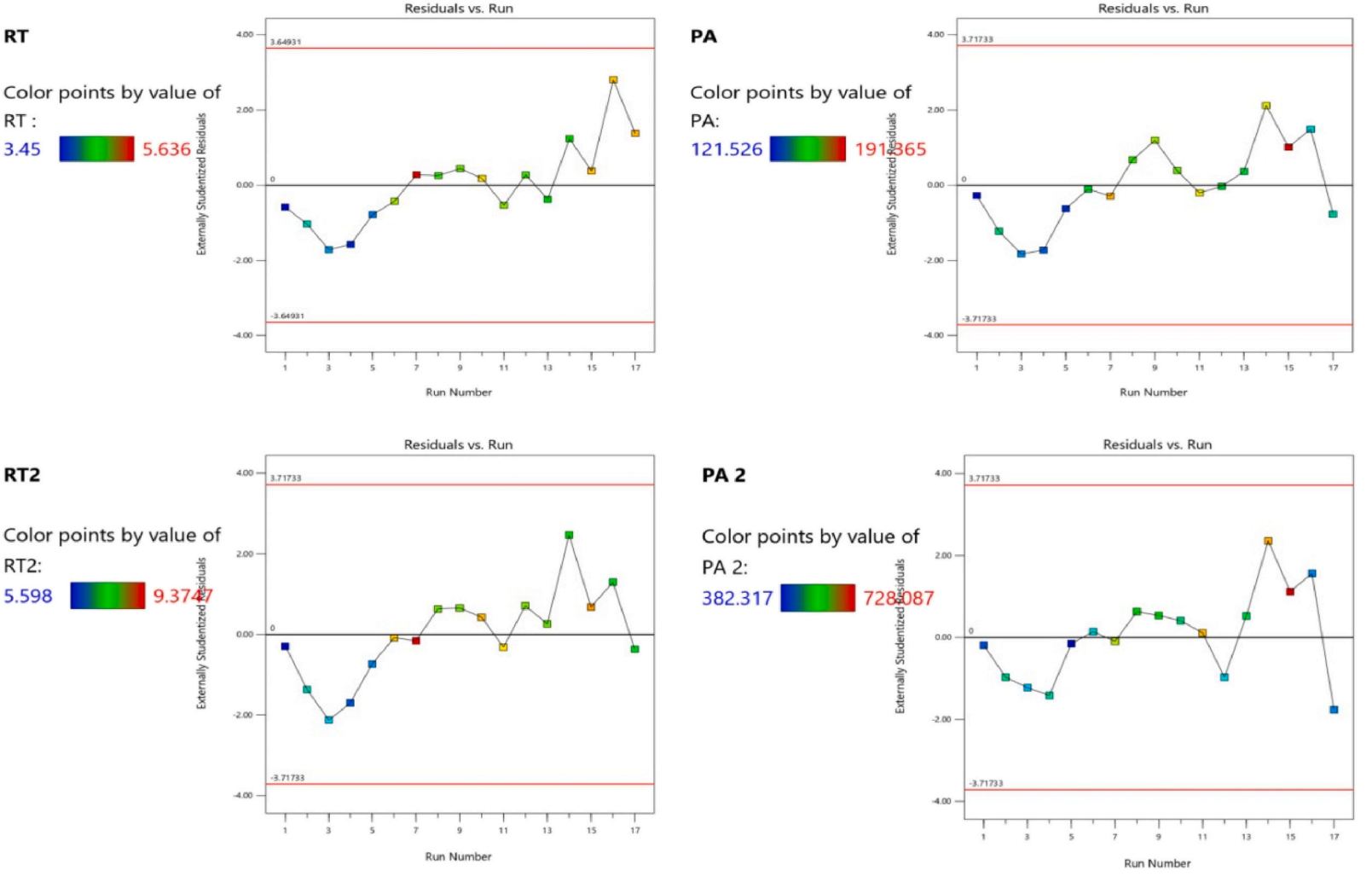

Fig. 2. Response surface methodology, residuals *vs.* run of retention time (RT) and peak area (PA) in BBD trials - RT and PA for FA and RT2 and PA2 for MTX.

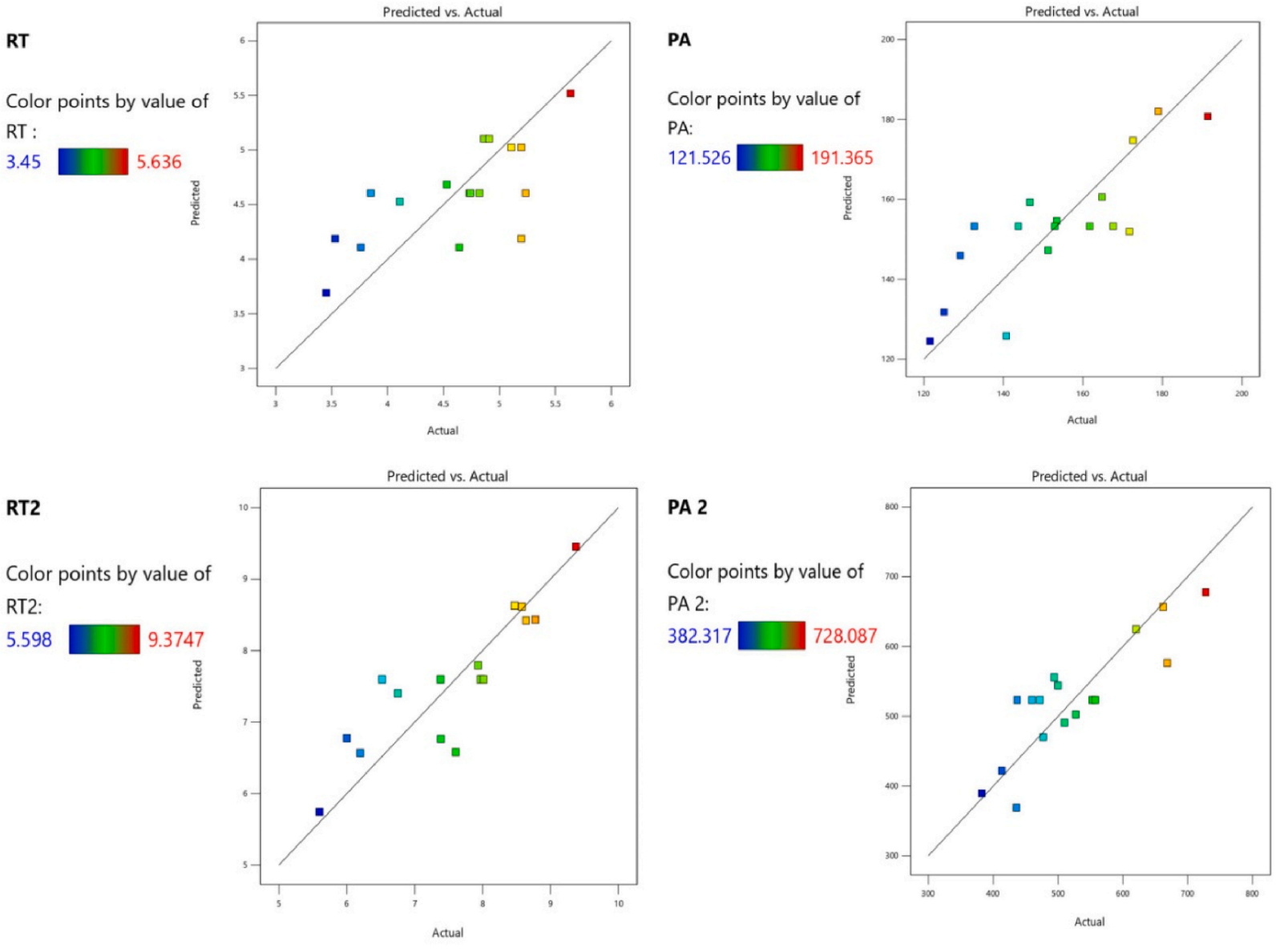

Fig. 3. Response surface methodology predicted *vs.* actual retention time (RT) and peak area (PA) in BBD trials - RT & PA for FA and RT & PA for MTX.

Table 3
System suitability.

	FA		MTX	
Sample	Plate count	Symmetry	Plate count	Symmetry
MCQ 1	7558	0.71	9778	0.73
MCQ 2	7554	0.72	9779	0.69
MCQ 3	7563	0.69	9785	0.71
MCQ 4	7567	0.72	9788	0.71
MCQ 5	7563	0.72	9789	0.72
MCQ 6	7561	0.72	9785	0.71
AVG	7561	0.71	9784	0.71
SD	4.12	0.01	4.16	0.01
%RSD	0.05	1.55	0.04	1.70

Table 4
Precision and accuracy of experiment's outcome.

	Precision								
	FA				MTX				
QC Sample	Intraday		Interday		Intraday			Interday	
	SD	%RSD	SD	%RSD		SD	%RSD	SD	%RSD
LQC (10 μg/ml)	0.04	0.02	0.83	0.43	LQC (75 μg/ml)	0.59	0.09	2.17	0.32
MQC (15 μg/ml)	1.7	0.59	3.53	0.28	MQC (112.5 μg/ml)	0.64	0.06	5.78	0.04
HQC (20 μg/ml)	0.42	0.11	5.03	0.30	HQC (150 μg/ml)	1.02	0.08	29.01	0.16

	Accuracy					
	FA			**MTX**		
% excess drug added	Avg.% recovered	SD	%RSD	Avg.% recovered	SD	%RSD
0%	100.56	0.96	0.96	99.56	0.95	0.91
50%	100.33	0.38	0.46	100.66	1.26	1.28
100%	99.8	0.23	0.28	100.52	0.42	0.46
150%	99.67	0.68	0.68	99.36	0.62	0.68

Table 5
Solution stability study outcomes.

	Two weeks at 25 °C		Four weeks at 2–8 °C	
Sample	% Amt.recovered (FA)	% Amt.recovered (MTX)	% Amt.recovered (FA)	% Amt.recovered (MTX)
MCQ 1	99.89	99.93	99.91	99.91
MCQ 2	99.72	99.98	99.89	99.83
MCQ 3	99.85	100.03	99.87	99.89
MCQ 4	99.91	99.93	100.05	99.84
MCQ 5	99.81	100.11	100.11	99.85
MCQ 6	99.98	99.95	99.89	99.88
AVG	99.86	99.99	99.95	99.87
SD	0.08	0.06	0.09	0.03
%RSD	0.08	0.06	0.09	0.03

results were interpreted in terms of the p value and f value of retention time and peak area of both drugs. Parallel, the model graphs of the response surface regarding residuals *vs.* run, overlay plot, desirability and contour.

4.2. FA retention time

The p value and F value were observed at 0.0073 and 7.14 in ANOVA (reduced linear model) analysis for FA retention time. The model is significant. A p value less than 0.05 indicates that the model terms are significant; here, model terms were considered (A) methanol percentage and (B) flow rate of the mobile phase. In fit statistics, the values of adjusted R^2 and predicted R^2 are 0.4344 and 0.2916, respectively. A difference between these values of less than 0.2 is reasonable. The adequate precision value is 8.96, which is more than 4, so this model can navigate the design space. The final equation in terms of coded factors (RT = A + B). The mean retention time was +4.61 min, and the values of the model terms were (−0.4965) and (−0.4177), respectively. The coded equation can determine the factors' relative merits by comparing the factor coefficients. Figs. 2a and 3a residuals *vs.* run and predicted *vs.* actual are presented by the color point value of retention time.

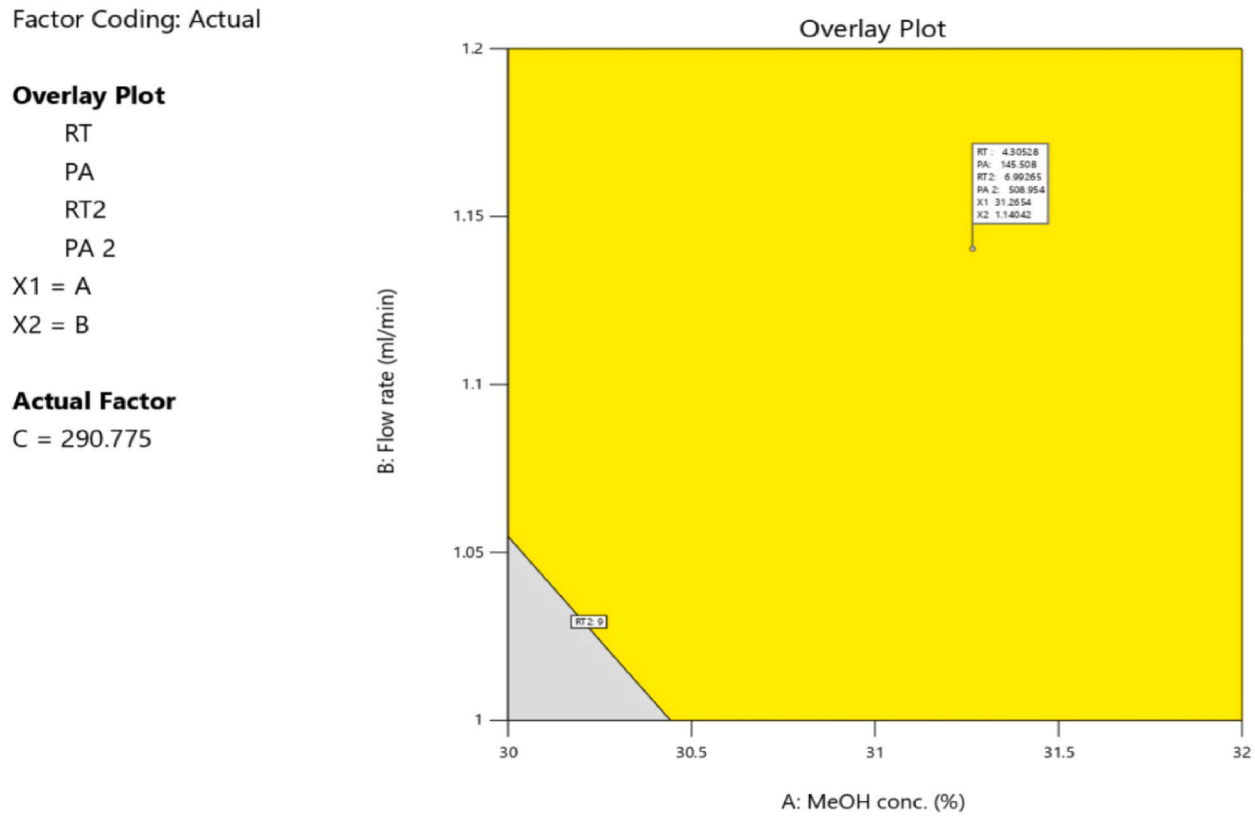

Fig. 4. Response surface methodology, overlay plot diagram between (A) MeOH conc. (%) *vs.* (B) Flow rate (ml/min).

4.3. FA peak area

ANOVA for linear model results for the peak area values of seventeen trials is presented in Table 1. The p value and f value of the model were observed at 0.0018 and 8.97, respectively. All three model terms are significant, as the p value is less than 0.0500. Overall, the model is significant. 0.73 The lack of fit f value implies that it is insignificant relative to the pure error. Nonsignificant lack of fit is good. The model fit statistic values were adjusted R^2 (0.5991) and predicted R^2 (0.4389). The difference is less than 0.2. The model can be used to navigate the design space because the value of adequate precision was 9.419. In the coded equation, the factor's coefficient value predicates the response value and a relative impact value of the individual's factor on response. The value of coefficient factors was observed in A (−11.40), B (−17.39), and C (−10.09). Figs. 2b and 3b residuals *vs.* run and predicted *vs.* actual revealed the peak area by color point value.

4.4. MTX retention time

A linear model of ANOVA interpreted the retention time value of MTX BBD in seventeen trials. The p value and f value were 0.0003 and 13.34, respectively, suggesting that the model is significant. The lack of fit value was 0.76, which implies that it is nonsignificant. Nonsignificant Lack of fit is good. The numerical value of adjusted R^2 (0.6983) and predicted R^2 (0.5745) value in fit statics analysis was reasonable. The difference was less than 0.2. The adequate precision value measures the signal-to-noise ratio. Here, the value was 12.96, suggesting navigating the design space because the value is greater than four. In the final equation, the coefficient values of the factors were (A = −1.03), (B = −0.8287), and (C = − 0.0058). Individual run-in trials were observed in Figs. 2c and 3c; the retention time color point value presented residuals *vs.* run and predicted *vs.* actual.

4.5. MTX peak area

The linear model of ANOVA analyzed the peak area of all seventeen trials. The model is significant because the p value and f value are 0.0004 and 12.66, respectively. The lack of fit value was 0.9436, suggesting nonsignificant, which is good for the model. In fit statistic data, the numerical value of adjusted R^2 (0.6861) and predicted R^2 (0.5692) is in reasonable agreement. The adequate precision value was 11.694, which is desirable for navigating the space. In the final coded equation, the coefficient values of the model terms were (A = −40.31), (B = −61.06), and (C = −93.46). Figs. 2d and 3d residuals *vs.* run and predicted *vs.* actual revealed the peak

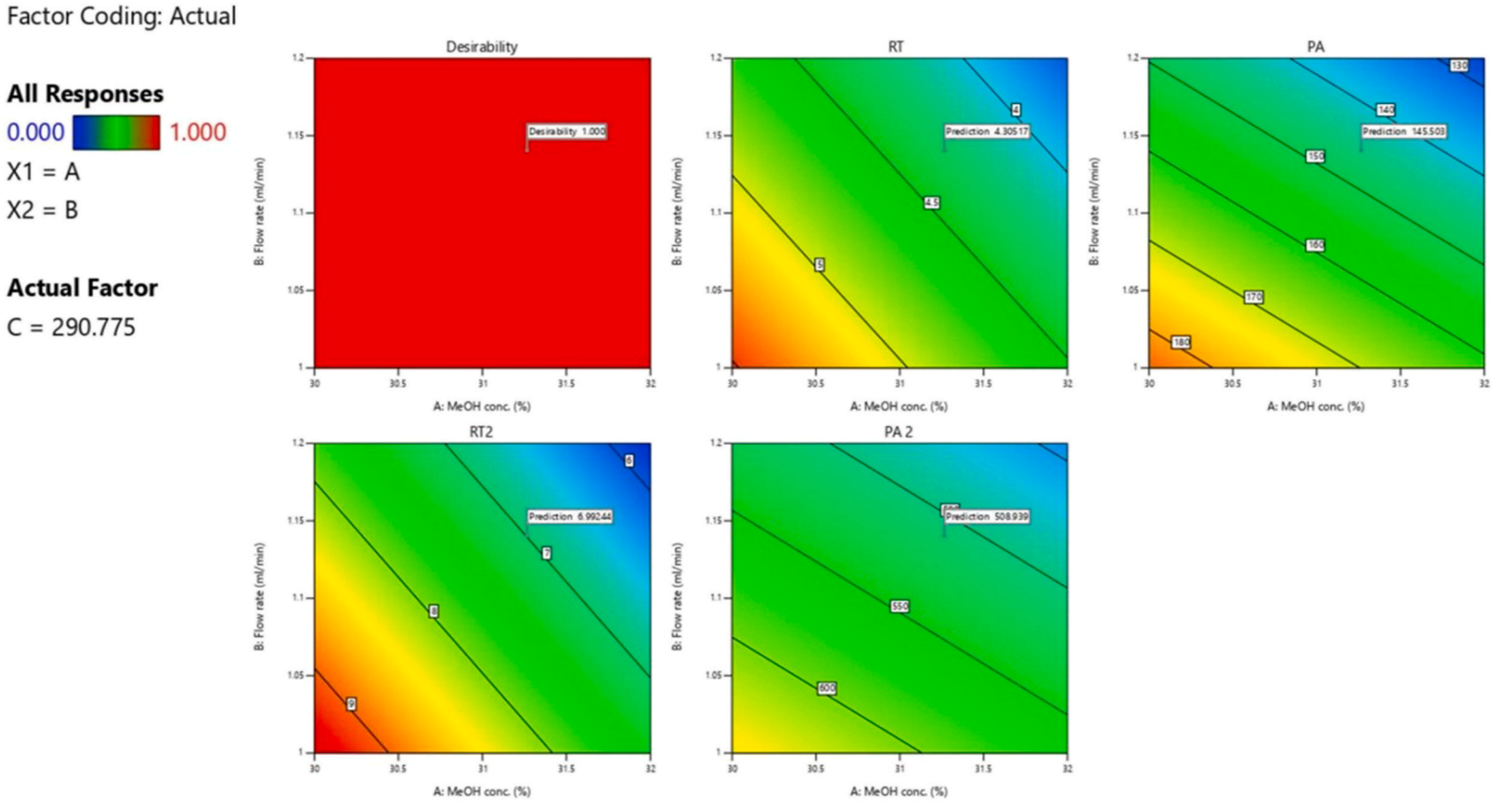

Fig. 5. Response surface graph of desirability and contour plot of FA (RT & PA) and MTX (RT2 & PA2).

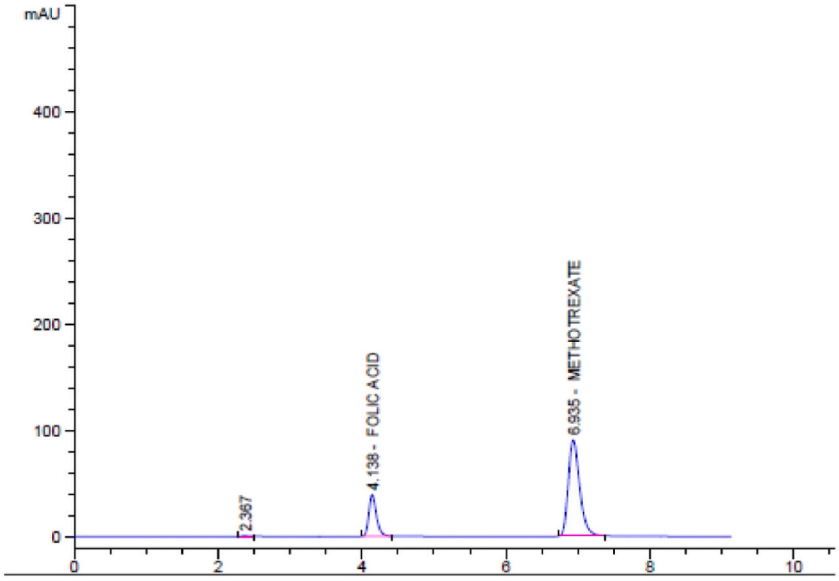

Fig. 6. Chromatogram of FA and MTX APIs in optimized chromatographic conditions.

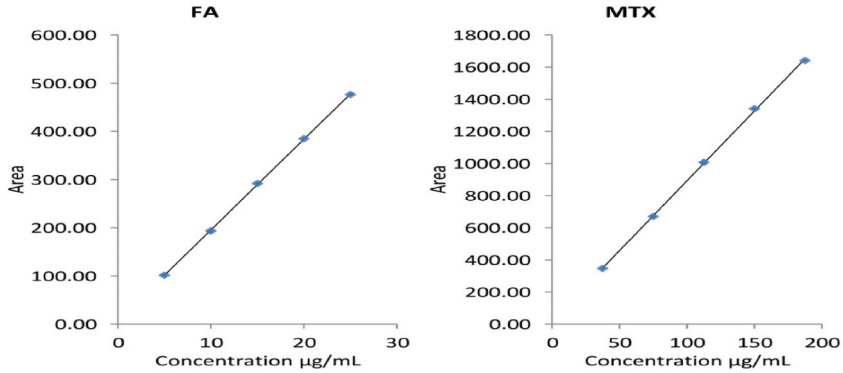

Fig. 7. Calibration curve of FA (5–25 µg/ml) and MTX (37.5–187.5 µg/ml).

area by color point value.

BBD seventeen trials with factors response 3^2 were interpreted with ANOVA. The response of individual drugs in terms of retention time and peak area values has helped to optimize the chromatographic conditions for method development. In addition, selected factors were shown to have effects on responses. The graphical overlay plot suggests the range of the factor's numerical value in Fig. 4 and the numerical optimization response surface regarding desirability and contour plot in Fig. 5. In Fig. 4, the yellow color of the overlay plot is the area where the effects of factors are within an acceptable range and shows their combined effects on the responses.

Method development: Under the optimized chromatographic conditions, both drugs' sharp and symmetric peaks in a 10-min run time were observed. The retention times for FA and MTX were 4.138 and 6.935 min, respectively. The areas of the peaks are FA (294.149 mAU*S) and MTX (1010.041 mAU*S). The chromatogram is shown in Fig. 6.

4.6. Method validation

(a) System suitability test: The experimental results are presented in Table 3. The average standard deviation and % RSD of both drugs were calculated. A % RSD value below two indicates that the instrument performs well.

(b) Linearity: The linearity of both drugs was validated. The results of the experiments (n = 3) are presented in terms of average standard deviation and % RSD. The average SD and % RSD values of FA were 0.62 and 0.11–0.59, respectively. For MTX, the average SD and % RSD were 1.79 and 0.09–0.30, respectively. Chromatograms of linearity are given in Fig. 7 and Table 2.

Table 6
Robustness experiment results.

	FA			MTX		
Conditions	RT	SD	% RSD	RT	SD	% RSD
Flow rate at 1 mL/min	4.123	0.03	0.01	6.898	0.03	0.44
Flow rate at 1.2 mL/min	4.139	0.62	0.20	6.877	0.02	0.33
Methanol (30%)	4.117	0.02	0.01	6.922	0.02	0.27
Methanol (32%)	4.132	0.01	0.15	6.916	0.03	0.49
Wavelength 290 nm	4.118	0.02	0.49	6.92	0.03	0.47
Wavelength 292 nm	4.12	0.02	0.46	6.918	0.03	0.39

Table 7
Stress degradation study results.

		FA			MTX		
Sr. No.	Degradation	1 h,	2 h.	8 h/24 h.	1 h.	2 h.	8 h/24 h.
		% of Degradation			% of Degradation		
1	Acidic	2.61	4.05	8.1	3.62	4.09	8.41
2	Alkali	0.42	1.84	5.81	0.75	3.68	6.77
3	H_2O_2	1.90	6.97	16.5	1.40	4.3	8.93
4	Neutral	0.86	1.19	1.2	0.5	1.05	1.14
5	Photolytic 24 h.	–	–	3.21	–	–	2.32
6	Thermal degradation	–	–	8.8			9.4

(c) Precision and accuracy: The developed method's precision and accuracy were determined per ICH guidelines. The results of the experiments are shown in Table 4. The precision values of both drugs in terms of intraday and interday were within acceptable limits. FA, % RSD values of intraday in all three levels were LQC (% 0.02), MQC (% 0.59), and HQC (% 0.11) and for interday LQC (% 0.43), MQC (% 0.28), and HQC (% 0.30). For MTX, the intraday RSD values at all three levels were LQC (% 0.09), MQC (% 0.06), and HQC (% 0.08), and the interday RSD values were LQC (% 0.32), MQC (% 0.04), and HQC (% 0.16). Inaccuracy, the % RSD range for FA was 0.28–0.96, and for MTX, it was 0.46–1.28.

(d) Solution stability: Quality control samples of level MCQ (n = 6) were analyzed. The % RSD of both drugs was determined under two conditions: first, two weeks at 25 °C and second, four weeks at 2–8 °C. Table 5 presents the results.

(e) Robustness: The robustness of the RP-HPLC-PDA method was determined. The factors were considered for the experiment. In the experimental results, the %RSD of intentionally changed factors is limited. The experimental results are presented in Table 6. The results indicate that the developed method was robust.

5. Stress stability studies

The results of the stress stability studies are presented in Table 7 in the numerical value of the degradation percentage. Selected chromatograms of these experiments are shown in Fig. 8 (a-f). In Tables 7 and it is summarized that the percentage degrades at different hours under different conditions. For example, under acidic conditions, after 1 h, two degradations occurred. One degradation was observed in the chromatogram after 2 h, and three degradations were observed after 8 h. Under alkali conditions, after 1 h, three degraded. After 2 h, two peaks were observed, and after 4 h, four peaks were observed in the chromatogram. A stress stability study was performed under oxidation conditions, where no prominent peak was found in the chromatogram. The chromatogram of the degradation study in neutral conditions indicated that two degradations were observed after 1 h, and after 2 h and 8 h, two degradations were observed. The chromatogram of the photolytic condition produced after 24 h has one degradation. The thermal degradation chromatogram produced after 40 h.

6. Assay of tablets

The developed method estimated three marketed tablet concentrations, and the chromatograms of MCQ level concentration are presented in Fig. 9(a–c) for the three brands. The concentrations of MGXT, FOLTNAX and TRUXOFOL were calculated with the standard calibration curve. The percentages of FA and MTX were 99.13% and 99.50 in MGXT, 99.17% and 99.47 in FOLTNAX, and 99.91 and 100.05 in TRUXOFOL tablets, respectively.

7. Conclusion

A new and novel reversed-phase HPLC-PDA analytical method was developed. The direct focus of dispersed light onto the detector

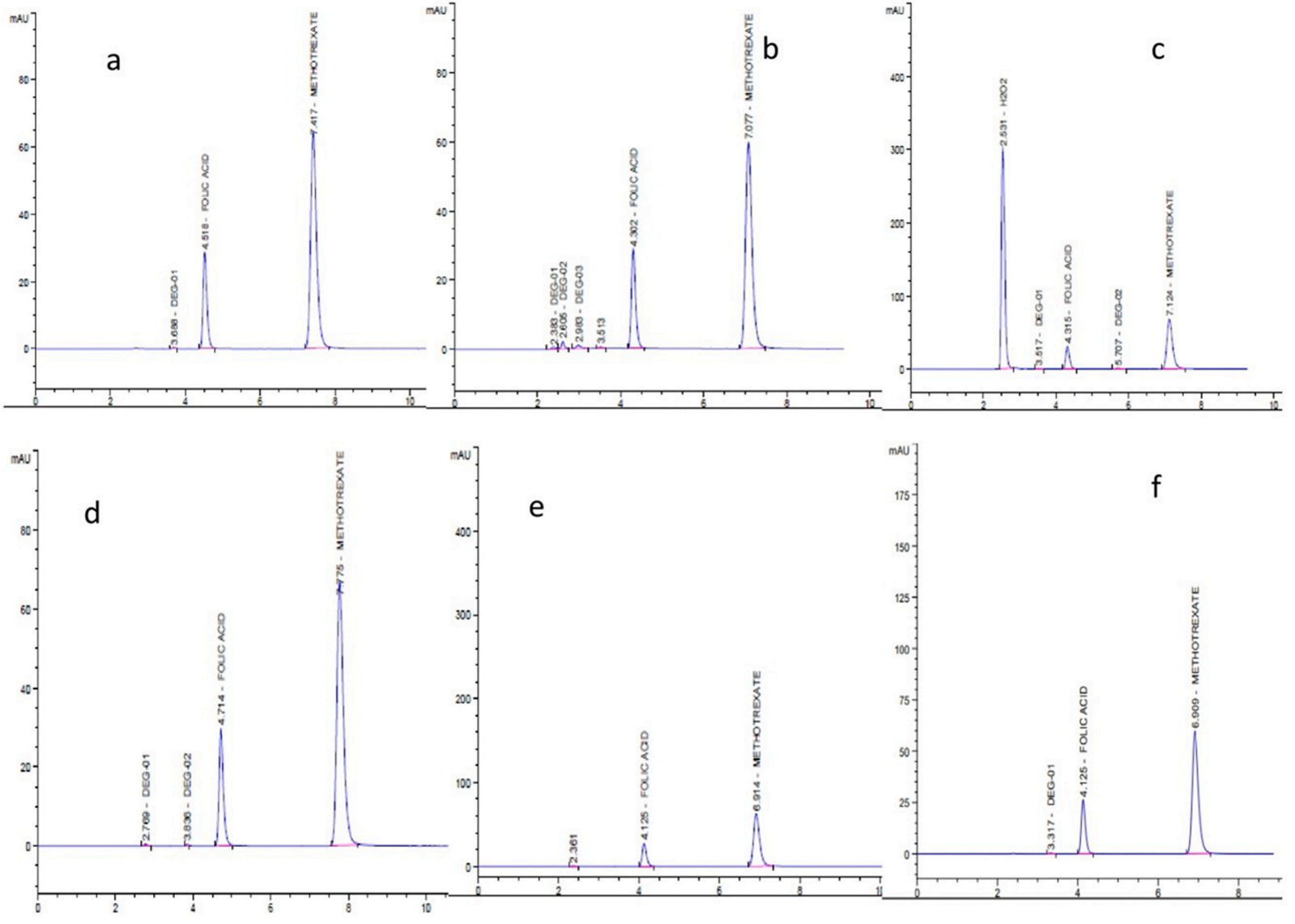

Fig. 8. Chromatogram stress stability studies (a) Acidic 8 h. (b) Alkali for 8 h. (c) Oxidation for 8 h. (d) Neutral 8 h. (e) Photolight 24 h. (f) Thermal degradation.

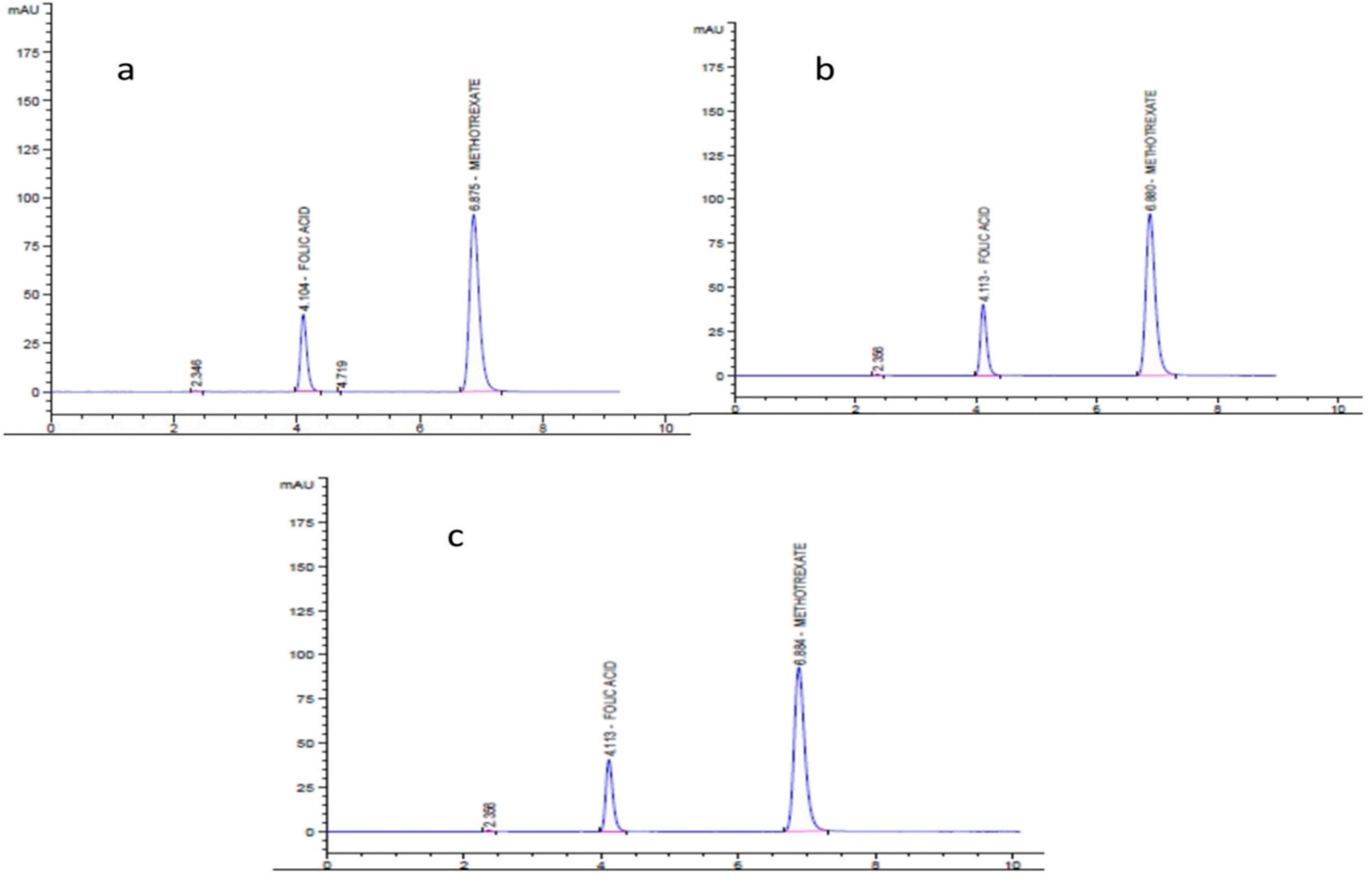

Fig. 9. Chromatogram of assay method (a) MGXT, (b) FOLTNAX, (c) TRUXOFOL.

array saves time and considerably reduces the complexity of the apparatus. Most spectrophotometers used today use a dispersion element and detector array combination. The present research has been done with a PDA detector with a QbD application. These research approaches make it novel and economical. The findings reported here are fascinating for routine analysis in the pharmaceutical industry. The chromatographic conditions were optimized via BBD. The effects of factors on the retention time and peak area were observed through the residuals *vs.* run and predicted *vs.* actual. In ANOVA, the numerical values of predicted R^2 and adjusted R^2 for both drugs were in the acceptable range. The model term value in terms of p value and f value indicated that the model was significant in both medicines. Finally, BBD's numerical values and graphical optimization suggested the appropriate chromatographic conditions. The overlay plot yellow color indicates the numerical values of the factors. From the BBD, the chromatographic conditions were optimized regarding the percentage of methanol in the mobile phase, flow rate of the mobile phase and elute detection wavelength. The responses of the drugs FA and MTX in terms of retention time and peak area were good. Validation of the developed method was covered in different parameters, and its results were within acceptable limits. The stress stability study results will help determine the formulation's shelf life. Quantitative analyses of the marketed formulations were performed. Furthermore, this novel method can be used for routine analysis in quality control.

Author contribution statement

Saad Ali Alshehri: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper. Shadma Wahab: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper. Mohammad Khalid: Performed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper. Mohammad Ali Abdullah Almoyad: Contributed reagents, materials, analysis tools or data; Wrote the paper.

Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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