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Optimization and Modelling of Novel RP-UPLC Method for Simultaneous Determination of Cefradine, Cefalexin, Sodium Benzoate and Methylparaben in Some Biological Fluids. Application to Experimental Design

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Abstract

Novel, economical, and eco-friendly UPLC method was optimized and validated for simultaneous determination of cefalexin (CFX) and preservative components namely, sodium benzoate (SB) and methylparaben (MP) in their dosage form, spiked human plasma, wastewater of poultry slaughter and in rabbit plasma using cefradine (CFR) as an internal standard (IS). Chromatographic system was optimized using design of experiments (DoE) such as Box-Behnken design (BBD) and response surface methodology (RSM) to improve the quality of the analytical methods, minimize the risk of method failure and provides a better solution for the defect results. Three input factors (independent variables) such as composition of methanol in the mobile phase, concentration of the phosphate buffer of the aqueous phase and its pH value were selected to study their effects on (dependent variables) as retention time, theoretical plate, and resolution. Isocratic chromatographic conditioning was created using mobile phase consisting of methanol: phosphate buffer (30mM) at pH 3.0 ± 0.1 (35:65, v/v), Agilent ZORBAX SB-C18 column (50 mm × 2.1 mm, 1.8 µm particle size) at flow rate 0.4 mL/min, injection volume 0.3 µL for RP-UPLC and UV detection at 230 nm; the retention time was 1.73 min for CFX, and column temperature was adjusted at 40ºC. The suggested method was validated as per the guidelines of the FDA for bioanalytical method validation and could be applied to quality control and laboratory prepared mixtures.

Keywords: RP-UPLC-UV; Box–Behnken Design; Cefradine; Cefalexin; rabbit plasma; wastewater of poultry slaughter

1. Introduction

Application of quality be design (QbD) and design of experiments (DoE) approaches is accepted by FDA and is described in pharmaceutical current good manufacturing practices cGMPs. [Scheme 1].

Many applications of DoE such as Box-Behnken design (BBD) have been extensively used in the pharmaceutical industry to optimize and minimize number of experimental trials of chromatographic system and achievement of a shorter time [1]. Cefradine (CFR), (**Fig.1a**.) A semi-synthetic product derived from a fermentation product that follows the first generation of the antibacterial cephalosporin group, CFR exerts microbial activity by intervening in later stages of the formation of the bacterial cell wall by deactivating one or more penicillin-related proteins and inhibiting the intercommunication of the peptidoglycan structure [2]. Cefalexin (CFX), (**Fig.1b**.), like CFR is a semisynthetic product follows the first generation of cephalosporin antibacterial group for oral administration [2]. Sodium Benzoate (SB) is chemically known as sodium benzene carboxylate (**Fig.1c**), the ingredient is used as an antimicrobial agent and as a flavoring agent used for food at levels not exceeding good manufacturing practices. Current use results in a maximu of 0.1 per cent for food [3]. Methylparaben (MP) also called methyl parahydroxybenzoate, (**Fig.1d**). It is used as antimicrobial preservative [2]. CFR, CFX, SB and MP are officially reported in the British Pharmacopeia (BP) which described HPLC methods for CFR, CFX, and MP, respectively and titrimetric method for SB [2]. While the United States Pharmacopeia (USP) has described an HPLC method for each of them [4].

Many analytical methods of analysis of the mentioned drugs have been published either alone or in blending with other components including high-performance liquid

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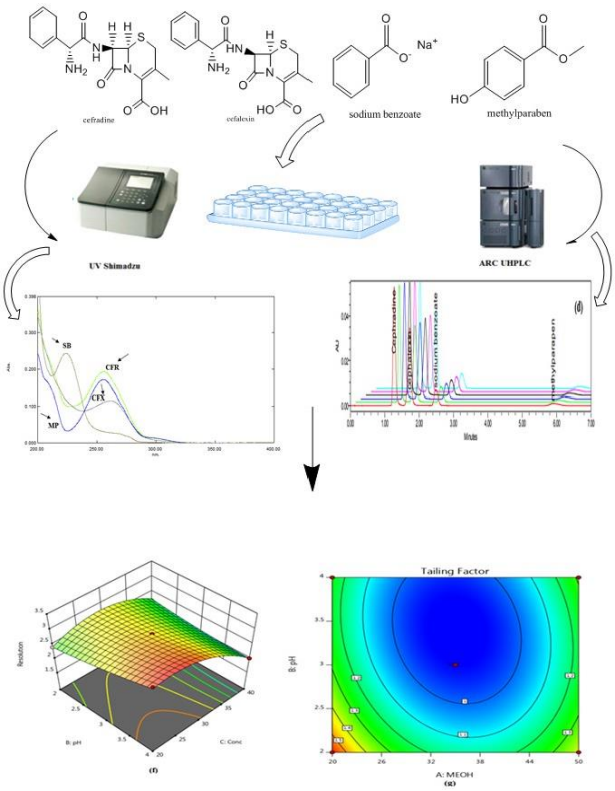
chromatography (HPLC) [5-21], capillary electrophoresis and electrochemical methods [22-25], thin layer chromatography (TLC) [26-30], HPTLC [31], colorimetric and spectrophotometric [32-39].



Scheme 1: Graphical Abstract for the proposed method

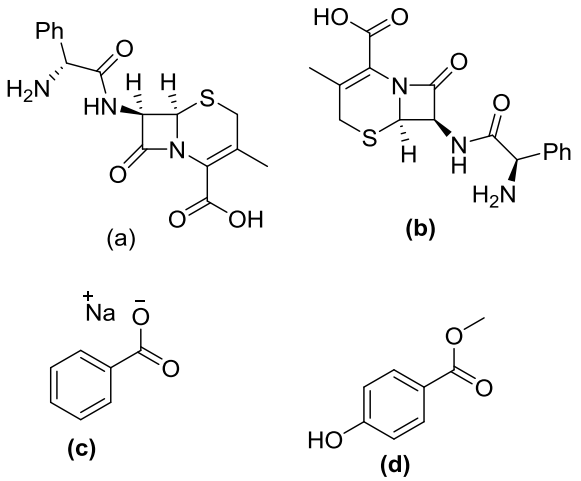


Fig. 1: Chemical structures of (a) CFR, (b) CFX, (c) SB, (d) MP.

UPLC is an update of liquid chromatography that keeps the experimental application of HPLC fundamentals and increasing the features of speed, sensitivity, and accuracy [40-44]. Different analytical methods have been reported using the DoE and BBD approaches to select the appropriate robustness for HPLC methods [45-49].

Comparison of the suggested methods and the issued ones are presented in Table 1, All of these reported methods concerned with CFX either alone or in combination with SB & MP separately, while the uniqueness of the developed method is concerned with resolution and estimation of CFX, SB and MP in the presence of internal standard CFR with good recovery and higher selectivity.

As far as we know, there's no RP-UPLC-UV method to simultaneously identify the quaternary mixture CFR, CFX, SB, and MP in their pharmaceutical dosage forms, spiked human plasma, in wastewater of poultry slaughter, rabbit plasma and application of Box-Behnken design. Therefore, the purpose of this research was the optimization and development of a novel, economic, rapid, accurate, selective, and validated UPLC method using design of experiment (DOE) approach for the determination of this quaternary mixture in dosage forms and biological fluids with good linearity and accuracy.

2. Experimental

2.1. Reagents

CFR, CFX, SB and MP were provided by hikma pharmaceutical industries company, Beni-Suef, Egypt. Methanol HPLC-grade, Ultra-purified water, potassium dihydrogen orthophosphate, and orthophosphoric acid analytical grade, were procured from (Scharlau, Spain).

Qualitative and quantitative composition of Lexin 125 mg powder for oral suspension. Each 5mL contains 125 mg of cephalexin (as Monohydrate) and include the following inactive ingredients: sucrose, colloidal silicon dioxide, croscarmellose sodium, methylparaben, propylparaben, saccharin sodium, sodium benzoate, color sunset yellow and flavour banana powder.

2.2. Standard sample

CFR, CFX, SB and MP stock solutions (1mg/mL) were prepared by transferring 100 mg of each drug to a 100-mL volumetric flask, add about 70 mL of the mobile phase, sonication till dissolve and avoid heating, complete volume with the same diluent. Various working standard solutions were prepared by further dilution of aliquots of their stock. Filter through 0.45 µm PTFE filter then store in refrigerator at 4ºC and protect from light.

2.3. Samples

For in use sample, constitute the bottles to the mark with water, transfer 5 mL aliquot to a 500-mL volumetric flask, freshly mix and free from air bubbles, add 350 mL diluent, sonicate, if necessary, complete to mark with diluent. Transfer 5 mL of this solution to 50-mL volumetric flask, dilute with solvent to volume and mix well. Filter

through 0.45 µm PTFE membrane filter. Surface water samples (Nile River, Giza Governorate) were treated with citrate buffer until reaching the pH value 3.1 and spiked with different aliquots of the standard solution.

2.4. Instrumentation

Waters ACQUITY® Arc™ UPLC System, a quaternary liquid chromatography supplied with Empower™ 3 Software. Stability chamber (VOSTCH VP 1300, Germany) with SIMPATI 4.06 Software. Autoclave, HX-150 (Systec, Germany). Incubator, BD-53 (Binder, England). Statistical analysis was established using Design Expert® (Version 11.1.2, Stat-Ease Inc., Minneapolis, MN, USA) for optimization the proposed method.

2.6. Procedures

2.6.1. *Mobile phase Preparation*

Mobile phase is consisting of methanol: (30 mM) potassium dihydrogen phosphate (35:65, v/v) and adjusted pH 3.0 ± 0.1 with orthophosphoric acid.

Diluent: Methanol: (30 mM) potassium dihydrogen phosphate (40:60, v/v) at pH 3.0.

2.6.2. Chromatographic Conditions (RP- UPLC)

Isocratic method was developed at ambient temperature using hplc column of Agilent Zorbax SB-C18 (50 mm × 2.1 mm, 1.8 µm particle size) at flow rate of 0.4 mL/min, injection volume of 0.3 µL for RP-UPLC and UV detection at 230 nm.

2.6.3. Biological preparations

2.6.3.1. Calibration standards and QC samples

Stock standard solutions of the studied drugs (each, 1 mg/mL) were prepared in the solvent. Appropriate series of dilutions were prepared from these stocks and accordingly solutions were used to construct the calibration curves to obtain concentration range levels from (5–50 µg/mL) for CFR, (3–35 µg/mL) for CFX, (3–30 µg/mL) for SB and (2–25 µg/mL) for MP.

2.6.3.2. Plasma samples preparation

The congealed plasma samples were dissolved at ambient temperature before performing analysis. Then 1 mL of blank plasma was added to each sample. Next, 1 mL of IS (CFR) has been added from its working solutions then vortex-mixed for 1 min. Precipitation technique was established by adding mixture of methanol: acetonitrile (1:9, v/v) and then vortex each for accurately one minute, after that the mixture was centrifuged at 4000 rpm for 10 min at ambient temperature. The clear supernatant was transferred to the HPLC vials, and then injected into the UPLC-UV system which was supplied with sample cooler at 5°C inside the autosampler.

2.6.4. Analysis of cephalosporin residues in poultry slaughter wastewater and rabbit plasma samples

The wastewater samples were collected from Cairo poultry slaughter company as per the environmental protection agency (EPA) guidelines in an amber glass bottle using Teflon lined caps and then protected from light at 4 °C after their filtration, while rabbit plasma samples were collected from a private rabbit farm in Al Qalyubiyah Governorate (which is situated 27 km to the north of Cairo in the Nile Delta region). Both types of samples were treated as follows:

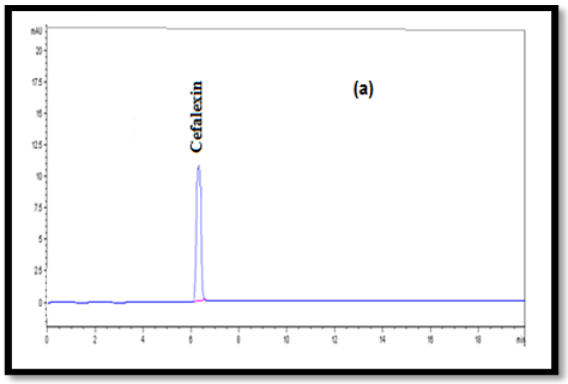
To 5 mL of the sample add 5 mL of acetonitrile to complete dissolution and deproteination, then vortex-mixing for 30 min and add 2 gm $MgSO_4$ & 0.5 gm of NaCl and vortex-mix for 1 min then centrifuge for 5 min at 5000 rpm. Transfer 1 mL of the supernatant to 10 mL centrifuge tube containing150 mg $MgSO_4$, 50 mg silica gel, and vortex-mix for one minute, then centrifuge for another one minute at 6000 rpm. Decant the supernatant and dissolve the residue in 1 mL of the mobile phase, filter through a 0.22 membrane filter before injection into the HPLC-DAD system as displayed in (**Fig. 2**.) and the obtained results were included in (**Table 1**).

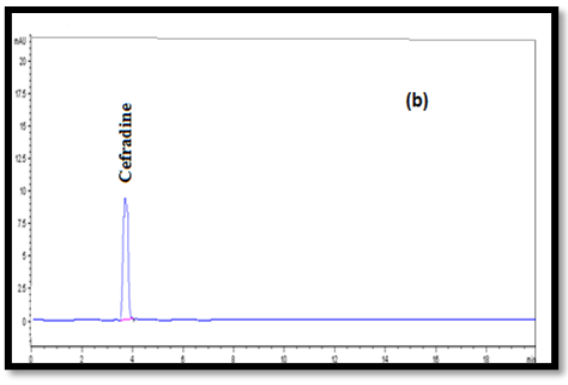
Table1: Determination of cephalosporin residues in poultry slaughter wastewater and rabbit plasma samples.

Sample	HPLC			
	CFR	CFX	SB	MP
1 Poultry slaughter wastewater	384.9 PPb [a]	362.1 PPb [a]	ND	ND
2 Poultry slaughter wastewater	387.8 PPb [a]	375.0 PPb [a]	ND	ND
3 Rabbit plasma	109.2 PPb [a]	98.2 PPb [a]	ND	ND
4 Rabbit plasma	110.1 PPb [a]	99.9 PPb [a]	ND	ND

N.D not detected

[a] The concentration was obtained by sample dilution (one part sample to three parts pure water)





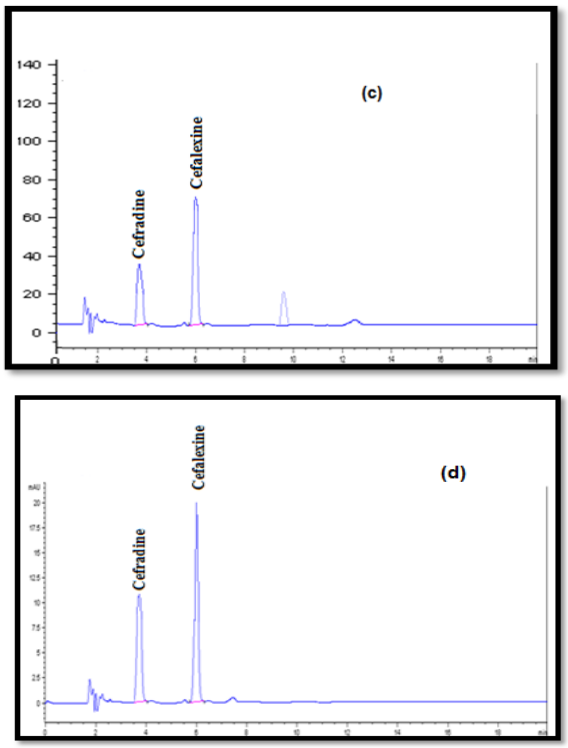


Fig.2: HPLC chromatograms of (a) 50 µg/mL of standard solution of CFX, (b) 50 µg/mL of standard solution of IS (CFR), (c) wastewater sample, (d) rabbit plasma sample.

2. Result and Discussion

3.1. Optimization of wavelength selection

Different wavelengths were scanned at (200 – 400 nm) for 15 µg/mL of each of the four drugs: CFR, CFX, SB and MP in their active material to achieve and optimize the wavelength with best sensitivity and minimum noise. As shown in supporting information (**Fig**.**3**) the wavelength value of 230 nm was the best option regarding sensitivity.

Fig. 3: Zero order absorption spectra of 15 µg/mL of each of CFR, CFX, SB and MP using solvent as blank.

3.2. Optimization of Filter Compatibility

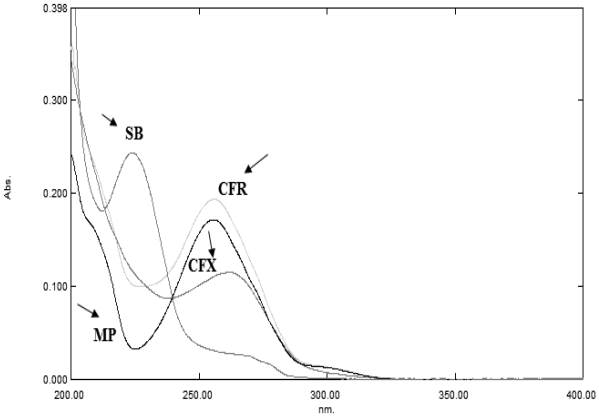
A study was performed to determine the effect of filtration procedure on the standard and sample solutions using two types of filters namely, syringe filters (PTFE) 0.45µm and syringe filters (nylon) 0.45µm. Two series of standard and sample solutions were prepared. The members of one of them were used unfiltered. The others were filtered, and two portions of filtrate of each four mL's and eight mL's were tested. The best recovery with minimum relative standard deviation was achieved using syringe filters (PTFE) of 0.45µm.

3.2. Optimization of Oven temperature and flow rates

To optimize the separation process, column temperature was tested at (25, 30, and 40ºC). Also, different flow rates (0.1, 0.2, 0.4, 0.5, 0.8 and 1.0 mL/min) were tried; the flow rate of 0.4 mL/min and temperature of 40ºC were the best couple for system with good resolution.

3.3. Optimization of Stationary phase

Precursory studies have been involved trying different stationary phases with different lengths and particle sizes, including Waters CORTECS® C18 column (50 mm × 4.6 mm, 2.7 µm particle size), Agilent ZORBAX SB-C18 column (50 mm × 2.1 mm, 1.8 µm particle size) and ACE Excel C18, 2µm, 2.1 x 50 mm, where that of Agilent ZORBAX SB-C18 column (50 mm × 2.1 mm, 1.8 µm particle size) provided better selectivity and resolution for the peaks of all components.



3.4. Optimization of RP-UPLC-PDA method

Box-Behnken design and response surface methodology have facilitated the development and improvement of the robustness of analytical methods, reduced the risk of method defect, introduced a better decision for decreasing trial and error and optimized the critical method. All the 17 experimental runs carried out to evaluate the impact of the three factors viz., methanol composition in the mobile phase, salt concentration of the phosphate buffer of the aqueous phase (mM) and pH of the aqueous phase on the output responses (dependent variables) as retention time, theoretical plate and resolution were shown in (**Table 2**).

The demonstrated data were submitted and validated by (ANOVA) using design expert software as exhibited in Table 3, for retention time response and supplementary (**Tables 4** & **5**) for resolution and tailing factor responses

Based on ANOVA results quadratic models were estimated for retention time resolution and tailing factor responses. In the retention time response, the model F-value of 68.0, P-values less than 0.05, also the lower standard deviation (% C.V) and the predicted R^2 of 0.8191 was in rational agreement with the adjusted R^2 of 0.9742; i.e., the variation is less than 0.2. The highly adjusted R-square value indicates that ideal terms are considerable. In this issue A, B, C, AC, BC, A^2, B^2, C^2 are considerable ideal terms. Results more than 0.1000 indicate that the ideal terms are

not considerable. Also, the resolution response for the model F-value of 194.49, P-values less than 0.05, the lower standard deviation (% C.V) and the predicted R^2 of 0.9369 was in rational agreement with the adjusted R^2 value of 0.9909; i.e., the variation is less than 0.2. Thus, the highly adjusted R-square value indicates that model terms are significant. In this issue A, B, C, AB, AC, BC, A^2, C^2 are considerable ideal terms.

Table 2: Box-Behnken design experimental runs with measured responses.

St	Runs	Variables			Responses for (CFX)		
		MeOH	pH	Salt	Rt	Rs	T
15	1	35	3	30	1.728	2.855	0.933
8	2	50	3	40	3.1	2.1	1.3
7	3	20	3	40	2.8	1.6	1.5
2	4	50	2	30	2.2	2.11	1.425
16	5	35	3	30	1.728	2.848	0.934
12	6	35	4	40	1.678	2	1.123
5	7	20	3	20	2.7	2.9	1.253
1	8	20	2	30	2.346	3	1.6
10	9	35	4	20	1.754	3.2	1
14	10	35	3	30	1.728	2.85	0.931
6	11	50	3	20	1.887	2	1.33
4	12	50	4	30	1.676	2.997	1.297
9	13	35	2	20	1.887	2.417	1.19
13	14	35	3	30	1.728	2.841	0.93
17	15	35	3	30	1.728	2.841	0.93
11	16	35	2	40	2.779	2.2	1.263
3	17	20	4	30	1.88	2.454	1.241

Table 3: ANOVA results for retention time response.

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	3.64	9	0.4045	68.00	< 0.0001
A-MeOH	0.0931	1	0.0931	15.65	0.0055
B-pH	0.6183	1	0.6183	103.94	< 0.0001
C-Conc	0.5666	1	0.5666	95.25	< 0.0001
AB	0.0008	1	0.0008	0.1414	0.7180
AC	0.3097	1	0.3097	52.06	0.0002
BC	0.2343	1	0.2343	39.38	0.0004
A^2	0.8427	1	0.8427	141.67	< 0.0001
B^2	0.0946	1	0.0946	15.90	0.0053
C^2	0.8390	1	0.8390	141.04	< 0.0001
Residual	0.0416	7	0.0059		
Lack of Fit	0.0416	3	0.0139		
Pure Error	0.0000	4	0.0000		
Cor Total	3.68	16			

Table 4. ANOVA results for resolution response.

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	3.43	9	0.3807	194.49	< 0.0001
A-MeOH	0.0698	1	0.0698	35.63	0.0006
B-pH	0.1067	1	0.1067	54.51	0.0002
C-Conc	0.8561	1	0.8561	437.30	< 0.0001
AB	0.5134	1	0.5134	262.24	< 0.0001
AC	0.4900	1	0.4900	250.30	< 0.0001
BC	0.2416	1	0.2416	123.40	< 0.0001
A^2	0.2749	1	0.2749	140.40	< 0.0001
B^2	0.0100	1	0.0100	5.11	0.0583
C^2	0.8207	1	0.8207	419.23	< 0.0001
Residual	0.0137	7	0.0020		
Lack of Fit	0.0136	3	0.0045	123.82	0.0002
Pure Error	0.0001	4	0.0000		
Cor Total	3.44	16			

Table 5. ANOVA results for tailing factor response.

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	0.7456	9	0.0828	184.02	< 0.0001
A-MeOH	0.0073	1	0.0073	16.26	0.0050
B-pH	0.0834	1	0.0834	185.33	< 0.0001
C-Conc	0.0213	1	0.0213	47.36	0.0002
AB	0.0133	1	0.0133	29.63	0.0010
AC	0.0192	1	0.0192	42.61	0.0003
BC	0.0006	1	0.0006	1.39	0.2772
A^2	0.4598	1	0.4598	1021.26	< 0.0001
B^2	0.0697	1	0.0697	154.91	< 0.0001
C^2	0.0295	1	0.0295	65.52	< 0.0001
Residual	0.0032	7	0.0005		
Lack of Fit	0.0031	3	0.0010	316.99	< 0.0001
Pure Error	0.0000	4	3.300E-06		
Cor Total	0.7488	16			

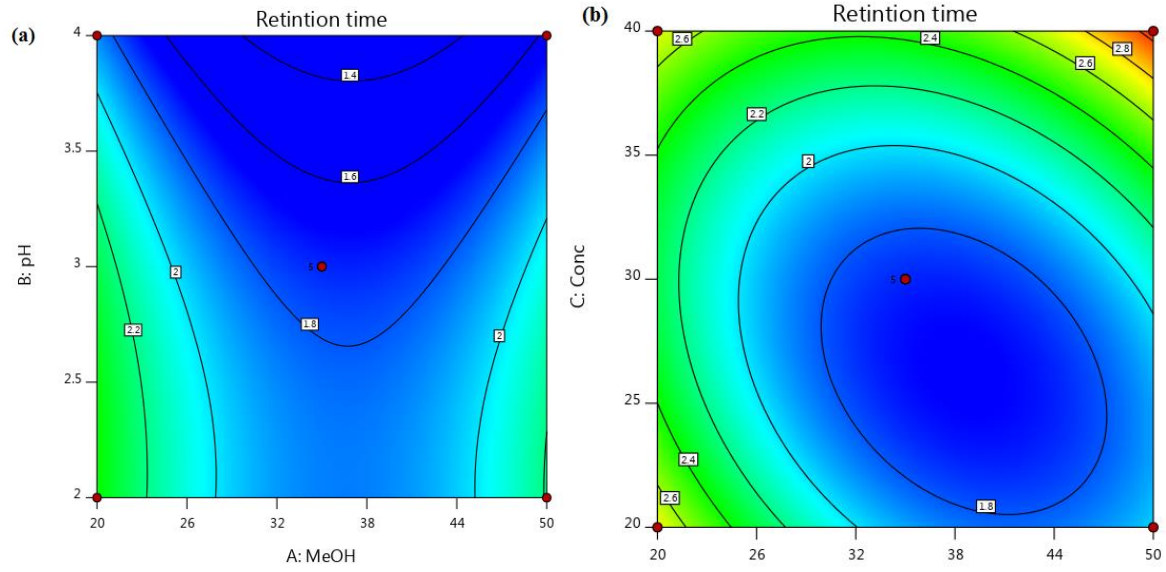
Finally in the tailing factor response the model F-value of 184.02, P-values less than 0.05, the lower standard deviation (% C.V) and the predicted R^2 of 0.9329 was in rational agreement with the adjusted R^2 value of 0.9904; i.e., the variation is, also, less than 0.2. Similarly, the highly adjusted R-square value indicates that ideal terms are considerable. Thus, in this issue A, B, C, AB, AC, A^2, B^2, C^2 are considerable ideal terms. Results greater than 0.1000 indicate that the ideal terms are not considerable, non-considerable terms are eliminated and the equation in terms of coded factors can be used to make augury about the responses (retention time, resolution and tailing factor responses) for given levels of each factor. The coded equation as shown below is useful for identifying the relative impact of the factors by comparing the factor coefficients.

Retention time = 1.73 - 0.108A- 0.278B + 0.266C - 0.015AB + 0.278 AC - 0.242BC + 0.447A2 -0.149 B2 + 0.446 C2 (I)

Resolution = 2.85 - 0.093A+ 0.115B - 0.327C + 0.358AB + 0.350 AC - 0.246BC - 0.255A2 +0.04 B2 - 0.442 C2 (II)

Tailing Factor = 0.93 - 0.030A- 0.102B + 0.052C + 0.058AB - 0.069 AC + 0.012BC +0.331A2 +0.129 B2 + 0.0842 C2 **(**III**)**

Outline plots which are shown in supporting information (**Fig. 4 a-i**) and 3-D response surface plots in (**Fig. 5 a-i**) showed that the effect of bared variation such as methanol composition as methanol content in the mobile phase, salt concentration in the aqueous phase (mM) and pH of the aqueous phase of HPLC system on the responses retention time, resolution and tailing factor which are strongly affected by the factors mentioned above in the equations (I, II & III).



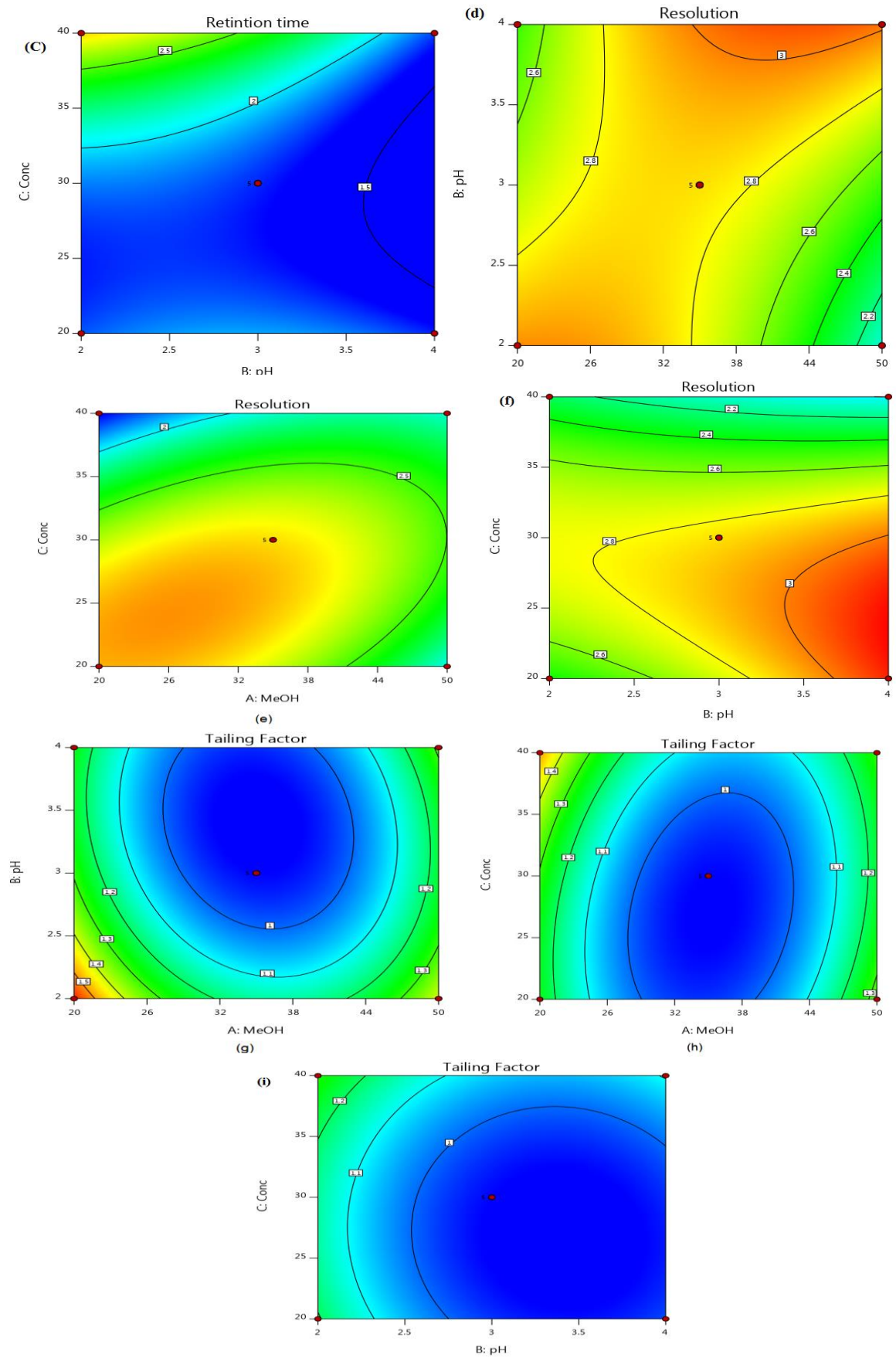


Fig.4: Contour plot of retention time, resolution and tailing factor responses.

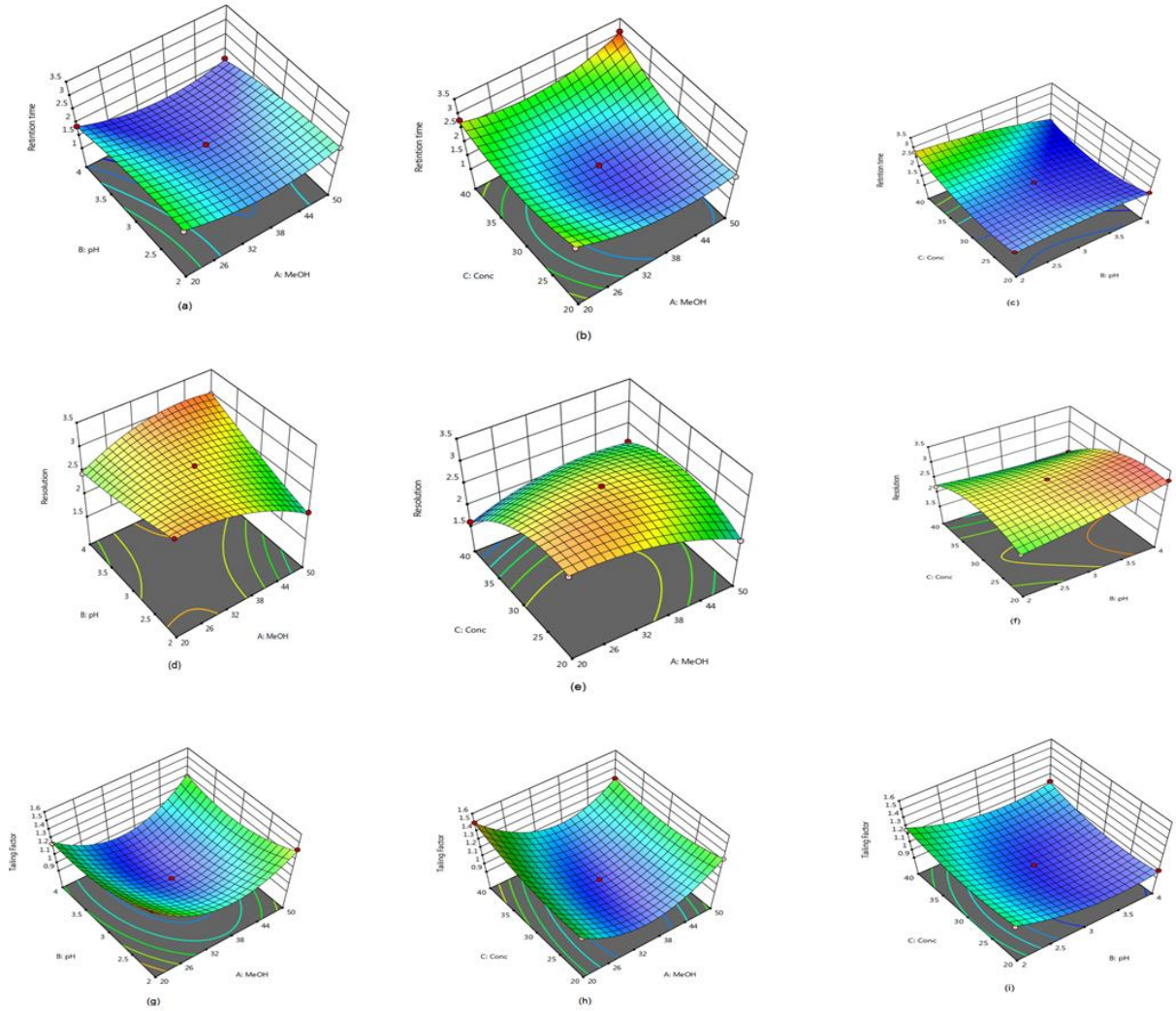


Fig.5: 3D BBD plots for retention time, resolution and tailing factor responses.

The interaction terms (A, B, C, AC, BC, A^2, B^2, C^2) display that the response (retention time) changes when two factors (A, B) are simultaneously changed at constant factor C. A positive value indicates a favoured effect on the retention time, while a negative value implies an inverse effect between the variables and the response. (**Fig.5a-c**) indicated that retention time increases with the decreasing of methanol and decreases with the increasing of methanol in the mobile phase. The increase or decrease in pH of aqueous mobile phase has no effect on the retention time, while the salt concentration of the aqueous mobile phase showed an inappropriate effect on the retention time pH. (**Fig.5d-f**). showed that resolution was affected by methanol and salt concentrations of the mobile phase, while the variation of pH didn't show any effect on the resolution. (**Fig.5g-i**). showed that tailing factor was affected by both the concentration of methanol and the pH of the aqueous mobile phase, while the salt concentration didn't show any effect on the tailing factor. As shown in (**Fig.6&7**), chromatographic conditions were optimized and reduced during numerical optimization in the Box–Behnken design to be one solution as the following: Organic phase (methanol) composition 35%, salt concentration (30mM), and pH of the aqueous mobile phase (3).

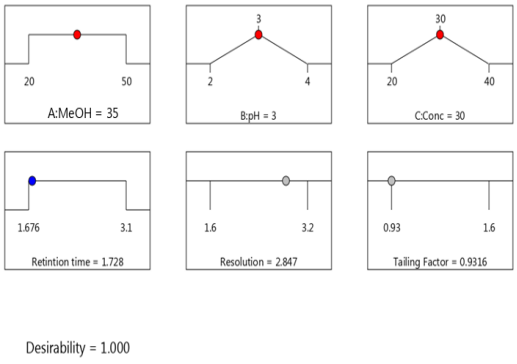


Fig.6. Numerical optimization for chromatographic conditions.

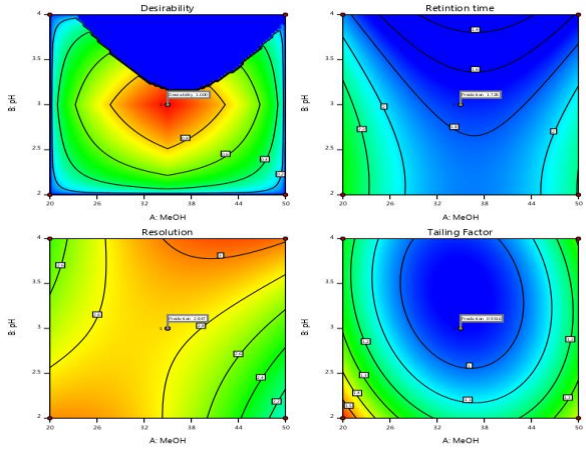


Fig.7: Numerical optimization for chromatographic conditions.

Finally, the best developing system was achieved as mentioned above and as shown in (**Fig.8**).

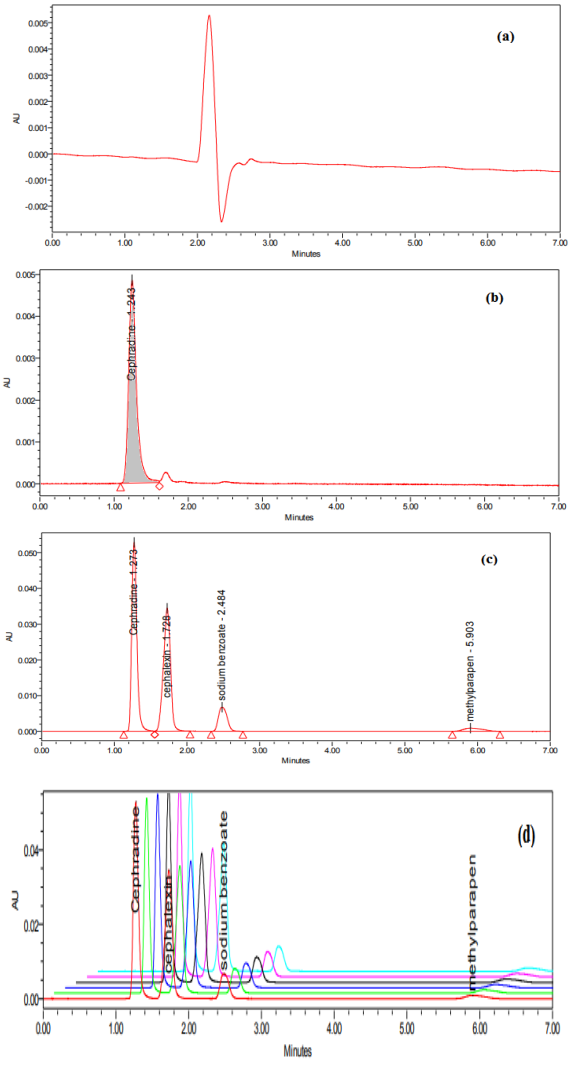


Fig.8: UPLC Chromatograms of (a) plasma blank, (b) plasma blank spiked with 25 µg/mL IS (CFR), (c) Standard solution of CFX, SB, MP spiked with IS (CFR), (d) overlay of standard solution.

3.2. Analytical Method Validation

Method validation was performed for the proposed UPLC method according to US-FDA guidelines [50].

3.2.1. Linearity and range

Linear calibration curve was achieved in the concentration range (3-35 ng/mL for CFX, 5-50 ng/mL for CFR, 3-30 ng/mL for SB and 2-25 ng/mL for MP) with coefficient of regression > 0.998. All calibration parameters are listed in (**Table 6**).

3.2.2. Lower limit of detection and quantification

The lower limits of detection (LLOD) and of quantitation (LLOQ) are calculated as LLOD=3.3×σ /S and LLOQ=10×σ /S, where σ = the standard deviation of the response, S = the slope of the calibration curve and can be estimated from the calibration curve of the analyte. The estimate of σ was carried out in three replicates of the blank of plasma, and then the standard deviation of these responses was calculated, as shown in (**Table 6**).

Table 6: Regression and validation parameters of the proposed UPLC method for determination of CFR, CFX, SB and MP.

Parameter	UPLC Method			
	CFR	CFX	SB	MP
Linear Range (ng/mL)	5-50	3-35	3-30	2-25
Wavelength (nm)	230	230	230	230
Slope	7616	6776.5	3622.9	1651.0
Intercept	9312.3	2121.1	411.56	77.14
Correlation coefficient	0.9990	0.9991	0.9993	0.9991
Repeatability	0.12	0.09	0.11	0.10
LLOD [a] (ng/mL)	1.2	0.63	0.55	0.65
LLOQ [a] (ng/mL)	3.7	1.92	1.66	1.96

3.2.3. Accuracy & precision

Accuracy of QC samples were evaluated with triplicate of three variable concentrations 3, 6 and 9 µg/mL for the studied drugs (low, middle, and high) as accuracy was represented by percentage recovery (R %) ± 15% (for QC samples) and 20% for (LLOQ). Intra-day precision was carried out by performing the analysis of spiked plasma in one day, while inter-day precision was achieved by measuring the spiked samples at three sequential days, respectively for the UPLC method and the recovery for the studied drugs were between 97.0 -103.0 %. The calculated RSD of inter and intra-day precision were less than 15% of the drugs as listed in (**Table 7**).

3.2.4. Stability

Short term (Bench-Top) stability of the drugs in human spiked plasma was evaluated at three levels of QC samples (low, middle and high) at room temperature (25°C) for 24 h, (long-term stability) showed that samples were stable when stored in the freezer at -30°C for 4 weeks, also (thaw and freeze) stability of the drugs in human spiked plasma

was estimated after three freeze-thaw cycles from frostiness at -30°C for 24 h at room temperature. The analytes were stable in plasma when compared with the freshly prepared samples and the variation was within 15%, while the mean recovery percentages were of 100 ± 15% as illustrated in (**Table 8**).

Table 7: Intra- and inter-day precision and accuracy for the determination of CFR, CFX, SB, and MP.

Added concentration (ng/mL)	Intra-day			Inter-day								
	1 day			1st day			2nd day			3rd day		
	3	6	9	3	6	9	3	6	9	3	6	9
CFR												
Mean	3.04	6.03	9.07	3.04	6.03	9.08	3.01	6.03	9.10	3.02	6.03	9.14
Recovery	101.47	100.51	100.76	101.46	100.50	100.93	100.36	100.44	100.14	100.53	100.25	101.50
RSD%	0.48	0.84	0.17	0.48	0.35	1.51	0.28	0.66	1.41	2.16	0.78	1.51
RE%	-1.47	-0.51	-0.76	-1.46	-0.50	-0.93	-0.36	-0.44	-1.14	-0.53	-0.25	-0.50
CFX												
Mean	3.08	6.01	8.98	3.08	6.01	9.10	3.06	6.01	9.02	2.98	5.99	9.04
Recovery	102.77	100.20	99.82	102.77	100.19	101.10	102.02	100.13	100.24	99.25	99.91	100.48
RSD%	0.06	0.95	0.20	0.06	0.40	2.03	0.31	0.75	0.05	0.21	0.89	1.00
RE%	-2.77	-0.20	0.18	-2.77	-0.19	-1.10	-2.02	-0.13	-0.24	0.75	0.09	-0.48
SB												
Mean	2.92	5.97	9.04	2.91	6.07	9.16	2.92	6.12	9.05	2.91	6.10	9.16
Recovery	97.47	99.55	100.43	97.15	101.22	101.80	97.28	102.02	100.60	97.02	101.61	101.77
RSD%	0.50	0.16	0.47	0.12	0.25	1.05	0.48	1.38	0.99	0.41	1.63	1.44
RE%	2.53	0.45	-0.43	2.85	-1.22	-1.80	2.72	-2.02	-0.60	2.98	-1.61	-1.77
MP												
Mean	3.03	5.98	9.01	2.98	5.98	8.96	2.95	5.98	8.97	2.96	5.99	8.97
Recovery	101.11	99.68	100.15	99.47	99.65	99.57	98.42	99.72	99.62	98.51	99.83	99.72
RSD%	3.14	0.35	0.54	0.91	0.11	0.85	0.15	0.01	0.64	0.89	0.18	0.29
RE%	-1.11	0.32	-0.15	0.53	0.35	0.43	1.58	0.28	0.38	1.49	0.17	0.28

Table 8: Stability of the studied drugs under different conditions.

Added concentration (ng/mL)	Three freezes–thaw cycles			Room temperature for 24 h			Long term after 4 weeks at -30 °C		
	3	6	9	3	6	9	3	6	9
CFR									
Mean	2.99	6.02	9.04	2.99	6.03	9.02	3.02	6.02	9.01
Recovery	99.78	100.31	100.48	99.76	100.54	100.19	100.72	100.30	100.11
RSD%	1.61	0.15	1.39	2.18	0.33	0.91	0.51	0.35	0.94
RE%	0.22	-0.31	-0.48	0.24	-0.54	-0.19	-0.72	-0.30	-0.11
CFX									
Mean	3.04	6.00	8.98	3.09	6.01	8.99	3.07	6.00	8.97
Recovery	101.36	99.97	99.82	102.98	100.23	99.94	102.42	99.96	99.63
RSD%	1.78	0.17	0.43	1.53	0.37	0.36	0.56	0.39	0.21
RE%	-1.36	0.03	0.18	-2.98	-0.23	0.06	-2.42	0.04	0.37
SB									
Mean	2.92	6.10	9.15	2.91	6.13	9.19	2.92	6.10	9.17
Recovery	97.28	101.73	101.66	96.94	102.21	102.08	97.41	101.71	101.90
RSD%	0.68	0.32	0.34	0.43	0.69	0.19	0.55	0.72	0.39
RE%	2.72	-1.73	-1.66	3.06	-2.21	-2.08	2.59	-1.71	-1.90
MP									
Mean	2.95	5.98	8.99	2.99	5.99	9.02	2.96	5.98	8.92
Recovery	98.41	99.74	99.93	99.67	99.79	100.17	98.70	99.71	99.12
RSD%	0.34	0.13	0.43	1.53	0.06	0.10	0.02	0.10	0.30
RE%	1.59	0.26	0.07	0.33	0.21	-0.17	1.30	0.29	0.88

3.2.5. Formulation assay

The developed method was determined by testing six samples from the dosage form using CFR as internal standard. The obtained results were included in (Table 9) indicating that the method is selective for the samples without interference from the excipients.

3.2.6. System suitability

System suitability is the established criteria that must be met by the method to accept the results that are generated. Precision, Theoretical Plates and Tailing Factor must be met at the beginning of the analysis, if specified in the method of analysis. Precision requirement can either be met at the beginning or it can be shown to be met throughout the run. All calculated parameters were found within the acceptable limits indicating good selectivity of the RP-UPLC method as listed in Supporting Information (**Table 10**).

Table 9. Assay results for the determination of CFR spiked with CFX, SB and MP in their dosage form by the proposed methods.

Pharmaceutical formulation	UPLC				Limit %
	CFR	CFX	SB	MP	
Lexin 125 mg POS	100.54	100.68	102.07	101.50	**90-120%**
	99.86	100.46	101.24	97.84	
	100.28	97.72	101.10	98.58	
	100.61	102.40	100.90	100.89	
	99.43	101.00	102.02	101.86	
	100.26	100.54	101.16	101.60	
Mean ± RSD	100.16±0.44	100.47±1.52	101.42±0.49	100.38±1.72	

Table 10. System suitability testing parameters of the developed methods.

Item	UPLC				Reference values
	CFR	CFX	SB	MP	
Tailing factor	1.3	0.9	1.3	1.2	T ≤ 2
Injection precision	0.9	0.7	0.8	0.7	RSD ≤1%
Number of theoretical plates (N)	2625	2800	2197	2084	N > 2000
Resolution	-	2.8	3.8	9.6	Rs>2
Retention time (R_t)	0.04	0.07	0.08	0.1	RSD ≤1%

Conclusion

Efficient and novel chromatographic method was developed and validated as per US-FDA guidelines for simultaneous determination of the quaternary mixture CFR, CFX, SB, and MP in their pharmaceutical dosage forms, wastewater of poultry Slaughter Company and in rabbit plasma. Application of Box-Behnken (BBD) was achieved to reduce the total experimental trials required for the optimization of UPLC and the robustness of the system can be used for regular routine analysis and stability study. The proposed method was demonstrated to achieve shorter time, high sensitivity and saves much of the cost of analysis and consumable reagents.

Conflicts of interest

"There are no conflicts to declare".

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