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ORIGINAL RESEARCH PAPER

Analytical quality by design approach for the control of potentially counterfeit chloroquine with some NSAIDs using HPLC with fluorescence detection in pharmaceutical preparation and breast milk

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ABSTRACT

Chloroquine phosphate (CQ) the antimalarial drug and suggested to treat the pandemic disease coronavirus (COVID-19) is often adulterated with some of the non-steroidal anti-inflammatory drugs (NSAIDs) such as paracetamol, aspirin (ASP), or both. The purpose of this study is to detect such counterfeited drugs, using a reversed phase high pressure liquid chromatography (RP-HPLC) method with fluorescence detection. Analysis was divided into three phases. In the first phase, a Plackett-Burman design (PBD) was used to screen five independent factors, namely, buffer pH, buffer concentration (mM), acetonitrile content (%), flow rate (mL/min) and triethylamine (TEA) content in the buffer preparation (%). The selected dependent variables were (resolution, symmetry of peaks and run time). The objective of the second phase was to optimize the method performance using Box-Behnken design (BBD) and desirability function for multiple response optimization to obtain the best chromatographic performance with the shortest run time. Optimal chromatographic separation was achieved on a YMC-pack pro C18 ODS-A column (15 cm × 4.6 mm, 5 μm) at room temperature. The optimum mobile phase consisted of acetonitrile and 5 mM sodium dihydrogen phosphate buffer containing 0.5% triethylamine (30:70, v/v) with the pH adjusted to 3.5 using an orthophosphoric acid solution. The flow rate was maintained at 1 mL/min, and the detection was performed with a fluorescence detector fixed at 380 nm ($\lambda_{\text{emission}}$) after excitation at 335 nm ($\lambda_{\text{excitation}}$). The third phase was method validation according to ICH guidelines, providing to be specific, precise, accurate, and robust. The method is linear over a range of 0.4–8 μg/mL for chloroquine and ASP, while for paracetamol it is linear over 16–48 μg/mL. The developed RP-HPLC method was used for quantitation of the three drugs in chloroquine dosage form samples. The method shows a great tendency in the classification between the genuine chloroquine and the adulterated ones in pharmaceutical preparations and breast milk.

KEYWORDS

breast milk, chloroquine phosphate, counterfeit, desirability function, fluorescence detection, quality by design

INTRODUCTION

Chloroquine phosphate (CQ); N⁴-(7-chloroquinolin-4-yl)-N¹, N¹-diethylpentane-1,4-diamine bis (dihydrogen phosphate) [1], is used to treat malaria and recently under investigations against COVID-19 [2–4]. Paracetamol (PAR); N-(4-hydroxyphenyl) acetamide [1], and aspirin (ASP); 2-(acetyloxy) benzoic acid [1], are both non-steroidal anti-inflammatory drugs (NSAIDs) that are used to relieve pain and reduce fever and inflammation.

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The World Health Organization (WHO) anticipated that almost half of the worldwide pharmaceutical market is involved with substandard drugs [5]. Among the most important drugs that are involved in such a problem are antimalarial drugs. In some developing African nations, the available malaria drugs are expired, substandard or fake [6]. Besides, some of the inappropriate antimalarial drugs in Thailand that were given to people as a medication contained a significant percentage of analgesic drugs (PAR and ASP) instead of the antimalarial agent [7] because of their lower price, and their antipyretic effect. It has been reported that ASP was used in the manufacture of fake CQ in Africa [8]. Unfortunately, the adulterated CQ with ASP is not only considered as a false fake treatment, but it is also thought to be an important contributor to acidosis in children with malaria and a cause of Reye's syndrome [9] to both children and infants if the drug is administered by a nursing mother. From this rising concern of safety, the detection of the studied drugs in breast milk was determined. PAR passes into milk in greater quantities compared to CQ and ASP as the protein binding of PAR appears at concentration greater than 60 µg/mL with percentage ranging from 15 to 21% [10]. While the protein binding of CQ and ASP is ranging from 50 to 67% for CQ [11] and 58.3% for ASP [12].

In the literature, CQ has been determined in potentially counterfeited anti-malarial drugs either by assessing CQ alone or in mixtures using a colorimetry and refractometry methods [13], and HPLC [14–17]. However, to the best of our knowledge, the simultaneous determination of CQ and the adulterants PAR and ASP in pharmaceutical preparations and breast milk has not been reported. The possibility of finding a combination of the two adulterants in the same counterfeit CQ drug is high [18]. Therefore, developing a method for simultaneous screening of the three compounds in a single run is necessary and will help to decrease the threat of counterfeit drugs.

Fluorescence detection in liquid chromatography is a powerful method that not only complements conventional UV absorption methods, but also, in many instances, achieves specificity that make it a more desirable method of adulteration detection [19]. In the mixture under study, all the three drugs possess native fluorescence.

Implementing quality by design approach is a fast method for optimizing the critical parameters in a relatively small number of experiments [20–22]. The experimental design for optimization process begins with a screening phase to assess the key variables influencing the response out of a large number of possible factors [23], consequently the response surface methodology with the desirability function [24–25] is used to estimate the relation between the studied responses and these key variables.

The objective of this study was to develop a simple and rapid reversed phase high pressure liquid chromatography (RP-HPLC) method with fluorescence detection by studying and optimizing the chromatographic separation with the aid of quality by design approach to simultaneously quantify CQ with its adulterants PAR and ASP to assist low-income

nations in distinguishing fake and substandard CQ drug in pharmaceutical preparation and breast milk.

EXPERIMENTAL

Material and reagents

The pharmaceutical-grade CQ, PAR and ASP used in this study were certified to be 99.7, 99.8 and 99.86% pure, respectively. CQ was supplied by Egyptian International Pharmaceutical Industries Company in Egypt, PAR was obtained from Hikma Pharma Company, and ASP was obtained from ADWIC (Egypt). Alexoquine® [labelled to contain 250 mg of CQ, B. N. 7107003] was purchased from a local drug store. Sodium dihydrogen phosphate, triethylamine (TEA), and orthophosphoric acid were supplied by ADWIC; (Egypt). Acetonitrile (HPLC grade) was purchased from Sigma-Aldrich (Germany).

Instrumentation

Chromatographic analyses were performed using an Agilent 1200 HPLC system with fluorescence detector model (G1321A). Samples were loaded into a manual Rheodyne injector (model G1328B, USA) equipped with an isocratic pump (G1310A) and an Eclipse C18 column (150 mm × 4.6 mm × 5 µm). The mobile phase was filtered through 0.45 µm membrane filters (Millipore, USA). The fluorescence detector was set at 380 nm ($\lambda_{\text{emission}}$) with excitation at 335 nm ($\lambda_{\text{excitation}}$). All determinations were made at room temperature.

A double-beam UV-visible spectrophotometer (Shimadzu, model: UV-1601 PC, Japan) was used to measure the absorbance spectrum of CQ, PAR and ASP in a 1 cm quartz cell over the range of 200–800 nm at room temperature.

Software

Statistical analysis of the data obtained from factor screening, optimization and method development calculations were performed using MODDE® 12.1 software.

Design of experiment

Plackett-Burman design (PBD) with five variables was used for screening the significant and non-significant variables. This design was selected based on the small number of experiments required. The dependent, independent variables and levels selected for the screening procedure are listed in Table 1. For method optimization, Box-Behnken design (BBD) was used with the 3 variables that were significant at the 95% confidence interval according to the ANOVA results obtained from PBD. The variables and levels selected for the optimization procedure were buffer pH (A; 3, 3.5, and 4), acetonitrile content (C; 20, 25, and 30%) and flow rate (D; 0.8, 1 and 1.2 mL/min). The resolution between peaks with run time were chosen as the responses for the separation of the compounds in the optimization phase.

Table 1. Variables used for the Screening testing

Independent variables	Lower value (−1)	Nominal value (0)	Upper value (1)	Constraints
A: Buffer pH	3	3.5	4	In the range
B: Buffer concentration (mM)	5	10	15	In the range
C: Acetonitrile content (%)	20	25	30	In the range
D: Flow rate (mL/min)	0.8	1	1.2	In the range
E: TEA content in buffer preparation (%)	0	0.5	1	In the range
Dependent variables				Constraints
Y ₁ : The resolution between CQ and PAR peak				Not less than 2
Y ₂ : The resolution between PAR and ASP peak				Not less than 2
Y ₃ : The symmetry of CQ peak				0.9–1.1
Y ₄ : The symmetry of PAR peak				0.9–1.1
Y ₅ : The symmetry of ASP peak				0.9–1.1
Y ₆ : Run time				2–4 min

Because of the nonlinearity of the BBD, a polynomial equation containing second-order model is given in Eq. (1).

$$Y_i = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 \quad (1)$$

where Y_i is the predicted response; β_0 is the model constant; X_1 , X_2 and X_3 represents the factors; β_1 , β_2 and β_3 is the regression coefficient calculated by the model by considering the average response of changing one variable at a time from its lower to higher level. The interaction terms: β_{12} , β_{13} and β_{23} demonstrates how the response changes when two variables are simultaneously changed; and β_{11} , β_{22} and β_{33} are the quadratic coefficients added to consider the nonlinearity.

Thus, for multiple response optimization of resolution and run time Derringer's desirability function was used. The values desirable to optimize one response differ than the other, therefore the desirability function combines the individual desirability for each response into an overall desirability function (D) for an optimal solution. Each response is related with its partial desirability function (d_i), where the desirable scale for each response ranges from zero to one, an undesirable response is assigned the value 0 and a desirable response value ranges between 0 and 1, depending on the closeness of the response to the sited target value.

When the target value T_i is desired for optimization, the desirability values are calculated using Eq. (2)

$$d_i = \begin{cases} \left[\frac{Y_i - Y_{\min}}{T_i - Y_{\min}} \right]^{wt_i} & \text{if } (Y_{\min} < Y_i < T_i) \\ \left[\frac{Y_i - Y_{\max}}{T_i - Y_{\min}} \right]^{wt_i} & \text{if } (T_i < Y_i < Y_{\max}) \end{cases} \quad (2)$$

Y_i is the predicted response obtained by the model, $Y_{\min}$ and $Y_{\max}$ represent the highest and lowest possible response and Wt_i represents the weight, the weight is adjusted by the analyst according to the importance of Y_i to be close to the desired value. Where, d_i is equal to zero when Y_i is less than $Y_{\min}$ or greater than $Y_{\max}$ and is equal to one when Y_i is equal to the target value. After the individual desirability

values are obtained, the goals are combined as a geometric mean to give an overall desirability value using Eq. (3).

$$D = (d_1 \times d_2 \times \dots \times d_n)^{1/n} \left(\prod_{i=1}^n d_i \right)^{1/n} \quad (3)$$

where, n represents the number of responses for optimization.

Chromatographic conditions

The optimal chromatographic separation of all the analytes was achieved with acetonitrile and 5 mM sodium dihydrogen phosphate buffer containing TEA (0.5% of the volume of the buffer) in a 30:70 ratio (v/v) with the buffer pH adjusted to 3.5 with orthophosphoric acid solution. The flow rate was maintained at 1 mL/min, and the injected volume was 20 μ L for all chromatographic runs. The detection was performed with a fluorescence detector fixed at 380 nm ($\lambda_{\text{emission}}$) after excitation at 335 nm ($\lambda_{\text{excitation}}$).

Preparation of the standard working solutions

A standard stock solution of each drug (0.1 mg/mL) was prepared by dissolving 10 mg of CQ in water and 10 mg of PAR and ASP in acetonitrile in 100 mL volumetric flasks and bringing the solutions to volume with the appropriate solvent. Fresh working solutions in the concentration range of 0.4–8 μ g/mL for CQ and ASP and 16–48 μ g/mL for PAR were prepared by diluting the standard stock solutions in the mobile phase.

Standard solutions of CQ and ASP with 8 and 40 μ g/mL PAR were freshly prepared in the appropriate solvent for screening and optimization.

Sample preparation of pharmaceutical dosage form

Ten tablets of Alexoquine[®] [labelled to contain 250 mg CQ] were accurately weighed and ground to fine powders. An accurate weight of powder equivalent to 10 mg CQ was transferred into a 100 mL volumetric flask, sonicated in 50 mL of distilled water for 15 min, and brought to volume

with the same solvent. The solution (100 µg/mL CQ) was filtered and further diluted to the desired concentration and made up to 25 mL with the mobile phase. The concentration of CQ in the tablet was calculated from a previously prepared calibration curve.

Determination of CQ in adulterated tablets by the standard addition technique

Standard addition technique was applied by adding different known concentrations of the pure CQ and adulterants; PAR and ASP to different known concentrations of CQ dosage form following the procedures mentioned above. The added concentrations for each drug were 2, 4, 8 µg/mL of both CQ, ASP and 20, 40, 80 µg/mL of PAR. The concentrations were calculated using the computed regression equations.

Sample preparation of the spiked human breast milk

Drug free breast milk samples were obtained from a healthy volunteer and the appropriate microliters of the stock solution of CQ, PAR and ASP were added to 1 mL of the breast milk. The added concentrations for each drug of 2, 4, 6 µg/mL of both CQ, ASP and 20, 40, 60 µg/mL of PAR. For deproteination, equal amounts of acetonitrile were added and centrifuged at 5,000 g for 10 min. Clear supernatant was diluted with water and injected for HPLC analysis under the optimized conditions. The concentrations were calculated using the computed regression equations.

Method validation

The proposed method was validated according to the guidelines set by the International Conference on Harmonization (ICH Q2R1) [26]. The evaluated parameters were, system suitability parameters, linearity of the calibration curve, specificity, limit of detection (LOD), limit of quantification (LOQ), precision, accuracy and robustness which was demonstrated using PBD. The RP-HPLC method for the determination of CQ and ASP was linear over a concentration range of 0.4–8 µg/mL and from 16–48 µg/mL for PAR. The LOD and LOQ were calculated as $LOD\ 3\sigma/S$ and $LOQ\ 10\sigma/S$, where σ is the standard deviation of the intercept and S is the slope. To test the prediction performance of the proposed method, the intra-day (repeated three times within the same day with the same conditions) and inter-day (repeated three times on three successive days) studies were performed at three different concentrations (4, 5 and 6 µg/mL for CQ and ASP) and (24, 28 and 32 µg/mL for PAR). The accuracy of the method was determined by a recovery study using the three compounds at the same concentrations used for precision analysis. PBD was used to determine robustness and the effect of 5 factors at different levels at 95% confidence interval. The 5 factors were, buffer pH (± 0.1 pH unit), buffer concentration (± 2 mM), acetonitrile content ($\pm 1\%$), flow rate (± 0.1 mL/min) and TEA content in the buffer preparation ($\pm 0.2\%$). System suitability parameter test performed according to USP37 [27],

involving injecting the standard solution six times, and was conducted to achieve adequate peak separation.

RESULTS AND DISCUSSION

The proposed method utilized an experimental design to optimize the conditions of the chromatographic separation between CQ, PAR and ASP. The optimization was carried out by using two designs: PBD to evaluate which variables were significant and BBD which had a great role in enhancing the performance of the chromatographic separation of the ternary mixture in a few steps obtaining a decrease in the retention time.

To adjust the excitation wavelength, a UV-visible spectrometer was first used to measure the absorbance spectrum of CQ, PAR and ASP individually from 200 to 800 nm. The wavelength that gave the maximum absorbance for the three drugs was expected to be near the excitation wavelength. Trial and error measurements around this wavelength resulted in the selection of 335 nm as the excitation wavelength for maximum emission. The emission wavelengths were detected over a range 350–600 nm, and CQ, PAR and ASP shows maximum emission at 380 nm.

Screening phase, using Plackett-Burman design (PBD)

PBD was used to evaluate the main effects of the 5 variables on the selected responses. The experimental design matrix used, and the results obtained by PBD are listed in Table 2. ANOVA test was applied to screen the significant from non-significant factors as shown in Table 3. The coefficient values obtained elucidate the impact of a factor in the model on the response. According to the coefficient values in Table 3, the resolution and run time are mostly affected by the acetonitrile content.

From the preliminary studies, Y_1 (resolution between CQ and PAR) and Y_3 (peak symmetry of CQ) through the different runs were not affected by any of the levels of the studied factors in a significant way obtaining a minor change in the results. On the other hand, Y_2 (resolution between PAR and ASP) was significantly affected by buffer pH and the percentage of organic solvent, it was significantly noticed that the retention time of ASP was the main influence.

The retention time of ASP was shortened with the increasing of pH, which may be explained by the ionized state of ASP. Being a weak acid with pKa 3.5 [28], ASP will be in the ionized form at any pH above 3.5. Therefore, ASP will become more hydrophilic and will be eluted faster in the RP-HPLC [29].

Y_2 (resolution between PAR and ASP) was also affected by the acetonitrile content which is probably due to the high eluting power of acetonitrile. TEA was added as an organic modifier to enhance the symmetry of the peaks by decreasing the tailing effect [30], TEA addition affected Y_4 (the peak symmetry of PAR) and Y_5 (the peak symmetry of ASP) positively. Moreover, the symmetry of PAR was also affected by acetonitrile content and the flow rate.

Table 2. The plan of Plackett-Burman design and experimentally obtained results

Run	A	B	C	D	E	Y ₁	Y ₂	Y ₃	Y ₄	Y ₅	Y ₆
1	1	−1	−1	1	−1	7.22	22.80	1.09	0.85	1.2	4.71
2	1	1	−1	−1	1	5.99	18.91	0.73	0.91	0.74	4.70
3	1	1	1	−1	−1	6.12	8.44	0.81	0.88	1.2	4.71
4	−1	1	1	1	−1	8.45	28.85	1.1	0.87	0.8	7.50
5	1	−1	1	1	1	5.47	4.36	0.86	1.04	0.82	2.90
6	−1	1	−1	1	1	6.87	26.00	0.70	0.91	0.91	6.30
7	−1	−1	1	−1	1	6.30	17.00	0.70	0.91	0.91	6.00
8	−1	−1	−1	−1	−1	6.16	24.80	1.00	0.60	1.79	10.25
9	0	0	0	0	0	6.16	19.80	0.93	0.92	0.83	4.83
10	0	0	0	0	0	6.25	19.10	0.93	0.92	0.82	4.83
11	0	0	0	0	0	6.83	19.00	0.93	0.93	0.82	4.83

The shaded part is the coded level pattern of Plackett-Burman design.

A: buffer pH, B: buffer concentration (mM), C: acetonitrile content (%), D: flow rate (mL/min), E: TEA content in the buffer preparation (%), Y₁: the resolution between CQ and PAR peak, Y₂: the resolution between PAR and ASP peak, Y_{3,4,5} the symmetry of CQ, PAR and ASP peaks, respectively and Y₆ is the run time.

Finally, the pH, acetonitrile content and flow rate influenced the run time. It was observed that any increase in the pH, acetonitrile content or flow rate will shorten the run time and vice versa.

Optimization phase, using BBD and desirability function

The BBD designs is used to elucidate the effects of interactions among variables and optimize the operating conditions of the experimental method by generating a response surface map that indicates the optimum conditions [31–32]. Ferreira et al. [33] and Bosque-Sendra et al. [34] described the advantages of BBD over other designs. Three independent variables were chosen to be optimized based on their significance on the selected responses. The selected responses were, resolution, run time and were chosen in the optimization phase based on their importance in chromatographic separation [35–36]. Y₁ (the resolution between CQ and PAR) was excluded as a response as it was not affected by any of the independent variables in the screening phase. The experimental design matrix used, and the results obtained by BBD are listed in Table 4. BBD was applied to

evaluate the main effects and the interactions among the three selected factors. All other factors were kept fixed at their optimum and minimum levels during the experiment (TEA content was 0.5% and the buffer concentration was 5 mM). Data of Y₂ and Y₆ were observed, the fitted model was quadratic. The developed regression equation is given in Eqs. (4) and (5).

The resolution between PAR and ASP is:

$$(Y_2) = 8.31 - 5.65A - 5.11C - 0.98D + 1.91AC + 0.28AD + 0.81CD + 2.64A^2 + 0.26C^2 + 0.68D^2 \quad (4)$$

Run time is:

$$(Y_6) = 2.68 - 0.80A - 0.75C - 0.65D + 0.34AC + 0.17AD + 0.15CD + 0.38A^2 + 0.13C^2 + 0.29D^2 \quad (5)$$

Interaction between factors

Other factor that contributes in developing the suggested design space of the model namely the interaction between factors. The term interaction is illustrated as the effect of a

Table 3. ANOVA results for Plackett-Burman design and 5% level of significance for the factors

Factors	Y ₁		Y ₂		Y ₃		Y ₄		Y ₅		Y ₆	
	Coeff.	P	Coeff.	P	Coeff.	P	Coeff.	P	Coeff.	P	Coeff.	P
A	−0.01	0.96	−2.55	0.03*	0.13	0.18	0.03	0.07	−0.007	0.90	−0.70	0.02*
B	0.28	0.43	0.87	0.34	−0.02	0.73	0.02	0.14	−0.11	0.09	0.03	0.87
C	−0.45	0.22	−5.00	6 × 10 ^{−4} *	−0.07	0.34	0.05	9 × 10 ^{−3} *	−0.10	0.11	−0.99	3 × 10 ^{−3} *
D	−0.34	0.39	−0.49	0.61	0.03	0.68	0.04	0.01*	−0.07	0.26	−0.96	6 × 10 ^{−3} *
E	0.09	0.78	−0.05	0.95	−0.02	0.74	0.04	0.02*	−0.14	0.03*	−0.25	0.30

A: buffer pH, B: buffer concentration (mM), C: acetonitrile content (%), D: flow rate (mL/min), E: TEA content in the buffer preparation (%), Y₁: the resolution between CQ and PAR peak, Y₂: the resolution between PAR and ASP peak, Y_{3,4,5} the symmetry of CQ, PAR and ASP peaks, respectively and Y₆ is the run time.

Coeff.: Is the coefficient values obtained by the model.

*P value less than 0.05 indicates a significant factor at 95% confidence interval.

Table 4. The plan of Box-Bhenken design and experimentally obtained results

Run	A	C	D	Y ₂	Y ₆
1	–1	–1	0	23.80	5.06
2	1	–1	0	8.94	2.85
3	–1	1	0	9.70	2.88
4	1	1	0	2.48	2.05
5	–1	0	–1	18.66	5.06
6	1	0	–1	6.50	3.01
7	–1	0	1	16.22	3.36
8	1	0	1	5.20	2.01
9	0	–1	–1	16.21	4.68
10	0	1	–1	4.40	2.81
11	0	–1	1	12.51	3.10
12	0	1	1	3.96	1.87
13	0	0	0	8.52	2.70
14	0	0	0	8.26	2.68
15	0	0	0	8.16	2.68

The shaded part is the coded level pattern of Box-Bhenken design.

A: buffer pH, C: acetonitrile content (%), D: flow rate (mL/min), Y₂: the resolution between PAR and ASP peak and Y₆ is the run time (min).

AC interactions

DC interactions

AD interactions

Fig. 1. Interaction plots demonstrating the relationship between A: buffer pH, C: acetonitrile content (%), D: flow rate (mL/min) on Y₂; resolution between PAR and ASP and b) Y₆; run time (min)

change in the level or value of one variable on the mean of the response depends upon the level or estimation of another [37].

In Eq. (4), the most impacted interaction coefficient on Y_2 is the interaction between buffer pH and acetonitrile content (AC), followed by the interaction between acetonitrile content and flow rate (CD), then the interaction between buffer pH and flow rate (AD), where the first two interactions were significant. The interpretation of AC interaction according to the study reveals that changing the buffer pH from high to low level results in a rapid increase in Y_2 both at the lowest and highest levels of acetonitrile content. The interaction CD can be illustrated as changing acetonitrile content from high to low results in a rapid increase in Y_2 both at the lowest and highest levels of flow rate. Therefore, according to the model to obtain the highest Y_2 , set all the factors at its lowest levels.

In Eq. (5), the interaction between all factors are significant. The interactions AC, CD and DC illustrated before in Eq. (4), but instead of Y_2 the response is Y_6 (run time) and in this situation the run time favoured to be shortened. To get a better understanding, the interactions plots are presented in Fig. 1. To compromise between the two responses, Derringer's desirability function was used. Derringer's desirability function allows simultaneous maximization of resolution with minimization of run time by setting the

desired target values for both responses. In case of multiple response optimization where the number of factors and responses is large and the value for each response is localized in different region, using the graphical optimization plots would be difficult to interpret.

The derringer's desirability function revealed the experimental conditions (factor levels) that corresponds to the minimum retention time with good resolution between peaks. Fig. 2 represents the desirability design space contour plots showing the robust set point suggested by the design according to the targeted values and weight to obtain the highest overall desirability (D) value and at the same time robust. The optimum conditions for the determination of CQ, PAR and ASP using the experimental design and the desirability function applied were; acetonitrile and 5 mM sodium dihydrogen phosphate buffer containing TEA (0.5% of the volume of the buffer) in a 30:70 ratio (v/v) with the buffer pH adjusted to 3.5. The flow rate was maintained at 1 mL/min. The deduced conditions were obtained from a relatively low number of experiments compared to that required by a traditional stepwise approach. The results showed that the run time was 2.73, which was within the 2–4 min. The resolution between peaks were greater than 2 were Y_1 and Y_2 obtained were 3.97, 5.81, respectively. The symmetry of peaks (Y_3 , Y_4 and Y_5) were 1.07, 0.92, and 0.98 for CQ, PAR and ASP, respectively, which were within 1.1 and 0.9.

Fig. 2. Contour plot of the overall desirability in the design space of the chromatographic factors: acetonitrile content (%) and buffer pH at flow rate 8.87 (mL/min) (a), acetonitrile content (%) and flow rate (mL/min) at buffer pH 3.65 (b), buffer pH and flow rate (mL/min) at acetonitrile content (%) 28.5 (c) with indication of probability of failure. Fixed factors: 0.5% TEA content and 5 mM buffer concentration

Fig. 3. Comparison between the predicted value by Box-Bhenken design and the actual experimental values of the two selected responses a) Y_2 ; resolution between PAR and ASP and b) Y_6 ; run time (min)

Validation of the optimized factors

The experimental model is well fit by the polynomial equations. The determination coefficient (R^2) in any model explains to which degree the independent variables in the model affects the dependant variables by measuring their variance. The model showed sufficiently good values of R^2 and adjusted R^2 within the acceptable limits ≥ 0.80 [38]. The determination coefficients (R^2) obtained were 1 and 0.999 and for the adjusted R^2 were 0.999 and 0.996 for the two responses Y_2 (resolution between PAR and ASP) and Y_6 (run time), respectively. In addition, reasonable prediction determination coefficients were obtained which were equal to 0.994, 0.977 for Y_2 (resolution between PAR and ASP) and Y_6 (run time), respectively, showing that the prediction performance of the proposed model is appropriate. As a result, there was a good agreement between the experimentally measured and expected values that indicates the high reliability and the validity of the experimental values, as shown in Fig. 3.

Chromatographic method validation

System suitability. The examined parameters were, the resolution between adjacent peaks, the peak symmetry, and the number of theoretical plates. The obtained results showed good system suitability, were the resolution between peaks (Y_1 and Y_2) were 3.97, 5.81, respectively. The symmetry factor of the three peaks (Y_3 , Y_4 and Y_5) were 1.07, 0.92, and 0.98 for CQ, PAR and ASP, respectively. Besides, the number of theoretical plates were 3,918.69, 8,243.12, 12,889.89 for CQ, PAR and ASP, respectively.

Table 5. Validation parameters obtained by applying the proposed HPLC method for the determination of CQ, ASP and PAR

Parameters	CQ	PAR	ASP
Concentration range, $\mu\text{g/mL}$	0.4–8	16–48	0.4–8
LOD*, $\mu\text{g/mL}$	0.414	2.556	0.371
LOQ*, $\mu\text{g/mL}$	1.255	7.746	1.126
Correlation coefficient (r)	0.9995	0.9996	0.9996
Slope	2.924	0.781	3.933
Intercept	0.676	6.905	0.157
SD of the residuals ($S_{y/x}$)	0.181	1.871	0.218
SD of the intercept (S_a)	0.367	0.605	0.443
SD of the slope (S_b)	0.077	0.018	0.093
Precision:			
Intra-day ^a			
% RSD	0.31,0.38,0.45	0.89,0.43,1.81	0.18,1.05,0.19
Inter-day ^b			
% RSD	1.40,1.61,1.81	1.54,1.66,1.74	1.60,1.34,1.68
Accuracy:			
% Recovery ^c	101.94,101.29,101.63	99.99,99.30,99.40	99.21,99.18,99.19

*The LOD and LOQ were calculated as $\text{LOD } 3x\sigma/S$ and $\text{LOQ } 10x\sigma/S$, where σ is the standard deviation of the intercept and S is the slope.

^aThe intra-day ($n = 3$), average of three concentrations of the standard solution of CQ and ASP (4,5,6 $\mu\text{g/mL}$), and PAR (24,28,32 $\mu\text{g/mL}$), repeated three times within the day.

^bThe inter-day ($n = 3$), average of three concentrations of the standard solution of CQ and ASP (4,5,6 $\mu\text{g/mL}$), and PAR (24,28,32 $\mu\text{g/mL}$), repeated three times in three successive days.

^cThe average % recovery ($n = 3$), of three concentrations of the standard solution CQ and ASP (4,5,6 $\mu\text{g/mL}$), and PAR (24,28,32 $\mu\text{g/mL}$).

Fig. 4. HPLC chromatogram of a laboratory prepared mixture of CQ (8 $\mu\text{g/mL}$, 1.77 min), PAR (40 $\mu\text{g/mL}$, 2.17 min) and ASP (8 $\mu\text{g/mL}$, 2.73 min)

Specificity. The specificity of method was analysed, and no interference of active drug materials was observed by performing separate injections of standard, placebo, and mobile phase, respectively.

Linearity and range. The method was linear over a concentration range of 0.4–8 $\mu\text{g/mL}$ for CQ and ASP, while the linear concentration range for PAR was 16–48 $\mu\text{g/mL}$. The high correlation coefficient confirms the good linearity of the relationship, which were, 0.9995, 0.9996 and 0.9996 for CQ, PAR and ASP, respectively. The parameters of the regression line were, the standard deviation of the slope (S_b), intercept (S_a) and residuals ($S_{y/x}$) confirmed the minimal widespread deviations in the estimation, which demonstrates acceptable accuracy with low deviations in the calibration points as shown in Table 5.

Accuracy and precision. The accuracy, inter-day and intra-day precision of the proposed method were determined by analysing standard solutions of CQ and ASP at concentrations of 4, 5 and 6 $\mu\text{g/mL}$, and satisfactory recovery percentages ranged from 99.18 to 101.94% were obtained. For PAR, the accuracy and precision were determined at concentrations of 24, 28 and 32 $\mu\text{g/mL}$ and the recovery percentages ranged from 99.30 to 99.99% showing good accuracy of the method. The inter-day and intra-day precisions were expressed as (RSD, %) and did not exceed 2%, indicating the high reproducibility of the results and the precision of the method.

Limits of detection (LOD) and quantitation (LOQ). The LOD and LOQ were determined according to the ICH guidelines. The LOD were found to be 0.41, 2.55 and 0.37 $\mu\text{g/mL}$, while LOQ were 1.25, 7.74 and 1.12 $\mu\text{g/mL}$ for CQ, PAR and ASP, respectively, proving the high sensitivity of the developed method.

Robustness using Plackett-Burman design (PBD). A second level PBD was used to determine the effect of 5 factors in

only 11 experiments. The robustness was assessed at buffer concentration and TEA volume although they gave non-significant results in the screening step to evaluate if an error occurred in the preparation of the buffer could affect the resolution between PAR and ASP (Y_2) and the run time (Y_6). The model was found to be statistically non-significant ($P > 0.05$) at 95% confidence interval.

Application of the proposed method for analysis of adulterated CQ samples in pharmaceutical preparations

A tablet of the commercial pharmaceutical product Alexoquine[®], labelled to contain 250 mg CQ, was treated according to the procedure described in the sample preparation section. Samples of CQ tablet formulation were analysed by the developed method. It was found that the commercially analysed tablets contained pure CQ; where no peaks that correspond to the 2 adulterants were found indicating 0% PAR and 0% ASP. To assess the accuracy of the method in determination of the three analytes

Table 6. Results obtained by applying the proposed method for the determination of CQ in Alexoquine[®] tablets and standard addition technique of CQ, PAR and ASP

Sample number	Authentic added, $\mu\text{g/mL}$			Recovery % of found ^a , $\mu\text{g/mL}$		
	CQ	PAR	ASP	CQ	PAR	ASP
1	2	20	2	100.00	99.13	99.00
2	4	40	4	99.75	99.48	97.00
3	8	80	8	100.86	102.35	100.19
Mean $\pm$ RSD				100.20 ± 0.57	100.32 ± 1.76	99.59 $\pm$ 0.84
Found of CQ in Alexoquine [®] [B. N. 7107003]labelled to contain 250 mg CQ				100 $\pm$ 1.98	0%	0%

^a Average of three determinations.

Fig. 5. HPLC chromatogram of spiked breast milk with CQ (2 µg/mL, 1.77 min), PAR (60 µg/mL, 2.17 min) and ASP (2 µg/mL, 2.73 min)

Table 7. Results obtained by applying the proposed method for the determination of CQ, PAR and ASP in breast milk

Sample number	Authentic added, µg/mL			Recovery % of found ^a , µg/mL		
	CQ	PAR	ASP	CQ	PAR	ASP
1	2	20	2	102.00	99.05	102.00
2	4	40	4	102.00	100.32	99.46
3	6	60	6	100.26	100.19	101.23
Mean				101.42	99.85	100.89
± RSD %				± 0.98	± 0.69	± 1.28

^aAverage of three determinations.

simultaneously in adulterated dosage forms, a standard addition technique was carried out. The chromatogram obtained of the laboratory prepared mixture is presented in Fig. 4. The results were satisfactory and indicated that the additives in the tablets did not interfere with the analysis, where the recovery percentages ranged from 99.59 to 100.32%. The results of the pharmaceutical preparation and standard addition are presented in Table 6.

Application of the proposed method for analysis of adulterated CQ samples in breast milk

Drug free breast milk was treated according to the procedure described in the sample preparation section using liquid-liquid extraction. The chromatogram obtained from analysis of spiked breast milk is presented in Fig. 5. The results were satisfactory with no interferences from milk matrix, where the recovery percentages ranged from 99.85 to 101.42%. The results are presented in Table 7. However, the proposed method is useful considering CQ and ASP in breast milk detection with minor concentrations due to their high sensitivity while PAR it is useful in case it was given in high dose due to its low sensitivity as it possess weak fluorescence signal in comparison to CQ and ASP.

CONCLUSION

In this study, a quality by design strategy was used to obtain a rapid, simple, and optimized RP-HPLC method for the determination of CQ, PAR and ASP simultaneously. PBD and BBD were selected based on the experimental objective of each to evaluate the significant factors influencing the chromatographic separation and to develop the best resolution between peaks with the shortest run time with the aid of the desirability function approach. The advantages of this method make it suitable for the analysis of the three drugs, in quality control laboratories for identifying counterfeit CQ dosage forms by its adulterants (ASP and PAR) and for easily distinguishing between real and fake pharmaceutical dosages on the commercial and illicit internet market in pharmaceutical preparations and breast milk.

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