Original Article

Simultaneous Estimation of Atovaquone and Mefloquine Hydrochloride: QbD based Method Development and Validation

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ABSTRACT

Background: Atovaquone are important drugs that are used in the treatment of malaria and in combination with mefloquine hydrochloride increases the efficacy and potency. Present study covers the development of UV-spectrophotometry based analytical techniques for the simultaneous estimation of mefloquine HCl and atovaquone employing the Quality by Design (QbD) methods. **Materials and Methods:** C-N-X method were employed for risk assessment study and Ishikawa fishbone diagram was plotted for understanding of various factors that affect method development. Central Composite Design (CCD) was utilized for selected significant factors optimization. Scanning speed and scanning interval were considered as independent variables and absorption at 222 nm and 251 nm were considered as responses. Response surface plots and design space were developed for mathematical modelling-based prediction of responses obtained within specified ranges and optimization. Developed simultaneous analytical method was further validated as per the ICH Q2 guidelines. **Results:** The method showed good linearity with R^2 > 0.9 and good recovery with % RSD less than 2. The LOD and LOQ of UV developed method was found to be satisfactory and well between the acceptable range. **Conclusion:** The developed method for simultaneous estimation of atovaquone and mefloquine hydrochloride may act as a quick, precise, accurate, and economical quality control method for regular and simultaneous estimation of atovaquone and mefloquine HCl in bulk and mixed dosage forms at industrial level.

Keywords: Accuracy, Analytical Method Development, ICH Q2 guidelines, Precision, Quality by Design, Risk Assessment.

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INTRODUCTION

Malaria is one of the most common parasitic infections throughout the world wide. Every year, there are more than 400 000 morbidities because of malaria, which can be avoided and curable disease by use of antimalarials.[1-3] There are several classes of antimalarials, which are used for prophylaxis and treatment of malaria. Mefloquine HCl and atovaquone (Figure 1) are popular antimalarial medicine utilized in the prophylaxis and management of malaria. Atovaquone is hydroxy nephthoquinone derivative and highly lipophillic in nature having antiprotozoal activity. It is a strong and effective inhibitor that causes mitochondrial dysfunction by reducing the electron transport chain in the bc1 complex.[2] Mefloquine HCl is a quinoline methanol derivative, phospholipid interacting anti-malarial agent. It is active against asexual stage of malaria and competes with complexing protein from heme binding and form drug-heme complex which is toxic to parasite.[4]

As the atovaquone is important antimalarial drug given in combination with other antimalarial drugs to increase the efficacy and mefloquine hydrochloride is one of the potent candidate to be given along with atovaquone to increase its efficacy. Both the

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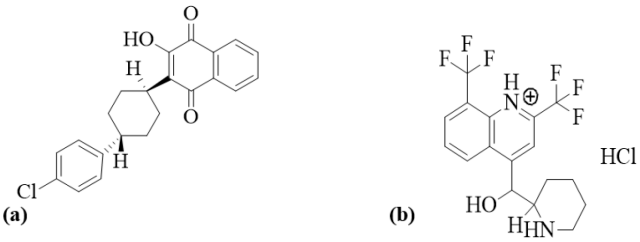

Figure 1: Basic structure of (a) Atovaquone (b) Mefloquine hydrochloride.

Figure 2: Typical steps of QbD involved in the development of robust analytical method.

drug works in synergism manner against the Plasmodium species that causes the malaria without showing any type of interaction as reported by FDA. So for designing a formulation consisting of atovaquone and mefloquine HCl as combination, it is important to have a method that simultaneously estimate the concentration of both the antimalarial drugs. As both drugs are estimated through the UV-spectrophotometric method, there is absence of any literature/documentation that simultaneously estimate both the drugs through the UV-spectrophotometric method.

As per USFDA directions and prevalent industrial practices, Quality by Design (QbD) is an important method employed frequently for the quality involvement during any method or product development. Accordingly in present study, endeavours were taken for creating QbD driven new UV-spectrophotometric strategy for simultaneous estimation of mefloquine Hydrochloride and atovaquone. QbD is an aggregate methodology, which guarantees quality is worked during the cycle to get the desired output. As indicated by ICH-Q8-(R^2) guidelines, QbD are defined as "*Based on sound science and quality risk management, a methodical approach to development that begins with established objectives and stresses product and process understanding and process control*".[5] Figure 2 illustrated the many processes involved in utilising the QbD during the development of a method for simultaneously estimating Atovaquone and Mefloquine Hydrochloride using a UV-spectrophotometric approach.

Application of QbD during the method development begins with identification of the Critical Analytical Attribute (CAA) and Analytical Target Profile (ATP) for selected analytical techniques.[6] Critical Method Variables (CMVs) are recognized for the selected analytical/method through risk assessment studies. Quality risk assessment are done through development of Ishikawa fishbone diagram depicting all the potential risk factors that affect the selected method/techniques. Optimization design of Design of Experiment (DoE) i.e., Central composite Design (CCD) offers a rationale-based approach for optimization of selected critical method variables (CMVs).[6] After that, method control strategy should be further employed for continual method improvement for robust method development. As per published reports, Goyal and co-workers developed HPLC techniques for simultaneous determination of mefloquine hydrochloride and atovaquone.[4] Viplava and Haritha developed HPLC method for estimation of atovaquone.[7] Rao and Challa developed HPLC techniques for determination of atovaquone in rat plasma.[8] Mangaonkar *et al.* developed LC-MS method for estimation of atovaquone in human plasma.[9] Magdum *et al.* developed HPLC method for simultaneous estimation of mefloquine hydrochloride and artesunate in bulk and marketed drug formulations.[10] Goyal and co-workers also developed simultaneous estimation method of arteether and mefloquine hydrochloride through UV-spectrophotometry.[11] No previous literature has been published regarding the simultaneous estimation of atovaquone and mefloquine hydrochloride through UV-spectrophotometric method. As atovaquone is an important drugs in treatment of malaria, whereas mefloquine hydrochloride increases its potency, there is strong possibility of any dosage form in upcoming times that contain both the drugs. So development of simple and validated simultaneous method for estimation of atovaquone and mefloquine hydrochloride is necessary. Therefore a validated and reliable UV-spectrophotometric techniques for simultaneous estimation of mefloquine hydrochloride and atovaquone could fill this void as UV method is simple and easy to perform analysis as compared to other methods such as HPLC, LC-MS etc.

Keeping this in view, present study was designed to develop UV-spectrophotometric techniques for simultaneous estimation of mefloquine hydrochloride and atovaquone by incorporating QbD and quality risk management and further validation using the methods

according to International Council of Harmonisation (ICH) guidelines Q2(R1) for industrial applicability.

MATERIALS AND METHODS

Materials

Pure drugs, mefloquine hydrochloride and atovaquone, were procured by Sanjay Biologicals, Amritsar, Punjab. Every others solvents used during the experimentation were of HPLC criteria and procured from S.D. Fine Chemical Ltd., India.

Methods

Instrumentation and Optical Characteristic

UV/Visible Double beam Spectrophotometer (Lab India 3000+) having UVWin software with 1 cm of quartz cuvettes were employed for this research purpose. Analytical weighing balance of Lab India was used for the weighing the reagents.

Preparation of Stock Solution

Stock solutions of both the drugs i.e., atovaquone and mefloquine hydrochloride containing 100µg/mL were prepared by using ethanol as solvent. Further the dilutions to 2,4,6,8,10 and 12 µg/mL, in triplicate were prepared by using stock solution.

Risk analysis-based model development

The use of a risk assessment approach is essential for gaining insight into potential high-risk elements and fostering risk protocols in order to determine the desired final product quality. The risk assessment was carried out in such a way that all potential high risk factors were identified, which were then given to a Create of Experimentation (DoE) research in order to design the experimentation area.[12] As indicated by ICH Q9 guidelines, identification of risk and risk analysis are the two significant elements for risk assessment methodology. Identification of the risk was performed by plotting Ishikawa fishbone diagram that delineate all potential factors that affect the method development. The next steps of risk assessment i.e., risk analysis was performed by utilizing the Control Noise Experiment (C-N-X) method.[6]

As initial step Critical Analyte Attribute (CAA) and variables involved during the method development were identified and Ishikawa fishbone diagram showing the relationship between these two was plotted (Figure 3). Afterwards, Control- Noise-Experiment (CNE) method was employed for identifying the critical risk factors from potential risk factors obtained from Ishikawa fishbone Diagram. The risky critical variables were further designated with scores as per severity and

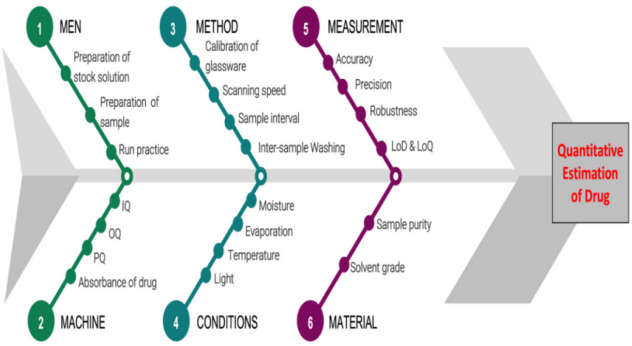

Figure 3: Ishikawa fishbone diagram for finding the probable factors that affects the method development.

[IQ-Installation qualification, OQ-Operational Qualification, PQ- Performance Qualification, LOD-Limit of Detection, LOQ- Limit of Quantification]

total scores were calculated to identify critical method variables (CMVs).

After analysis of Ishikawa fishbone diagram, various method variables viz., scanning speed of sample/ instrument, types of solvent, sampling interval, integrity of sample etc., were studied through C-N-X method for risk analysis during method development. Important critical factors were further subjected to optimal optimization design for determining the optimal level of variables for achieving the predetermined analytical target profile.[6]

Quality by Design (QbD) based approach for optimization of critical method variables (CMV)

After determining the CMV through risk analysis methodology, two important method variables i.e., scanning speed and scanning interval were selected for further optimization and rest other method variables were set to the constant value(s) at their lowest or optimum value.[12]

Central composite design (CCD) of Design of Experiment (DoE) with alpha value equal to one was employed to find the optimum level of method variables to make the method robust. Method variables i.e., scanning speed (A) and scanning interval (B) were selected for optimization through CCD (the basis of C,N,X method of risk assessment, as shown in Table 2). CCD gave 13 runs for two selected variables consisting of 5 centred points and same has been depicted in Table 2. Two responses absorbance at 251 nm for atovaquone and at 222 nm for mefloquine hydrochloride were selected for analysing the selected independent variables. Design Expert Software Version 11 (Stat-Ease Inc., Minneapolis, MN) was used for determining the relationship among the independent and dependent variables. All the experiments performed during the optimization studies were done in triplicate.

Suitable mathematical model in the form of coded equation (Equation 1) were generated by employing the Design Expert software by feeding the data in the software for showing the relationship between the independent method variables and dependent variables or responses.

$$Y = X_0 + X_1A + X_2B + X_3AB + X_4A^2 + X_5B^2$$

Equation 1

Where Y depicts the response, X_0 presents the intercept, X_1 and X_2 are the coefficients of the two factors A and B, and X_3 presents the coefficients of an corelation term of factor A and B and X_4 and X_5 represents the coefficients of quadratic of selected factors.

Design Space

According to the ICH Q8 recommendations, "Design space or working space is defined as a multidimensional mix of input factors (method variables) and process parameters that have been shown to provide quality assurance". Optimization in association with response surface methods were applied for the designing the design or working space. Design space comprised of area having operative range of selected method variables i.e., scanning speed and scanning interval. Applied experimental design i.e., central composite design (CCD) gave 13 experimental runs for 2 selected method variables (scanning speed and scanning interval) as given in Table 1.

Table 1: Experimental design matrix obtained through central composite design.

	Factor 1	Factor 2
Run	**A: Scanning speed**	**B: Sampling Interval**
1	0	-1
2	0	0
3	0	0
4	-1	0
5	-1	-1
6	0	0
7	1	-1
8	0	1
9	1	0
10	0	0
11	0	0
12	1	1
13	-1	1
Coded value	**Scanning Speed**	**Scanning Interval**
-1	Slow	0.5 nm
0	Medium	1.0 nm
1	Fast	2.0 nm

Analysis of Variance (ANOVA) with the utilization of Design Expert software was applied for generating various important parameters such as p-value, *f*-value, R^2 value, adequate precision, etc. Different polynomial equations were generated having significant p value less than 0.5.[12] Different plots such as Normal plot, Predicted vs Actual plots, Liverges vs Run plot, Residual vs Run Plot, Residual vs Predicted plot, Cooks Distance plot for both the critical method variables were generated using the software for analysing the behaviour or effect of scanning speed and scanning interval as independent variable, on the dependent variables or responses (i.e., Absorbance at 251 nm and Absorbance at 222 nm). 2D contour plots and 3D response surface plot were generated for depicting the relationship between the selected independent method variables and response variables. Further overlay plot were generated for showing the design space from all experimental area.

Model Validation

For analysing the suitability or working of the developed model in lab scale, point prediction feature was explored to get the value of the responses at particular value of method variables in design space for further optimisation of the method. The optimized value of responses as obtained were further compared to the values of responses obtained by performing the experiment at lab scale at same value of method variables. This comparison validated the developed polynomial model-based predictions under real experimentation condition in analytical lab.[13]

Validation of Developed Method

Validation is one of the important parameters for formation of a reliable, robust and reproducible UV-spectrophotometric analytical method to attain industrial acceptance. Hence, the optimized UV-spectrophotometric method was validated for specificity, linearity, accuracy, precision, repeatability, intermediate precision, system suitability and robustness according to the ICH Q2(R1) guideline.[14] All experiments were carried out in triplicate.

Specificity

Specificity was determined to examine the possible interaction of excipients with our target drugs. For specificity study, solvent's spectrum was taken and interference at the λ_{max} of diluted atovaquone and mefloquine HCl standard solutions was checked.[15-16]

Linearity

Linearity refers to an analytical technique' ability to deliver results that are proportional to the amount (quantity) of analyte in the sample (within a given range). Atovaquone and mefloquine HCl had linearity ranges of 2-12 g/mL for both drugs, the correlation coefficient (R^2) were also calculated.[15-16]

Accuracy

The accuracy of any analytical technique is defined as the degree of concordance between both the amount recognised as a traditional actual value or an established reference value and the values calculated. In a blind research, accuracy is determined by the proportion of analyte recovered by assay, followed by spiking samples. The percent recovery by assay of a known additional amount of analyte in the sample or the difference between the mean and the recognised actual value were used to calculate accuracy. Recovery experiments at 80 percent, 100 percent, and 120 percent of the test concentration of both medications were used to test the method's accuracy. The samples were formulated in tripclate and the percent recovery was calculated.[15-17]

Precision

The precision of a technique is defined as the degree of uniformity (degree of variance) between a set of observations acquired from repetitive sampling of a homogenous sample under the specific circumstances. Dependability and intermediate precision are used to achieve accuracy (ruggedness).[15-17]

Intermediate Precision

Variations within laboratories are expressed by intermediate precision; various days (inter-day and intra-day precision), different analyzers, different equipment, and so on. The goal of intermediate precision validation is to ensure that after the development phase is completed, the technique will provide the same findings in the same laboratory. The goal is also to make sure that the approach will provide the same findings in different labs. To determine inter-day and intraday precision of atovaquone and mefloquine HCl, three duplicates from the stock solution were produced in three concentrations (2,4, 6 g/mL) and absorbance was collected. The intraday results were taken at an interval of 1 hr for 3 times. For inter-day precision same procedure was followed for another two days.[15-17]

Repeatability

The term "repeatability" refers to the precision of a measurement made under the same operating conditions over a short period of time. Intra-assay precision is another synonym for repeatability. Repeatability of atovaquone and mefloquine HCl was done by taking minimum of 6 determinants of 6 µg/mL concentration and the absorbance were taken to determine the % RSD.[15-17]

Robustness

The assessment of robustness should be done throughout the development process and is dependent on the technique under investigation. It should demonstrate the validity of an analysis in the face of deliberate changes in technique parameters.[18]

Limit of detection (LOD) and Limit of Quantification (LOQ)

The sensitivity of the devised approach was evaluated in terms of LOQ and LOD, which are dependent on the standard deviation (σ) and slope (s) of the calibration curve (n=3), as per the ICH Q2 (R1) criteria. LOD and LOQ were determined experimentally by injecting each drug concentration twice and computing the values from the slope and standard deviation, as shown in Equation 2:[4]

$$LOD = 3.3 * \sigma/s \quad LOQ = 10* \sigma/s \qquad \text{Equation 2}$$

where, "σ" = standard deviation,
"s" = slope.

RESULTS AND DISCUSSION

Wavelength Determination

As no previous literature was available on simultaneous estimation using the UV-spectrophotometry of both the drugs i.e., atovaquone and mefloquine hydrochloride, both drugs were scanned in UV-spectrophotometer separately and maximum wavelength (s) were observed at 251 nm and 222 nm for atovaquone and mefloquine hydrochloride respectively. The spectra showing the maximum wavelengths (T_{max}) for both the drugs are shown in Figure 4 (a) and (b). Both the drugs were analysed simultaneously and overlay spectra of both the drugs i.e., atovaquone and mefloquine hydrochloride is given in Figure 5.

Risk Assessment Study

Defining the ATP and CAAs

As a requirement for UV-spectrophotometric analytical method development QbD approach, Analytical Target Profile (ATP) was defined by identifying the quality ascribes of the proposed UV-spectrophotometric method.[19] Table 2 enlist the possible ATP for current

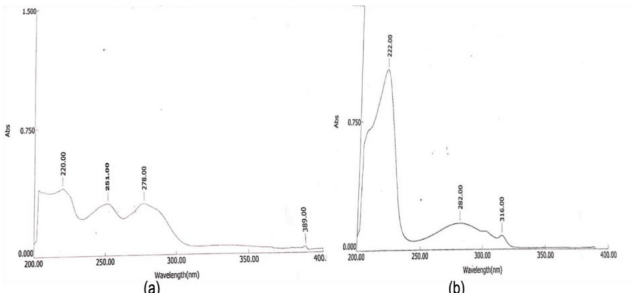

Figure 4: (a) λ of atovaquone and (b) λ of mefloquine hydrochloride.

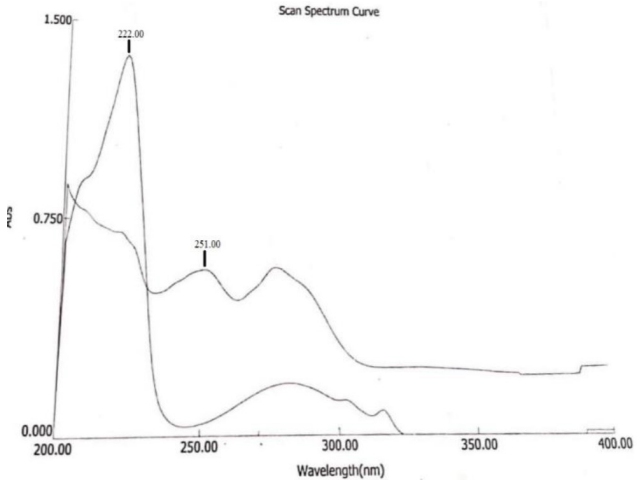

Figure 5: Overlay spectra of atovaquone and mefloquine hydrochloride.

UV-spectrophotometric method involving atovaquone and mefloquine hydrochloride.

The rationale for formulation of new UV-spectrophotometric techniques was for quick and easy analysis of drug as compared to other complex analytical methods i.e., HPLC and LC-MS. For achieving the fixed target profile, absorbance of atovaquone and mefloquine hydrochloride was selected as Critical Analyte Attribute (CAA).[6]

Ishikawa fishbone diagram (Figure 3) has been drawn for showing corelation among the various process variables and critical analytical attributes.[16] On the basis of previous relevant literature and discussion among the research team, various factors affecting the selected response i.e., absorbance were outlined.

Risk Factor Analysis through C-N-X method

Criticality analysis of the factors identified through the Ishikawa fishbone diagram (Figure 3) were done through C-N-X method. Various level and criticality of each of the process parameter and analytical attributes have been shown in Table 3. Severity scores were given to each of variables according to their ability to affect the final quality attributes. The ability of each of variables affecting the final quality parameter were outlined on the basis of previous scientific literature analysis and brainstorming.

Table 3 also depicts the strategy for controlling each of variables. After analysis, scanning speed and sampling interval were selected as most significant risk factors based on severity, which can be controlled by applying the DoE methodology. Rest of moderate and less

Table 2: ATP for UV-spectrophotometric method of atovaquone and mefloquine hydrochloride.

S. No.	ATP parameter	Target	Justification
1.	Sample	Active pharmaceutical ingredients (API)	Quantifiable assessment of a pharmacological molecule in a pharmaceutical dose form utilising a well-established analytical approach.
2.	Method category	UV-spectrophotometric method	Drug molecules are easily estimated using the UV-spectrophotometric.
3.	Instrument requirement	UV-spectrophotometer	UV-spectrophotometer with high accuracy and precision.
4.	Type of analyte	Liquid (Solution)	There is uniform miscibility in the case of liquid analyte.
5.	Standard stock solution preparation	Dilution of main drug in linear manner.	Solution of drug should have complete miscibility with requisite solvent for its suitable dilution.
6.	Formulation of sample	Handling	Various concentration of solution containing drugs were prepared by manual weighing requisite excipients and drug molecules with great care and precision as per SoP.
7.	Application of Method	Estimation of atovaquone and mefloquine hydrochloride	The developed method has been used to estimate atovaquone and mefloquine hydrochloride in a variety of pharmaceutical formulations.

Table 3: C,N,X based risk assessment of various process variables and analytical attributes.

Reason	The impact of risk on absorbance (Score)	Total Score	C-N-X	Strategy for controlling the risk
Scanning speed	10	100	X	Design of Experiment
Solvent	5	50	C	Can be controlled during the experimentation
Wavelength selection	4	40	C	251 nm for atovaquone and 222 nm for mefloquine hydrochloride.
Sample Purity	4	40	N	Quality can be assessed
Sample Preparation	4	40	C	Can be controlled by following SoP.
Equilibrium of detector	3	30	C	Can be controlled by performing the qualification of equipment.
Sampling Interval	10	100	X	Design of Experiment

C: Control, N: Noise and E: Experimental
Determination of total score- (Risk level of the selected factors X 10),
Score was given on scale of 1-10 to express Low (1), moderate (5) and high risk (10).

effective risk factor were fixed on constant value, as they have comparatively less or moderate effects on the selected risk factors.[20]

DoE based Optimization of Selected Critical Variables

Optimization of critical analytical variables were performed through statistical design i.e., central composite design. A design recipe consisting of 13 runs for selected independent factors i.e., scanning speed and sampling interval were generated through CCD with the utilization of Design Expert software and shown in Table 2. Absorbance at 251 nm and Absorbance at 222 nm were selected as responses or dependent variables.

QbD based Statistical Optimization Data Analysis

Multiple linear regression analysis technique was used for evaluating the experimental data after depicting the mathematical model with the help of software. Data analysis were done through the second order quadratic model generated with the help of coded equation which forecasts the relationship between the selected variables and response.[21]

Table 4: ANOVA analysis of response i.e., absorbance of 251 nm and 222 nm.

Responses	Absorbance at 251 nm		Absorbance of 222 nm		Remarks
Source	*F*-value	*p*-value	*F*-value	*p*-value	
Model	34.86	< 0.0001	42.86	< 0.0001	significant
A-Scanning speed	47.92	0.0002	4.29	0.0771	
B-Sampling Interval	96.18	< 0.0001	80.53	< 0.0001	
AB	2.00	0.2005	0.2894	0.6072	
A^2	3.03	0.1252	18.29	0.0037	
B^2	28.13	0.0011	65.75	< 0.0001	
Residual					
Lack of Fit	0.3361	0.8015	0.6417	0.6273	not significant

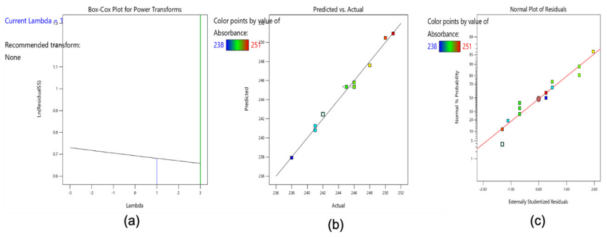

Figure 6: (a) Box-Cox plot (b) Predicted vs actual plot (c) Normal plot generated with the help of Design Expert software.

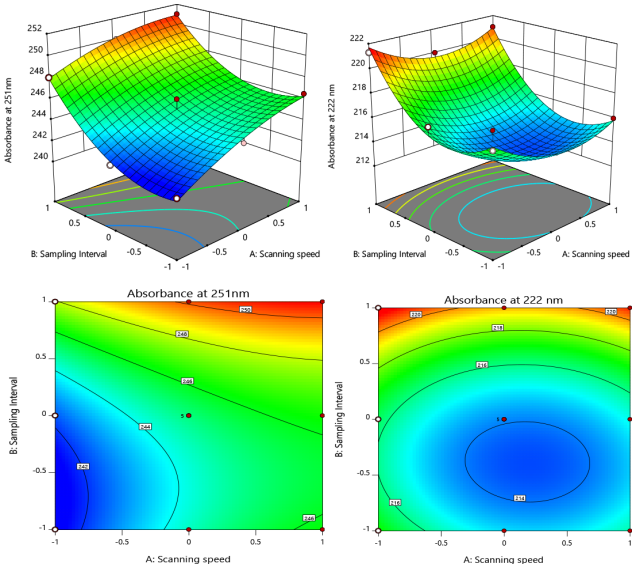

Figure 7: (a) 3D Response surface plot (b) 2D contour plot showing the corelation between the selected variables i.e., scanning speed and sampling interval and response.

Responses Equation in Coded form

The equations in the form of coded factors were employed for predicting the correlation across the

independent and dependent variables in terms of different levels. Different levels were distinguished as lowest and elevated levels which were further denoted as -1 (low level) and +1 (higher levels). The equation of selected responses are shown as Equation 3 and 4.

Absorbance at 251nm = +245.07 + 2.00A + 2.83B - 0.5000 AB - 0.7414A^2 + 2.26B^2 Equation 3.

Absorbance at 222nm = +214.09-0.550A+2.38B+0.1750AB+1.67A^2+3.17B^2 Equation 4.

Where, A is scanning speed, and B is scanning interval. Statistical analysis of selected factors were performed with the help of Analysis of Variance (ANOVA). The lack of fit, R^2, and adjusted R^2 values were used to determine a significant model with a p-value less than 0.05 were used for model's adequacy. Model having significant 'Lack of fit' showed that the change assessed by the imitates doesn't explain the gap between anticipated and exploratory data information points. R^2 values in ANOVA near to 1 showed that how good anticipated model suit the experimental model and the value should be near to 1.[12] Table 4 denotes various ANOVA analysis of selected responses i.e., Absorbance at 251 nm and 222 nm with respect to various parameters like p-value, Lack of fit and R^2 value.

As shown in Table 4, the *p*-value for model as well for the factors were found to be less than 0.05, exhibiting the significancy of developed model. The *F*-value of both the responses were found to be 34.86 and 42.86 respectively, which implies that developed model were significant. Chance of error on the basis of *F*-value was found to be 0.01% in the developed model. The analysis also suggested the second order quadratic model with adjusted R^2 was found to be 0.97 which is near to1.

Different plots such as (predicted vs actual plot, box cox plot, and normal plot generated in design were shown in Figure 6 (a, b, c). All the plots are showing the acceptable criteria of response in comparison of variables.

For depicting the corelation between the variables and response, 2D contour plot and 3D response surface plots were constructed employing the Design Expert software and shown in Figure 7 (a) and (b). With the analysis of above plots, it was concluded that the absorbance starts decreasing with middle and low value of both the selected variables i.e., scanning speed and sampling interval. It is important to select the design space from the experimental area to optimize selected critical factors (sampling interval and scanning speed) in respect to selected response (absorbance at 251 nm and 222 nm). Figure 8 depicts the overlay plot highlighting the design space in dark yellow from experimental area in grey colour. The region in light yellow colour represents the area through which various factor can varied.

Validation of developed experimental model

Validation of experimental design is also an important criteria involved in the risk assessment and practical applicability of any analytical method development involving the QbD and QRM principles. After comparing the predicted value to that of in experimental value, developed model was found to be 96% similar to that of experimental value, confirming that the mathematical model can be acceptable to define the interrelation of

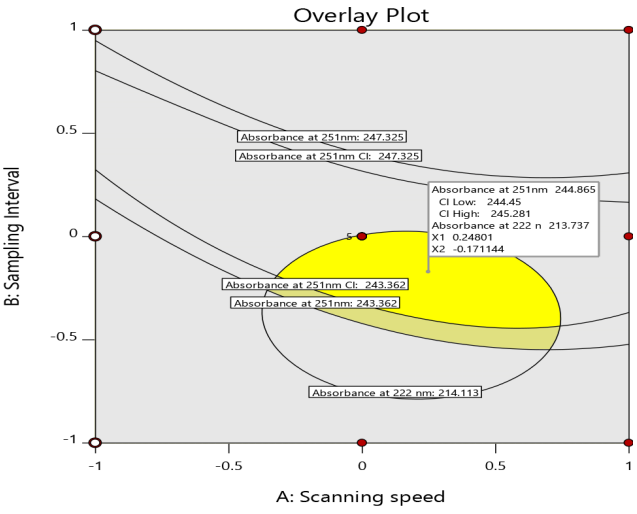

Figure 8: Overlay plot showing the design space (dark yellow) from experimental area.

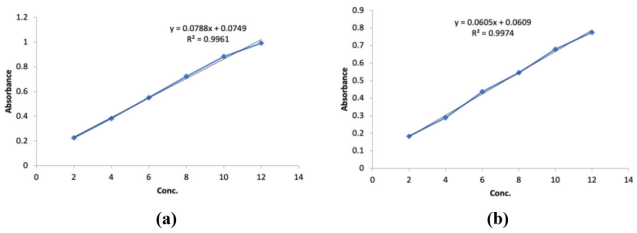

Figure 9: Linearity curve of atovaquone at (a) wavelength 251 nm and (b) wavelength 222 nm.

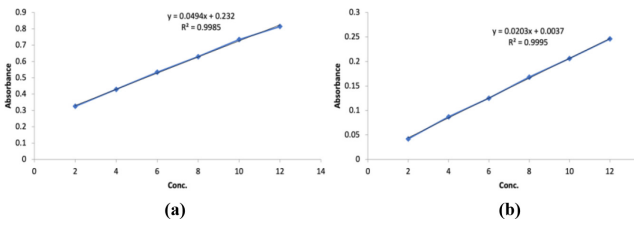

Figure 10: Linearity curve of mefloquine HCl at (a) wavelength 222 nm and (b) wavelength 251 nm.

selected variables to get the desired response within the identified design space.[13]

Analytical Method Validation

Specificity

For checking the specificity of the developed analytical method, absorbance of diluted samples of both drugs i.e., Atovaquone and Mefloquine HCl were observed. Overlay spectra (Figure 5) of both the drugs (i.e., Atovaquone and Mefloquine HCl) showed no interaction of drugs with excipients and each of drugs shown unique peak confirmed the specificity of developed method.[12]

Linearity

For method validation of simultaneous estimation of both drugs i.e., atovaquone and mefloquine HCl, linearity studies were done separately at 251 nm and 222 nm at concentration ranges of 2, 4, 6, 8 and 10 µg/mL. The R^2 value for both wavelength for both the drug atovaquone and mefloquine HCl were found to be 0.9961, 0.9974 and 0.9985, 0.9995 respectively [shown in Figure 9 (a, b) and Figure 10 (a, b)], which was in the acceptable range of not less than 0.99 (ICH Q2 Guidelines) confirming the linearity of developed method.[22-23]

Table 5: Recovery studies analysis at 80%, 100 % and 120% of atovaquone test sample at 251 nm.

Sl. No.	Unfortified sample			Fortified sample			% Recovery
	Conc. (µg/mL)	Absorbance	Mean Absorbance	Conc. (µg/mL)	Absorbance	Mean Absorbance	
1	2	0.182	0.183	2+4	0.402	0.402	98.64
		0.184			0.402		
2	4	0.224	0.222	4+4	0.441	0.442	99.09
		0.221			0.443		
3	6	0.448	0.447	6+4	0.668	0.668	99.54
		0.446			0.669		

Table 6: Recovery studies analysis at 80%, 100 % and 120% of atovaquone test sample at 222 nm.

Sl. No.	Unfortified sample			Fortified sample			% Recovery
	Conc. (µg/mL)	Absorbance	Mean Absorbance	Conc. (µg/mL)	Absorbance	Mean Absorbance	
1	2	0.239	0.239	2+4	0.583	0.583	100.58
		0.240			0.583		
2	4	0.341	0.342	4+4	0.683	0.682	99.41
		0.343			0.682		
3	6	0.504	0.503	6+4	0.845	0.846	100.29
		0.503			0.847		

Table 7: Recovery studies analysis at 80%, 100 % and 120% of mefloquine HCl test sample at 222 nm.

Sl. No.	Unfortified sample			Fortified sample			% Recovery
	Conc. (µg/mL)	Absorbance	Mean Absorbance	Conc. (µg/mL)	Abs.	Mean Absorbance	
1	2	0.326	0.325	2+4	0.753	0.752	99.53
		0.324			0.751		
2	4	0.428	0.429	4+4	0.853	0.852	98.60
		0.430			0.851		
3	6	0.534	0.534	6+4	0.962	0.962	99.76
		0.534			0.962		

Table 8: Recovery studies analysis at 80%, 100 % 120% of mefloquine HCl test sample at 251 nm.

Sl. No.	Unfortified sample			Fortified sample			% Recovery
	Conc. (µg/ml)	Absorbance	Mean Absorbance	Conc. (µg/ml)	Absorbance	Mean Absorbance	
1	4	0.084	0.083	4+6	0.218	0.217	98.52
		0.082			0.216		
2	6	0.135	0.136	6+6	0.268	0.268	97
		0.137			0.268		
3	8	0.166	0.167	8+6	0.297	0.296	94.85
		0.168			0.295		

Accuracy

Following the creation of an analytical technique, it is critical to ensure that the procedure consistently produces the desired and accurate result with a high level of accuracy. This creates are requirement to validate the analytical procedures. Accuracy communicates the closeness of arrangement between the value observed and the reference value. Recovery experiments at 80, 100, and 120 percent of the test concentrations of both medications were used to test the method's accuracy. At the 251 and 222 nm wavelengths, the samples were generated in triplicate, and percent recovery was calculated for each of the medications. The average % recovery for both the drugs were 100.06% and 99.33% respectively [shown in Tables 5-8]. The observed values were well within the acceptable range of 98%-102% as per ICH Q2 guidelines and also reported in previous

Table 9: Intraday and Intraday precision of atovaquone and mefloquine HCl at wavelength 251 and 222 nm.

	Absorbance of atovaquone						Absorbance of mefloquine HCl					
Wavelength	251 nm			222 nm			251 nm			222 nm		
Conc. (µg/mL)	4	6	8	4	6	8	4	6	8	4	6	8
Day 1												
Absorbance	0.381 0.380 0.382	0.555 0.553 0.557	0.724 0.722 0.725	0.380 0.383 0.383	0.552 0.550 0.551	0.724 0.720 0.722	0.085 0.087 0.086	0.133 0.129 0.131	0.168 0.169 0.167	0.486 0.484 0.486	0.644 0.647 0.645	0.829 0.827 0.825
Mean	0.381	0.555	0.723	0.382	0.551	0.722	0.086	0.131	0.168	0.485	0.645	0.827
S.D.	0.001	0.002	0.001	0.001	0.001	0.002	0.001	0.002	0.001	0.001	0.001	0.002
%RSD	0.262	0.360	0.211	0.453	0.181	0.277	0.162	1.522	0.595	0.237	0.236	0.241
Day 2												
Absorbance	0.387 0.389 0.382	0.555 0.553 0.554	0.725 0.722 0.725	0.381 0.383 0.385	0.553 0.555 0.553	0.725 0.723 0.723	0.085 0.086 0.085	0.135 0.139 0.137	0.168 0.169 0.169	0.485 0.485 0.486	0.646 0.645 0.646	0.827 0.825 0.825
Mean	0.386	0.553	0.724	0.383	0.553	0.723	0.085	0.137	0.168	0.485	0.645	0.825
S.D.	0.003	0.007	0.001	0.002	0.001	0.001	0.0005	0.002	0.005	0.005	0.005	0.001
%RSD	0.934	0.127	0.239	0.522	0.208	0.159	0.676	1.459	0.346	0.118	0.089	0.139
Day 3												
Absorbance	0.388 0.385 0.385	0.557 0.557 0.559	0.728 0.729 0.730	0.388 0.387 0.386	0.555 0.554 0.553	0.725 0.727 0.725	0.089 0.087 0.088	0.137 0.135 0.137	0.167 0.169 0.167	0.487 0.487 0.485	0.645 0.647 0.644	0.826 0.827 0.826
Mean	0.386	0.558	0.729	0.387	0.554	0.725	0.088	0.136	0.167	0.486	0.645	0.826
S.D.	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.0005
%RSD	0.448	0.253	0.137	0.258	0.180	0.159	1.136	0.846	0.688	0.237	0.239	0.069

Table 10: Repeatability of atovaquone and mefloquine HCl at 251 and 222 nm.

Sl. No.	Conc. (µg/mL)	Absorbance of atovaquone		Absorbance of mefloquine HCl	
		251 nm	222 nm	251 nm	222 nm
1	6	0.550	0.553	0.132	0.638
2	6	0.548	0.552	0.133	0.633
3	6	0.547	0.554	0.133	0.635
4	6	0.549	0.552	0.135	0.638
5	6	0.551	0.554	0.133	0.637
6	6	0.548	0.553	0.135	0.637
Mean		0.548	0.553	0.133	0.636
±S. D.		0.0014	0.0008	0.001	0.0019
% R.S.D.		0.268	0.161	0.917	0.309

published literature,[24-25] which confirming the accuracy of developed method.

Precision

Precision represents the repeatability and reproducibility of developed model in various unchanged conditions. The intraday precision for both the drugs (Atovaquone and Mefloquine HCl) were taken at an interval of 1 hr in triplicate. Table 9 represent the intraday precision for various concentration of selected drug samples. The average % RSD for Atovaquone at 251 and 222 nm were found to be 0.276% and 0.303% respectively while % RSD for Mefloquine HCl for both the wavelengths (251 nm and 222 nm) were found to be 0.238% and 1.093% respectively. The results of intraday precision for both the drugs were in the acceptable range of < 2% (ICH Q2 guidelines) and also reported in previously literature.[24-29]

Intermediate Precision

As shown in Table 9, intermediate precision of both the drugs were analysed for 3 days and results found were well in the acceptable range (% RSD < 2%) which confirmed that the developed model were reproducible and reliable in various unchanged conditions.

Repeatability

Repeatability was measured by running by minimum of 6 samples of concentration of 6 µg/ml of both the drugs i.e., Atovaquone and Mefloquine HCl. Results of repeatability are shown in Table 10. % RSD for Atovaquone at both the wavelength were 0.268 and 0.161 respectively. While % RSD for Mefloquine HCl were 0.309 and 0.917. The observed results were found to be in limit prescribed in ICH Q2 guidelines (less than

Table 11: Robustness of atovaquone and mefloquine HCl at 251 and 222 nm.

Conc. (µg/mL)	Absorbance of atovaquone at 251±2 nm			Absorbance of atovaquone at 222±2 nm			Absorbance of mefloquine HCl at 251±2 nm			Absorbance of mefloquine HCl at 222±2 nm		
	249 nm	251 nm	253 nm	220 nm	222 nm	224 nm	249 nm	251 nm	253 nm	220nm	222 nm	224 nm
6	0.544	0.553	0.558	0.540	0.553	0.561	0.544	0.553	0.558	0.129	0.132	0.139
6	0.547	0.551	0.557	0.542	0.554	0.562	0.547	0.551	0.557	0.128	0.137	0.138
6	0.544	0.557	0.559	0.542	0.552	0.560	0.544	0.557	0.559	0.128	0.135	0.139
Mean	0.545	0.553	0.558	0.138	0.541	0.553	0.545	0.553	0.558	0.128	0.134	0.138
SD	0.001	0.003	0.001	0.005	0.001	0.001	0.001	0.003	0.001	0.005	0.002	0.005
%RSD	0.317	0.551	0.179	0.416	0.213	0.180	0.317	0.551	0.179	0.449	1.868	0.416

2%) and also reported in previously published research work related to method development and validation.[30]

Robustness

Robustness was calculated by observing the absorbance of both the drugs at different wavelength. The robustness were determined by calculating the % RSD for both atovaquone and mefloquine HCl. Results were well within the acceptable range of ICH Q2 guidelines (< 2%) which validate the robustness of developed method.[30] The results are shown in Table 11.

LOD and LOQ

The LOD of the created analytical method for both drugs was 0.452 and 0.729, respectively, and LOD was 0.748 and 1.939, respectively, indicating that the developed method for estimation of both drugs has a high degree of sensitivity.[12]

CONCLUSION

The present study highlight the simple, reproducible, sensible and economical analytical UV -spectrophotometric techniques for the simultaneous determination of Atovaquone and Mefloquine HCl, employing the systematic Quality by Design paradigms. Following the establishment of ATP and CAAs, the CMVs were further selected by using risk assessment study through the Ishikawa fish bone diagram and C-N-X method. Risk surface methodology were further employed for the optimization of selected model by using CCD as optimization design presenting the complete understanding of particular response factor relationship and also the interaction among them.

Developed analytical method was further validated through the various validation parameters according the ICH Q2 guidelines such as linearity, accuracy, precision, robustness, repeatability, LOD, and LOQ. The results developed method novelty, linearity, accuracy and preciseness. Moreover, the recorded value of LOQ and LOQ suggested the higher sensitivity of developed model.

The application of QbD and various parameters of ICH Q2 guidelines advocate the potential application of developed optimized analytical method in quality control laboratories for the simultaneous determination of the both the drugs in the pharmaceutical industries. The developed method is suitable for routine estimation of the both drugs with any pharmaceutical formulation without any interference with the commonly used excipients in economic and simple way. The method has enormous potential to be accepted at industrial level for the estimation of both drugs i.e., atovaquone and mefloquine HCl in simultaneous way or individually due to involvement of QbD and ICH guidelines.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

***RSD:** Residual Standard Deviation; **%:** Percent; **2D:** 2 Dimensional; **3D:** 3 Dimensional; **Abs.:** Absorbance; **ANOVA:** Analysis of Variance; **ATP:** Analytical Target Profile; **C-N-X:** Control-Noise-Experimental; **C18:** Column having Octadecyl chain of carbon atom; **CAA:** Critical Analyte Attribute; **CCD:** Central Composite Design; **Cm:** Centimeter; **CMVs:** Critical Method Variables; **conc.:** Concentration; **DOE:** Design of Experiment; **Fig.:** Figure; **ICH:** International Conference on Harmonization; **IP:** Indian pharmacopoeia; **KBr:** Potassium Bromide; **LOD:** Limit of Detection; **LOQ:**

Limit of Quantitation; **M:** Molarity; **min.:** Minute; **mL:** Mille liter; **C:** Centigrade; **pH:** Power of hydrogen; **QbD:** Quality by Design; **QC:** Quality Control; R^2**:** Correlation coefficient; **RP-HPLC:** Reverse Phase – High Performance Liquid Chromatography; **RSD:** Relative Standard Deviation; **Rt:** Retention time; **SD:** Standard Deviation; **sec.:** Second; **USFDA:** United States Food Drug Administration; **UV:** Ultra-Violet; **v/v:** Volume by volume; **µ:** Micro; **µg:** Micro gram.

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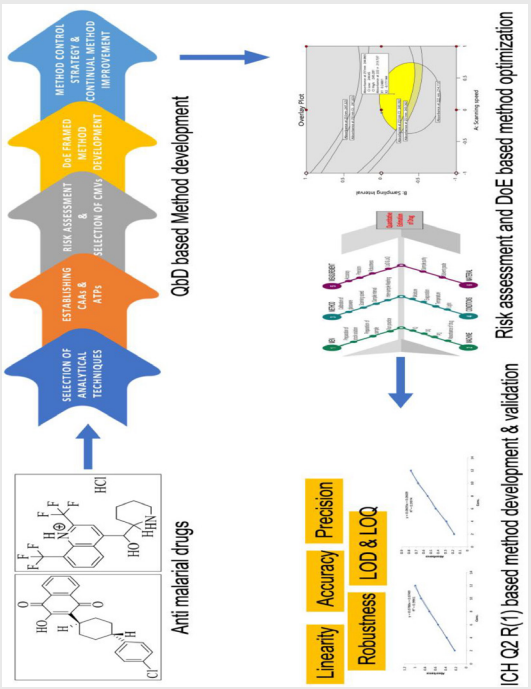
PICTORIAL ABSTRACT



SUMMARY

Malaria is one of the deadly disease the world is facing. Nearly malaria lead to more that 400,000 death worldwide. So, research related to treatment of malaria gains high popularity among the research community. Atovaquone is one of the proved drug for the treatment of malaria but due to of low solubility and low permeability, it's usage for the treatment has been restricted. But many research concludes that the atovaquone with combination of mefloquine hydrochloride have higher efficacy and therapeutic effect. So, simultaneous estimation of both the drug is an important parameter for the formulation of dosage form for treatment of the malaria. Research related to simultaneous estimation through the HPLC method has been widely done. But there are absence of research related to the simultaneous estimation through the UV spectrophotometric method. This paper presented the simultaneous method for the estimation of the atovaquone and mefloquine hydrochloride through the UV spectrophotometric method. The concept of Quality by Design has also been utilized for making a robust simultaneous estimation of both drugs. Risk assessment for estimating the risk involved in the process of simultaneous estimation has been estimated with the help of C-N-X method. 2 D contour plot and 3D response surface plot has been plotted for the showing the relationship between the independent and dependent variables. Out lay plot has been plotted and model of QbD has been validated by comparing the results with the experimental results. Various standard parameters as per ICH guidelines such as LOD, LOQ, Accuracy, Precision, Robustness, Repeatability etc. has been applied for the validating the simultaneous method for estimation of the atovaquone and mefloquine hydrochloride. This research can be applied for the robust method validation of various other drugs by applying the concept of QbD.

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