Stability Indicating LC Method Development for Hydroxychloroquine Sulfate Impurities as Available for Treatment of COVID-19 and Evaluation of Risk Assessment Prior to Method Validation by Quality by Design Approach

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Abstract
A quality by design-based stability indicating HPLC method has been developed for hydroxychloroquine sulfate impurities. The optimized HPLC method can detect and quantify the hydroxychloroquine sulfate and related organic impurities in pharmaceutical solid oral dosage forms. Nowadays, for the quantification of impurities in drug products demands more comprehensive way of analytical method development. The quality by design approach allows the assessment of different analytical parameters and their effects with minimum number of experiments. A highly sensitive and stability indicating RP-HPLC method was developed and evaluated the risk assessment prior to method validation. The chromatographic separation was achieved with X-terra phenyl column (250 × 4.6 mm, 5 µm) using phosphate buffer (0.3 M and pH 2.5). The gradient method flow rate was 1.5 mL min⁻¹ and UV detection was made at 220 nm. The calibration curve of hydroxychloroquine sulfate and related impurities were linear from LOQ to 150% and correlation coefficient was found more than 0.999. The precision and intermediate precision % RSD values were found less than 2.0. In all forced degradation conditions, the purity angle of HCQ was found less than purity threshold. The optimized method found to be specific, accurate, rugged, and robust for determination of hydroxychloroquine sulfate impurities in the solid oral dosage forms. Finally, the method was applied successfully in quality control lab for stability analysis.

Keywords Hydroxychloroquine sulfate · Quality by design · Design of experiments · HPLC · Validation

Introduction

Hydroxychloroquine sulfate (HCQ) is one of a large series of 4-aminquinolines with antimalarial activity [1, 2]. Moreover, it is used for the treatment of rheumatoid arthritis. Sometimes HCQ is very effective for the treatment of autoimmune diseases. Some of the experiments on HCQ are being performed in terms of its capability to inhibit some of the corona virus. There is a wide range of interest in the use of HCQ, which is commonly available for the potential interventions for COVID-19. Health agencies was employed HCQ for the tentative medication of COVID-19 in China, Italy, South Korea, USA, India, and Turkey [3–7].

The literature survey revealed different types of analytical techniques are used for determination of HCQ, such as HPLC with PDA detector [8, 9], HPLC with UV detector [10–14], chromatography-tandem mass spectrometry [15, 16], LC/IT/MS [17] and some other techniques [18, 19]. The majority of the literature was reported in pharmacokinetics of HCQ in biological fluids. Saini et al. [20] was identified and characterized only photo-degradation
impurities of HCQ and they are not reported comprehensive method development and validation results. To the best our knowledge, there was no stability indicating method with force degradation studies by using HPLC. The Forced degradation studies (FDS) is play an important role for development of stability indicating method [21–23]. Further, it is useful to understand the intrinsic stability of the drug substance and drug product. The RP-HPLC is powerful and simple technique for quantification impurities in drug product. Moreover, in the regulated pharmaceutical industry, drug products released into the global market after determination quality safety and efficacy. Hence, stability indicating HPLC methods are required with suitable precision, accuracy, and sensitivity level.

Nowadays, most of the pharmaceutical industries are including the quality by design (QbD)-based analytical methods in dossiers to meet the regulatory expectations. QbD approach in regulated pharmaceutical industry defining critical process parameters (CPP) and critical quality attributes (CQA) for assessment of risk and for establishment design space for method operating conditions. The current work demonstrates a simple way expounding the mathematical inferences to establish the acceptable range for the chromatographic conditions. A scientific way to explain design of experiments (DoE) approach by performing 15 series of experiments under the fractional factorial design (FFD) to evaluate the risk assessment before method validation. Further, the FDS was performed to know the possible degradation impurities and degradation pathways of HCQ. The HCQ has formed four new process impurities during the manufacturing process. These are 4,7-dichloroquinoline (DHC), desethyl hydroxychloroquine (DHC), hydroxychloroquine-O-acetate (HCA), and hydroxychloroquine-O-sulfate (HCS), respectively.

Chemicals structures are listed in Fig. 1.

Materials and Methods

Instrumentation

The Agilent HPLC 1260 Infinity-II consists four channels, pressure range up to 600 bar, degasser with integrated purge valve, thermostatic sampler, and column compartment. The photo-diode array detector (PDA) connected to empower 3 software (Build 3471 SPs Installed: Feature Release 3 DB ID: 2639633283) to monitor the output signal. The column is X-terra phenyl, 250 × 4.6 mm, 5 µm (Part No: 186001147). Sartorius semi-micro and micro-balances were used for weighing of impurities, standards, and samples. Bio-technics ultra sonicator was used to extract drug from the sample matrix.

Materials and Reagents

Procured the HCQ with certified purity of 99.2% from the SCI pharma. Desethyl hydroxychloroquine (DHC) (Lot No. SP-027-145) with purity of 96.07%, hydroxychloroquine-O-acetate (HCA) (Lot No. AVN-1804-HCQ-03) with purity of 99.68%, hydroxychloroquine-O-sulfate (HCS) (Lot No. AVN-1804-HCQ-02) with purity of 95.78%, and 4, 7-dichloroquinoline (DCQ) (Lot No. AVN-1804-HCQ-01) with purity of 99.36% were purchased from the SimSon Pharma, Mumbai, India. AR grade potassium dihydrogen phosphate and ortho phosphoric acid were procured from VWR chemicals, Radnor, PA, USA. HPLC grade acetonitrile (99.9%) from J.T. Baker was procured from VWR chemicals, Radnor, PA, USA. High quality HPLC grade water was used.

Chromatographic Conditions

The separation of impurities was achieved by using a gradient mobile phase-A containing 0.3 M of potassium dihydrogen phosphate buffer (pH 2.5) and filtered through 0.45-µm membrane filter. Mobile phase-B comprises mixture of acetonitrile and buffer in the ratio 70:30 v/v respectively. The ratio of gradient program was shown in Table 1. Used X-terra phenyl, 250 × 4.6 mm, 5 µm column with a with 1.5 mL min⁻¹. The detection was made at 220 nm with 10 µL injection volume.

Preparation of Diluent

To prepare the standard and sample solutions, 1.0% of orthophosphoric acid and acetonitrile in the ratio of 90:10 v/v were optimized, respectively, as a diluent, based on the solubility of the HCQ and impurities.

Preparation of System Suitability and Standard Solution

Initially, prepare 100 µg mL⁻¹ of DHC, HCA, HCS, DCQ impurity stock solutions and 5000 µg mL⁻¹ HCQ standard in 100% acetonitrile. The individual impurity and standard stocks were further diluted to 10 µg mL⁻¹ of DHC, 4 µg mL⁻¹ of HCA, HCS, DCQ, and 2000 µg mL⁻¹ to get system suitability solution.

Preparation of Sample Solution

The sample consists of HCQ as main active and different types of inactive ingredients in matrix formulation. 20 tablets were taken and crushed into a fine powder and transferred equivalent to 100 mg of HCQ into a 50 mL volumetric flask, to this added about 35 mL of diluent, and sonicated for 20 min with intermittent shaking then diluted volume with
diluent and mixed well. Centrifuge 10 mL of sample solution at 3500 rpm for 10 min and injected into HPLC.

### Possible Degradation Pathways for HCQ

Based on the route synthesis and chemical properties of HCQ, drug substance is able to produce four process-related impurities. Qiu et al. [24] explained the formation of impurities during the synthesis of pharmaceutical products and forced degradation studies. In the current study, we tried to explain how these HCQ impurities are generated during the manufacturing process. 4,7-Dichloroquinoline is the key

![Chemical structures and IUPAC names: a hydroxychloroquine sulfate; b 4,7-dichloroquinoline; c desethyl hydroxychloroquine; d hydroxychloroquine-O-acetate; e hydroxychloroquine-O-sulfate](image)
starting material of HCQ, which is the limit reagent, after the purification process also, some residual content of material may present in the final drug substances so that we termed as process-related impurity. HCA impurity could come from trans-esterification reaction of HCQ with ethyl acetate during the manufacturing process [24]. HCS impurity come from the esterification reaction of HCQ with sulfuric acid during the manufacturing process. Desethyl hydroxy chloroquine could come from the AEHPA impurity of 2-(4-aminopentyl) amino) ethanol or typical Hofmann elimination reaction form hydroxychloroquine substance. The HCQ N-oxide impurity (EP impurity-A) come from the oxidation reaction of HCQ with oxygen during the manufacturing the process. The EP impurity E comes from the AEHPA impurity of 4-amino-pentanol. The EP impurity D comes from the AEHPA impurity of N-(4-aminopentyl) ethylamine. The EP impurity F could come from AEHPA impurity of 2-methylpyrrolidone. The chemical reactions and chemical structures were shown in Fig. 2.

**Forced Degradation**

Forced degradation (FD) is required to demonstrate the specificity of the stability indicating methods and it provides the possible degradation impurities of the drug substance and drug products. Stress studies help in generating the impurities in very less time. Therefore, formulation scientist can make stable formulations within shorter time [25]. Nowadays, FD studies are mandatory requirements for the global regulated markets to fill the dossier and to understand the drug development process [22, 26, 27]. As per the ICH guidelines, stress studies must be included with acid and base hydrolysis, thermal degradation, photostability, and oxidation conditions. No specific regulation was mentioned regarding the conditions of pH range, concentrations of acid, base, and temperature; and, it can be assessed based on the chemical properties of drug substance. Photostability conditions have specifications in the ICH Q1B. The stress conditions should get a minimum of 1–10% of degradation of drug substance. It is not necessary that all stress conditions would result in 10% of impurities. Out of all different types of conditions, any one stress condition must get a minimum of 5% degradation so that the stressed sample would be used for the development of stability indicating methods.

To prove the specificity of the current method, the stress conditions for drug product and placebo in different conditions like acidic (2 N HCl, 30 min at 70 °C), basic (2 N NaOH, 30 min at 70 °C), oxidation (10% Hydrogen peroxide, 6 h at 70 °C) were performed [28]. For photo-stability condition, exposed samples and placebo in photolytic chamber for 200-W h m−2 for UV light and 1.2 million Lux hours for visible light to meet the ICH Q1B guidelines. To perform the thermal degradation, solid samples were exposed directly (7 days at 105 °C in hot air oven) [29]. To know the impact of humidity, exposed samples were kept at 25 °C/85% RH, 7 days in a desiccator. The acid and base samples and placebo were neutralized with equal concentrations of base and acid to stop the degradation reaction.

**Results and Discussion**

For the separation and the estimation of HCQ and impurities, method development was started with selection of buffer and pH. Based on the pKa value (3.5) of HCQ, we selected potassium di hydrogen phosphate buffer (0.3 M) and adjusted pH 2.5 (± 1 of pKa) with orthophosphoric acid. Initially, HPLC started isocratic method with 1.0 mL min−1 by using 100% buffer as mobile phase. Prepared samples by spiking the impurities (10 µg mL−1 of DHC, HCA, HCS, DCQ, and HCQ 2000 µg mL−1) and injected in to HPLC. The DCQ impurity not eluted even after 60 min of run time due to high non-polarity nature. Thereafter, mobile phase was prepared with combination of acetonitrile and phosphate buffer (0.3 M, pH 2.5) in the ratio of 20:80 and 40:60 v/v. As the amount of acetonitrile increased, the DHC and other impurities retention time decreased to less than 30 min. However, it leads to merging of DHC with HCQ and HCA with HCS. Thereafter, we decided to go with gradient method instead of isocratic method to get the more resolution between the adjacent peaks within short run time. The mobile phase A (MP-A) was prepared with 100% phosphate buffer (0.3 M, pH 2.5), and mobile phase B (MP-B) was prepared with the mixture of phosphate buffer (0.3 M, pH 2.5) and acetonitrile in the ratio of 70:30 v/v, respectively. Initially, gradient started with 95% of MP-A, then gradually increased to MP-B to elute the impurities. Optimized gradient program with 1.5 mL min−1 by using the mid-polar stationary phase column (X-terra phenyl, 250 × 4.6 mm, 5 µm). System suitability parameters were evaluated (retention time, tailing factor, and resolution) at 220 nm by using PDA detector. The method development optimization process was shown in Table 2. The chromatograms of individual impurities and HCQ were shown in Fig. 3.

**Design of Experiments (DoE)**

The optimized chromatographic conditions were subjected for risk assessment prior to method validation using statistical design of experiments for evaluating the critical quality attributes (CQA) and critical process parameters (CPP). In the current gradient method, the CPPs are flow rate (1.3–1.7 mL min−1), acetonitrile composition in mobile phase B (630–770 mL) and column temperature (20–30 °C). The current DoE section, fraction factorial design was used for linear and interaction models. Compared to generic
Fig. 2 Possible degradation pathways of HCQ: 

a) trans-esterification reaction of HCQ with ethyl acetate and formation of HCA; b) esterification reaction of HCQ with sulfuric acid and formation of HCS; c) typical Hofmann elimination reaction of hydroxychloroquine sulfate will give DHC; d) oxidation reaction of HCQ with oxygen will give N-oxide impurity; e) AEHP impurity reaction with 4-amino-pentanol will give EP impurity-E; f) AEHP impurity reaction with N-(4-aminopentyl) ethylamine will give EP impurity-D; g) AEHP impurity reaction with 2-methylpyrrolidone will give EP impurity-F.
analytical method development, varying one factor at a time, application of DoE where all the factors are altered simultaneously to accelerate the risk assessment with greater influence [30, 31]. By considering all these response factors (Table 3), the statistical experimental design chosen was fractional factorial design (FFD), with 15 experiments at two levels and three central points.

The main aim of the study was to identify the critical factors contributing to the resolution of the impurities with minimum number of experiments. The current method is used for the analysis of stability of the samples of HCQ; hence, the method should show stability, indicating the nature and spectral purity. Because the multiple factors are altering simultaneously, separation between the impurities and HCQ peaks may diminish. The poor resolution of impurities disputes the peak purity and quantification. For this reason, in the current DoE study, we evaluated resolution between the DHC and HCQ (R1). Other response factors, such as tailing factor and theoretical plates were not changed significantly, and the results are shown in Table 4, so that we can neglect DoE.

**Statistical Analysis and Inferences**

The impact of resolution was represented with half-normal plots and pareto charts (Fig. 4). Mainly two factors viz., varying the acetonitrile composition and HPLC flow rate were significantly affected the resolutions of DHC and HCQ. ANOVA and F test also confirmed the high degree of significance for chosen model with \( p < 0.05 \) (Table 5). The response surface diagrams (3D graphs) were exercised to understand the interaction of variables for R1. Out of the 15 DoE experiments, the std no. 8, 12, and 14 were shown more resolution when compared with other experiments. Hence, the flow rate 1.5 mL min\(^{-1}\), acetonitrile composition 700 Ml, and column temp 25 °C were finalized for the method validation.

**Method Validation**

The analytical method validation [32, 33] of HCQ and related impurities (DHC, HCA, HCS, DCQ) was performed as per the current ICH guidelines.

**Specificity**

To determine the specificity of the method prepared HCQ standard (2000 µg mL\(^{-1}\)) and impurities (10 µg mL\(^{-1}\) DHC, HCA, HCS, DCQ) and injected into HPLC. The HPLC chromatogram was verified interference placebo peaks corresponding to HCQ. Further, specificity of the HCQ and impurities were determined by using forced degradation of acid (Fig. 5), base, oxidation, photolytic, thermal (Fig. 6), and humidity samples. All the stressed chromatograms demonstrated no interference of any other peaks corresponding to excipients present in the formulations. The major degradation was shown in the oxidation sample (> 10%), remaining all conditions are shown less than 1% of degradation. In the majority of stressed conditions, the resolution between the peaks was adequate. Further, the peak purity was verified by using the empower software. In all the conditions, peaks were shown purity angle less than that of purity threshold. Then, the mass balance was calculated and it was found near to 100%, and the results are shown in Table 6.
Fig. 3 Typical individual chromatograms of a DHC; b HCQ; c HCA; d HCS; e DCQ
Precision and Ruggedness

To determine the precision of the test method, six individual sample solutions were prepared from homogenous mixture of tablet powder, further spiked the impurity mixture at 100% level (DHC, HCA, HCS, DCQ) and injected in to HPLC. To prove the ruggedness (intermediate precision) of the method, the samples prepared on the different day, different chemist, and different column used to know the impact of inter-lab variability. The % of RSD was calculated for each individual spiked impurity from precision and intermediate precision, and it was found less than 2.0%. The individual RSD values were shown in Table 7 and the chromatogram was shown in Fig. 7.

Accuracy

The accuracy of the test method was demonstrated by standard addition method. The placebo was prepared as per the test method and spiked the HCQ and impurities in three different concentration levels (50, 100, and 150%), and for each level, triplicate samples were prepared. The % recovery was calculated for HCQ and impurities, and the results are found satisfactory. The results are shown in Table 7.

Limit of Quantification and Limit of Detection

A study was conducted to establish limit of detection (LOD) and limit of quantification (LOQ) for HCQ, DHC, HCA, HCS, and DCQ by slope method. The minimum concentration was established at which all these impurities were quantified with acceptable precision and accuracy. The results are tabulated in Table 7.

Linearity Range

To demonstrate the linearity of the optimized method, different concentrations of HCQ solutions and DHC (not less than 7 concentrations) range from 0.201 to 6.03 µg mL⁻¹, HCA 0.500 to 6.004 µg mL⁻¹, HCS 0.300 to 5.999 µg mL⁻¹ and DCQ 0.302 to 6.032 µg mL⁻¹ were prepared. Each concentration peak area was recorded in triplicate, and taken average area from triplicate injections. The linearity of each component was determined by the linear regression method, where all the seven calibration curve levels were found to be in linear concentration range from LOQ to 150% of target specification level. The linear regression equation for the calibration curve was shown in Fig. 8, and the results are tabulated in Table 7.

Conclusion

A Qbd-based simple and stability indicating LC method has been developed successfully for HCQ and its related impurities. The optimized HPLC method can detect and estimate the HCQ impurities in its bulk and finished solid oral dosage forms. In the current QbD process, evaluated factors significantly influencing on the resolution of impurities by performing simultaneous variation of factors under the DoE approach. In addition to the resolution of impurities, acceptable design space was established for chromatographic conditions. By using the simple
**Fig. 4**  
(a) Half-normal plots, (b) pareto charts, (c) 3-D response surface plots, (d) cube model graph depicting the impact of R1 factor (resolution between DHC and HCQ)

**Table 5**  
ANOVA results obtained for factorial model

| Source       | Sum of squares | df  | Mean square | F value | p value  |  
|--------------|----------------|-----|-------------|---------|----------|  
| Model        | 0.0264         | 2   | 0.0132      | 4.46    | 0.0356   | Significant  
| A-Flow rate  | 0.0021         | 1   | 0.0021      | 0.7203  | 0.4127   |  
| C-ACN ratio  | 0.0243         | 1   | 0.0243      | 8.20    | 0.0142   |  
| Residual     | 0.0355         | 12  | 0.0030      |         |          |  
| Lack of fit  | 0.0341         | 2   | 0.0171      | 121.93  | <0.0001  | Significant  
| Pure error   | 0.0014         | 10  | 0.0001      |         |          |  
| Cor total    | 0.0620         | 14  |             |         |          |  

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Fig. 5  Forced degradation chromatograms of acid sample stressed at 2 N HCl at 70 °C for about 21 h

Fig. 6  Forced degradation chromatograms of thermal sample stressed at 7 days for 105 °C

Table 6  Forced degradation conditions as per ICH and results

| Nature of stress | Condition                                      | Total impurities | % Assay of stressed sample | Purity angle | Purity threshold | Purity flag |
|------------------|------------------------------------------------|------------------|-----------------------------|--------------|------------------|-------------|
| Unstressed       | NA                                             | 0.34             | 99.7                        | 0.092        | 0.273            | No          |
| Acid             | 2 N HCl at 70 °C for about 21 h                | 0.25             | 97.2                        | 0.088        | 0.275            | No          |
| Base             | 2 N NaOH at 70 °C for about 30 min             | 0.32             | 97.8                        | 0.097        | 0.277            | No          |
| Peroxide         | 10% H₂O₂ at 70 °C for about 6 h                | 11.41            | 88.3                        | 0.122        | 0.327            | No          |
| Photolytic       | 200watt hour/m² for UV light and 1.2 million lux hours for visible light | 0.33             | 99.8                        | 0.086        | 0.276            | No          |
| Thermal          | 7 Days for 105 °C                              | 0.47             | 98.8                        | 0.089        | 0.273            | No          |
| Humidity         | About 85% RH at 25 °C for 7 days               | 0.36             | 101.5                       | 0.086        | 0.274            | No          |
chromatographic conditions, the optimum resolution was achieved with good peak symmetry. We tried to explain the possible degradation pathways and performed the forced degradation to prove the stability indicating the nature of the method. No interference was observed in the FDS conditions, and which was specific for the estimation of impurities. The lower LOD and LOQ values demonstrated high sensitivity of the method. The optimized method was validated as per the ICH guidelines, and the results are found satisfactory. Finally, developed method was used in the quality control lab for the analysis of stability samples.

Table 7  Linearity and other validation parameters results obtained by the proposed HPLC with HCQ and related impurities

| Parameters            | DHC    | HCQ    | HCA    | HCS    | DCQ    |
|-----------------------|--------|--------|--------|--------|--------|
| Retention time (Rt)   | 9.701  | 11.644 | 16.200 | 19.629 | 36.527 |
| Relative retention time (RRt) | 0.888  | 1.00   | 1.239  | 1.319  | 2.306  |
| Relative response (RR) | 0.93   | 1.00   | 1.00   | 0.74   | 2.38   |
| Linearity             |        |        |        |        |        |
| Range (µg mL⁻¹)       | 0.201–6.034 | 0.200–6.004 | 0.500–6.004 | 0.300–5.999 | 0.302–6.032 |
| Slope                 | 26,427.93 | 29,236.95 | 27,598.21 | 21,329.40 | 69,212.61 |
| Intercept             | −139.06 | 528.45 | 164.11 | −463.92 | 8672.17 |
| Correlation coefficient | 0.9999 | 0.9999 | 0.9999 | 0.9999 | 0.9999 |
| LOD (µg mL⁻¹)         | 0.067  | 0.066  | 0.166  | 0.101  | 0.101  |
| LOQ (µg mL⁻¹)         | 0.201  | 0.200  | 0.500  | 0.300  | 0.302  |
| Accuracy (% of recovery) (a) | 101.2 ± 2.35 | 101.22 ± 1.54 | 98.52 ± 0.98 | 97.25 ± 2.06 | 102.65 ± 1.61 |
| 50% Mean ± SD         | 101.2 ± 2.35 | 101.22 ± 1.54 | 98.52 ± 0.98 | 97.25 ± 2.06 | 102.65 ± 1.61 |
| 100% Mean ± SD        | 98.5 ± 1.89 | 101.25 ± 2.92 | 102.58 ± 2.55 | 102.58 ± 3.58 | 98.59 ± 2.28 |
| 150% Mean ± SD        | 99.2 ± 3.6  | 98.25 ± 1.05 | 99.21 ± 1.81 | 98.25 ± 3.14 | 99.85 ± 0.68 |
| Precision (%RSD) (b)  |        |        |        |        |        |
| Repeatability         | 0.62   | 0.26   | 0.89   | 0.43   | 0.91   |
| Intermediate precision| 1.25   | 0.68   | 1.05   | 1.47   | 1.68   |

(a) Average of three determinations of each concentration levels
(b) RSD of six determinations of each component

Fig. 7 Typical chromatograms of spiked sample with impurities for precision
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Compliance with Ethical Standards

Conflict of Interest  The authors declare the absence of conflicts of interest.

Ethical Approval  This article does not contain any studies with human participants performed by any of the authors.

Fig. 8  Linearity curves of HCQ and related impurities

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