ANALYTICAL METHODS FOR THE DETERMINATION OF HYDROXYCHLOROQUINE IN VARIOUS MATRICES

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Received: 26 May 2020, Revised and Accepted: 20 Jun 2020

ABSTRACT
Hydroxychloroquine (HCQ) is classified under the class of drugs called antimalarials. This is used for prevention and treatment of malaria. HCQ is also used in the treatment of DLE (Discoid Lupus Erythematosus) or SLE (Systemic Lupus Erythematosus) and RA (Rheumatoid Arthritis). Recently, this drug attracts its attention by scientists of all of the worlds for its potential activity in the improvement of conditions of covid patients. There are many clinical trials are under process to prove its activity against this dangerous virus. The presented review describes different analytical procedures for the analysis of HCQ in various components available in the currently available literature. The paper will be certainly helpful for the scientists and researchers engaged in research, especially in the development of formulation or quality assurance of HCQ. The results of any clinical trial also includes the determination of drug in body fluids for interpretation of data. The analytical methods described here are explained in three parts; spectrophotometry, chromatography and other (including capillary electrophoresis and electroanalytical methods)

Keywords: Hydroxychloroquine, Spectrophotometry, Chromatography methods, Electroanalytical methods, Methods for determination of Hydroxychloroquine, Determination of hydroxychloroquine

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DOI: http://dx.doi.org/10.22159/ijap.2020v12i4.34304. Journal homepage: https://innovareacademics.in/journals/index.php/ijap

INTRODUCTION
Hydroxychloroquine (HCQ) is more commonly used than chloroquine, is a well-tolerated Disease-modifying anti-rheumatic drugs (DMARD) that is commonly used in combination therapy regimens for RA [1]. The IUPAC name of HCQ (C18H26ClN3O) is (RS)-2-[(7-Chloro-4-quinolylamino)pentyl(ethyl)amino]-ethanol [2].

The HCQ is synthesized by an antimalarial drug, chloroquine (CQ) with incorporating hydroxyl group in its structure, in the year 1946. After the discovery of HCQ, it was found to be two to three times less toxic than CQ [3]. Hydroxychloroquine (fig. 1) is a 4-aminoquinoline shown to possess the above-described activities. This is also known to be an immunosuppressant drug. The abnormalities caused by HCQ therapy is rare compared with CQ, which is also associated with acute liver lesion [4]. HCQ is available in the market in the form of tablet formulations. The dose of HCQ ranges from 100 mg to 1.2 grams per day, quickly absorbed in two to four hours. The absorbed portion is about 74% ±13%. The blood concentration of HCQ rises shortly after absorption but fall rapidly due to fast distribution into organs of the body [5].

Antimalarial drugs modulate the immune system through their known ability to influence the pH in intracytoplasmic vesicles. As discussed in the previous section, after fast absorption, the deposits of HCQ and CQ extensively distributes in the tissues. The drug is concentrated intracellularly, particularly in acidic cytoplasmic vesicles. The deposition of HCQ in immune system cells is the reason of its antirheumatic effect [6].

The novel virus spread (Covid-19) from Wuhan province of China in the year 2019 is now a threat for economy and health infrastructure [7]. The world’s apex health organization, WHO declared this as pandemic because of rapid spread in almost every part of the world [8, 9]. In the stage of writing this article, the available literature indicates more than 300 clinical trails (ongoing), and different drugs are in the phase of evaluation for potential effects against covid-19 [10].

One of the advantages of HCQ over its parent drug CQ is tolerance in higher doses form longer periods. The US FDA approved HCQ for its marketing in their country in 1994 [11]. The effect of HCQ and CQ on...
viral replication goes beyond the inhibition of cytokines. These drugs are weak bases known to influence acidic vesicles and inhibition of many enzymes. The enzyme glycosyltransferases is inhibited by HQC and decreasing the occurrence of viral replication of some of its families. This is believed to be one of the potential explanations of its antiviral mechanism of these drugs [12].

**HCQ and covid-19**

The fast outbreak of new coronavirus (SARS-CoV-2) has now become a serious concern for health for almost all countries of the world [13, 14]. The mechanism of entry of this virus into the host cell is believed to be the result of interaction between ACE2 (Angiotensin-converting enzyme) and spiral protein of virus [15]. This is now established that elderly people and people with other comorbidities e.g. diabetes (TYPE 2) are in high risk with a high death rate [16].

In spite of best efforts by almost all health-related researchers, till the mid of June of this year, we have not found any therapeutic option for this virus and this is the reason of declared “public health emergency” in most of the countries, including Indian subcontinent. Researchers are investigating many drugs for treatment, including these low-cost antimalarials; HCQ and CQ [17].

The reputed government research organization of India, Indian Council of Medical Research (ICMR) recommended the dose of HCQ 400 mg twice on day 1 and once/week after that) for chemoprophylaxis to health care workers handling patients of covid in both confirmed and suspected infections cases. Also recommended for household contacts (asymptomatic) for any confirmed cases [18].

The healthcare system of the whole world is facing an alarming situation because of spread of new coronavirus. Thus, there is an urgent requirement for therapeutic option for control of new coronavirus. HQC is a derivative of Chloroquine (CQ), currently under investigation may be therapeutic option against COVID-19 infection. HQC was found to have less acute poisoning than CQ and share a similar mechanism of action and structure [19].

The mechanism of action of HCQ against covid 19 virus is still under investigation. It is believed to change the pH of endosomes. Because of this the entry of virus, its transportation and postentry events are believed to be prevented [17]. The report suggests halting replication and modification of virus and also inhibition of glycosyltransferases [20]. In vivo, hydroxychloroquine is metabolized into chloroquine [21].

The HQC and its parent drug CQ both have comparable pharmacological activity as antimalarial agents, but former is preferred due to less ocular toxicity. The available literature suggested the prolongation of QT interval by using CQ in combination with other antiretroviral drugs such as ritonavir or lopinavir, but this problem is less in HCQ and this is the reason of its selection for covid patients [22].

Thus, apart from the development of medication in this pandemic, there is a definite need of the literature presented here to help scientists or researchers involved in the investigation of development of a formulation of HQC. This article is a comprehensive review of analytical methods published so far for the analysis of HQC in a different mixture of components.

**MATERIALS AND METHODS**

Author searched various online libraries available online related to determination of Hydroxychloroquine. The databases searched are, pubmed, wiley, sciencedirect, taylor and francis, nature, BMJ and google scholar. The keywords used for search are ‘determination of Hydroxychloroquine’, ‘estimation of Hydroxychloroquine’, and ‘Hydroxychloroquine in covid-19’.

**Analytical methods**

**Spectrophotometry methods**

The quantitative analysis performed by spectrophotometry is due to the interaction of radiations of ultraviolet and some part of infrared region with sample and has an influence on variety of different fields of science and technology. There are many changes in a spectrophotometer in last few decades due to advancement in technology with inclusion of many new features for different type of samples and optical properties [23].

Five spectrophotometric methods [24-27] were found from various sources available in internet. Feraz et al. [24] used USP method [26] and performed quality by design in the determination of hydroxychloroquine in tablet formulations. The limit of quantitation and limit of quantitation found are 1.27 and 0.38 µg/ml respectively.

In another method 0.1 N HCl was used as diluent and determination performed at same wavelength as in the previously described method. Pharmacopical method found cited here is referred from United States Pharmacopoeia [25].

Two methods in single paper in which one involves simple spectrophotometry method and another first derivative method developed by Mehta and Patel. In this method water is used as diluent for the preparation of sample in contrast to other methods using acidic medium [27].

**Chromatography methods**

There are three different characteristics of chromatography separations: (a) these are a physical method for separation of components; (b) involvement of two different phases, stationary phase and mobile phase; and (c) separation of components on the basis of distribution constants of individual components in stationary and mobile phase. There are several important factors responsible for the acceptable separation of sample [28].

The first paper related to the chromatography method was found to be published in 1985. HQC and its metabolites are basic compounds and fluoresce at high pH, facilitating sensitive detection using chromatographic methods. This may be the reason that most of the methods utilizing spectrometry detection are based on fluorescence technique. First published paper is assay for HQC and three major metabolites (fig. 1), using fluorescence detection in blood and plasma of RA patients. In this method chloroquine was used as an internal standard. Nine different anti-inflammatory drugs indicated in rheumatoid arthritis administered together with HQC were found not interfere with the method [29].

The next method is related to quantitation for HQC and three major metabolites (fig. 1), in human plasma using a fluorescence detection method. Determinations were performed in two sets of samples (1) fresh and immediately prepared sample, (2) stored for 18 d in

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**Table 1: Summary of UV spectrophotometry method**

| Calibration curve/Target concentration | Solvent/Diluent | Wavelength | LOD and LOQ | Matrix | Reference |
|---------------------------------------|----------------|------------|-------------|--------|-----------|
| 8-12 µg/ml                            | 0.01 M HCl     | 342 nm     | 0.38 and 1.27 µg/ml | Raw and formulation | [24]       |
| 1-20 µg/ml                            | 0.1 N HCl      | 343 nm     | Not mentioned | Raw and formulation | [25]       |
| 10 µg/ml                              | Dil. HCl (1 in 100) | 251-261 nm | 0.37, 1.1212 µg/ml | Raw material | [26]       |
| 2-12 µg/ml                            | Water          | 347.80 nm (First derivative) | 0.3145, 0.9530 µg/ml | Bulk drug and formulation | [27]       |
freezer prior for analysis. This method is useful for application in clinical samples, as claimed by researchers [30].

Determination of HCQ and its three metabolites in blood and urine samples is also available with the claimed to be without the interference of chloroquine or quinine in the sample. Analyses was performed in whole blood and urine samples from two healthy, male, adult volunteers during weekly antimalarial chemoprophylaxis. Fluorescence detector was used at $\lambda_{ex} = 320$ nm and $\lambda_{em} = 380$ nm [31].

Wainer and Iredale (1992) developed method for the determination of the enantiomers of hydroxychloroquine, and three major metabolites (fig. 1) in plasma, using a Chiral-AGP chiral stationary phase. Method validated for the pharmacokinetic of HCQ in rabbit and includes two steps (1) achiral chromatography and (2) enantioselective chromatography using two different mobile phases [32].

Another method is achiral-chiral HPLC system has been developed for the quantitative analysis of HCQ in urine. Determinations performed for HCQ and the same three metabolites discussed earlier. This study compiles the analysis in single oral dose pharmacokinetic study (200 mg HCQ sulfate) [33].

Analysis and separation method of HCQ enantiomers and metabolites in two steps, developed by Wei et al. [34]. In this method authors found unsatisfactory separation using chiral column for HCQ enantiomers and thus developed method in two stages. In the first step achiral separation is performed and racemates were collected. The second step is the separation of enantiomers using chiral column for drugs and metabolites [34].

Another HPLC method for analysis of quinine, HCQ, CQ and DCQ is also available. The method is applied in whole blood, serum, and blood (dried) adsorbed in filter paper. The method is useful for analysis in a particularly small volume. Method described fluorescence detection and claimed to obtained within an hour of receipt of the sample [35].

Another HPLC method in serum samples for simultaneous determination of HCQ, CQ and corticosteroids from rheumatoid arthritis patients developed by Volin [36]. Patients received HCQ sulphate or CQ diphosphate and then determination performed in serum samples using diode array detection [36].

Method to quantify enantiomers is described and applied in determination of HCQ in rat liver microsomes. This is a single-step method for chiral separation using UV detection ($\lambda = 434$ nm) [37].

The LC-MS/MS determination is performed by using hydroxychloroquine-d4 as the internal standard. A high throughput analysis is claimed, as run time is just three minutes and applied in human plasma [38].

The LC-MS/MS analysis is performed for the separation of three metabolites and parent drug (HCQ) in human plasma. Method may be applied for monitoring blood concentration of drug and metabolites, especially in the case of poisoning [39].

Plasma determination of HCQ was developed and validated by using the RP-HPLC-UV method using chloroquine as an internal standard [40].

Fluorescence detection clubbed with HPLC method is also available and applied used to monitor the blood level of HCQ in Systemic Lupus Erythematosus (SLE) patients [41].

An UPLC method using UV detection is developed, aimed for analysis of HCQ in serum samples. Method is applied for monitoring drug concentration in patients suffering from Whipple’s disease and Q fever [42].

A VAMS method (volumetric absorptive microsampling) based LC-MS/MS method is also reported for therapeutic drug monitoring. The absorption of a fixed volume of blood (10 μl) is possible by using VAMS method, with decreased variations in sampling especially problems related to dried blood spot are its additional advantages [43].

An HPLC-FL ion-pairing method for the determination of HCQ with its three metabolites i.e. DHQ, DCQ, and BDCQ is also available. The method was applied for monitoring drug concentration in in patients suffering from Systemic Lupus Erythematosus (SLE). The method was also compared with the already available LC/MS/MS method and claimed to be applied for routine clinical monitoring [44].

The quantitation of HCQ and its three metabolites (DHQ, DCQ, and BDCQ) in blood and tissues through LC-MS/MS is one of the recent publication. Method can also be utilized for pharmacokinetic monitoring of HCQ [45].

A recent published HPTLC with UV detection method is for the simultaneous determination of three different drugs in blood and urine samples of RA patients. The principles of Quality by Design were also used. Therapeutic monitoring and pharmacokinetic determination of drug are applications of this method [46].

**Electroanalytical methods**

The recent development of electrical methods for analytical determinations makes them more selective, sensitive, rapid and easy compared to the equipment available after its development and now applicable for most of the fields of pharmaceutical and chemical analysis. Electrochemical methods nowadays enables usage for trace analysis also for pharmaceuticals with a sufficient degree of precision, accuracy, selectivity, sensitivity and reproducibility [47].

A differential pulse voltammetry method using glassy carbon electrodes developed by Arguelho et al. [48]. This is the first electrochemical method found in the available literature. Best current for analysis of HCQ was obtained at pH 4 and scan rate between 2 and 8 mV/s. In the width higher than 100 mV researchers observed loss of resolution. The detection limit reported is 11.29±2.6 mg mL$^{-1}$ [48].

The first capillary electrophoresis method for simultaneous analysis of hydroxychloroquine (HCQ) with its three metabolites, in micromolar fraction of liver homogenates. The quantitation limit reported is 125 ng/ml, and detection performed at 220 nm. The researchers found that major metabolite of HCQ by microsomal enzymes is (R)-(R)-deethylhydroxychloroquine (DHQ) [49]. Another capillary electrophoresis method for determination of HCQ, its enantiomers and metabolites in the concentration range of 10–1000 ng/ml for parent drug applied in human urine. The limit of detection and quantitation values reported were 10 and 21 ng/ml respectively [50].

In other electrochemical method determination was performed by using glassy carbon electrode prepared in laboratory by using cyclic voltammetry, double potential step chronocoulometry and linear sweep voltammetry (LSV) techniques. The detection limit reported in this method is 4.65 nM. The prepared modified electrode was then utilized for determining of HCQ in human body fluids [51].

Determination of HCQ in synthetic urine and in pharmaceutical tablets by the square-wave voltammetry method is also available. The reported detection limit is 0.06 μmol L$^{-1}$. The electrode utilized was cathodically pretreated (CPT) boron-doped diamond (BDD) electrode [52].

Potentiometric determination of HCQ in pure form, tablets and human urine samples using standard addition method. Silver-silver chloride and modified paste electrodes were used for analysis. The limit of detection reported was up to 5.0× 10$^{-6}$ mol L$^{-1}$ [53]. Similar method by using coated graphite electrode (GGE) is also reported. The detection limit found is 1.8×10$^{-5}$ mol L$^{-1}$ and response time ≤ 10 s [54].
PO
PO
HPO
1% triethylamine and 1
determination of HCQ with nearly the same procedures and
than chromatography. There are limited methods available for the
determination of HCQ, a member  of the group of 4-aminoquinolines [57] and is a
slow-acting antirheumatic drug [58]. It is administered as a racemic
mixture [rac-HCQ] of two isomers, R(-)-HCQ and S(+)-HCQ. The
hepatic metabolism generates three metabolites, DHCQ, BDCQ and
DQ. These three molecules are also chiral molecules [59].

There are five spectrophotometry methods found in the available
literature. These all methods are for routine quality control of HCQ
in bulk or formulations. Spectrophotometry methods are cheaper
than chromatography. There are limited methods available for the
determination of HCQ with nearly the same procedures and
dilutions. The summary is provided under table 1.

| Method   | Column                  | Mobile phase          | pH | Flow rate | LOD  | LOQ   | Matrix     | Reference |
|----------|-------------------------|-----------------------|----|-----------|------|-------|------------|-----------|
| HPLC-F   | Poly(styrene
divinylbenzen e) packing | Methanol (80): Water(20) | 11 | 1 ml/min  | -    | -     | Blood and plasma | [29]      |
| HPLC-UV  | CN                      | 0.06 M Di-butylamine Phosphate (40): 0.05 M Monobasic sodium Phosphate (60) | 3.5 | 1.2 ml/min | -    | -     | Plasma [30] |           |
| HPLC-F   | ODS                     | Hexane: tert butyl ether: 0.5% n-butylamine in methanol (1:1:1) | -  | 1 ml/min  | 10 ppb | -      | Blood and urine | [31]      |
| HPLC-UV  | CN                      | 0.03 M sodium phosphate buffer, pH 7.0-ethanol-acetonitrile (79:20:1) | -  | 0.9 ml/min | 10 ng/ml | -     | Plasma [32] |           |
| HPLC-UV  | CN                      | Na2PO4 Buffer (0.03 mol/l): ethanol: and acetonitrile (7:20:1) | -  | 0.7 ml/min | -    | -     | Plasma and in urine | [33]      |
| HPLC-UV  | ODS                     | Phosphate buffer-methanol: ethanol: Triethanolamine (78:22.1:0.8, v/v) | -  | 0.75-0.8 ml/min | -    | -     | Plasma | [34]      |
| HPLC-F   | CN                      | Buffer pH 3.5          | -  | 2 ml/min  | <1 ng | -     | Blood [35] |           |
| HPLC-F   | Silica                  | Methanol: water (98.5:1.5)+4.705 g NH4Cl | 8.9 | 1.5 ml/min | -     | 0.005-0.01 mg/l | Serum, whole blood or filter paper-adsorbed dry blood | [36]      |

| Method   | Column                  | Mobile phase          | pH | Flow rate | LOD  | LOQ   | Matrix     | Reference |
|----------|-------------------------|-----------------------|----|-----------|------|-------|------------|-----------|
| HPLC-PDA | ODS                     | 50 mmol monobasic NaH2PO4 buffer (7 g/l)+6 mmol heptanesulfonic acid (sodium salt) (1.3 g/l) | 3.1 | 1.0 ml/min | 2 ng/ml | -     | Serum [37] |           |
| HPLC-UV  | C8                      | Hexane: Isopropanol (92.8)+0.1% Diethyl amine | -  | 1.0 ml/min | -     | 125 ngmL⁻¹ | Rat liver microsomes | Blood | [38]      |
| LC-MS/MS | C8                      | Formic acid (0.1%): ACN (94.6: v/v) | -  | 0.5 ml/min | 2 ng/ml | -     | Blood [39] |           |
| LC-MS/MS | ODS                     | Water and methanol (Gradient)+0.1% formic acid | -  | 0.5 ml/min | 25 ng/ml | -     | Blood [40] |           |
| HPLC-UV  | ODS                     | Water: Solvent (ACN: methanol: 50:50) in ratio 7:5:25 | 3.0 | 2.0 ml/min | 0.24 μg/mL | 0.84 μg/mL | Plasma | [41]      |
| HPLC-F   | ODS                     | Glycine buffer/NaCl (pH 9.7, 1.00 mmol) and methanol (46:54; v/v) | -  | 1.2 ml/min | 25 ng/mL | -     | Blood [42] |           |
| UHPLC-UV | Cl8                     | 1% triethylamine and 1 mmol oxalic acid | 2.4 | 0.5 ml/min | -     | 0.25 μg/mL | Serum | [43]      |
| LC-MS/MS | C8                      | 0.1% HCOOH and 0.01% triethylamine in water (or acetonitrile) | 0.5 ml/min | 5 ng/mL | 10 ng/mL | Blood | [44]      |
| HPLC-FL  | ODS                     | Water: methanol: ACN (47:10:43) | 9.4 | 1.0 ml/min | -     | 20 ng/mL | Blood | [45]      |
| LC-ESI-MS/MS | Cl8 | Gradient: Part A: 0.2% formic acid in water, Part B: 0.1% formic acid in methanol | 2.0 L/min | 1 ng/mL | -     | -     | Blood and tissues. |           |
| HPTLC    | -                       | Ethyl acetate: methanol: 25% ammonia, (8: 2: 3) | -  | 260,64 ng/ml | 1810.01 μg/mL | -     | Serum and urine samples | [47]      |

DISCUSSION
The Q and its hydroxyl derivative HCQ are weak bases with five
decades usage as antimalarial agents [55]. HCQ is one of the drugs
generated significant interest for the treatment of COVID-19
infection. There are research published providing some idea about
the activity of HCQ against this novel coronavirus [56].

HCQ, a member of the group of 4-aminoquinolines [57] and is a
slow-acting antirheumatic drug [58]. It is administered as a racemic
mixture [rac-HCQ] of two isomers, R(-)-HCQ and S(+)-HCQ. The
hepatic metabolism generates three metabolites, DHCQ, BDCQ and
DQ. These three molecules are also chiral molecules [59].

The HPLC methods are versatile and can be used as both analytical and
preparative technique. Organic molecules are detected after exit from
column using absorption pattern in UV/Visible radiation. As described
in spectrophotometry method, HCQ showing maximum absorption at
342 nm in UV region, many chromatography coupled with
spectrophotometry detection method were developed [60]. The
testing of enantiomers are quite often with HPLC in last few decade.

The research-based on photochemical degradation of HCQ, followed
by HPLC is also available. Degradation performed by an immersion
lamp with emission wavelengths of 240-600 nm, 120 W. Degradation products were isolated first by using preparative TLC and isolated fractions were then identified using MS detection method [63].

There is another paper described forced degradation studies of HCQ. The stress conditions applied for degradation of drug through hydrolysis, dry heat and photolysis. The researchers found six different degradation products when photolytic degradation was attempted under alkaline conditions. The products were characterized through LC-MS-TOF, LC-ESI-MS and LC-PDA studies [64].

In another study the samples used were HCQ solutions in water from a different source; spring, sea, river and ultrapure water and photolytic decomposition of HCQ investigated using simulated solar radiation (300-800 nm). It was found that humic acids, nitrate and iron (III) enhanced the photodegradation of HCQ due to the formation of hydroxyl radicals and their attack on the molecule of HCQ. Contrary to this, chloride, bromide and sulfate inhibited the photodegradation of HCQ [65].

**Future aspects**

There are a limited number of spectrophotometry methods available in the literature. There are many methods available in which chromophore is added in molecule by any chemical reaction to increase the absorbance in UV region. This is one of the ways to increase the sensitivity of method. While going through this particular class of analytical method for determination of HCQ, and to the best of knowledge of author, no such method has been developed and validated. Stability indicating method using spectrophotometry analysis is also not found. However, this is also well evident that the separation of analytes using simple UV-Vis is difficult as compared to chromatographic methods. There are different types of chromatographic methods available in the literature including HPTLC method. The HPTLC method for the determination of HCQ is still needs to be explored. The stability-indicating HPTLC method is also not available.

**CONCLUSION**

The FDA of United States approved HCQ in 1955 and licensed for a treatment option for SLE and inflammatory arthritis before the outbreak of new coronavirus. This drug in the current scenario HCQ is receiving the attention of our scientists because of its action in immune system and antiretroviral effect. India has enormous potential and capacity of manufacturing HCQ tablets in large scale. There is a surge in demand of HCQ after covid pandemic from almost every part of world due to its potential activity against this virus. However, more studies are required to establish its activity in improving conditions of covid patients. The activity of HCQ against covid 19 virus is already discussed in the introductory section. In the current scenario, covid is biggest enemy of humanity. The socio-economic implications due to lockdown is believed to be huge. Thus, there is a sudden need for any drug to save the life of covid patients and scientists in almost all parts of the world are engaged in the development of suitable drug. The review is presented with an aim to help scientists or researchers involved in the development of the formulation of HCQ. Different spectrophotometry, chromatography and electroanalytical methods are presented here in a systematic way and easy understandable language.

**FUNDING**

Nil

**AUTHOR CONTRIBUTION**

The whole work carried by me.

**CONFLICT OF INTERESTS**

None

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