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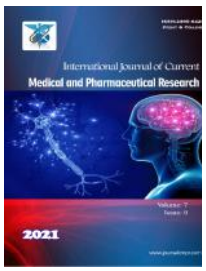

Research Article

MEASUREMENT OF HYDROXYCHLOROQUINE (HCQ) IN BLOOD IN ASYMPTOMATIC HEALTHCARE WORKERS ON PROPHYLACTIC REGIMEN FOR COVID-19 INFECTION - AN OBSERVATIONAL STUDY

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ABSTRACT

Background: Hydroxychloroquine (HCQ) is an inhibitor of COVID-19, demonstrated by in-vitro data. There is no pharmacokinetic data on HCQ for pre-exposure prophylaxis in Indians. We estimated the peak and trough blood levels achieved on prophylactic dose HCQ administration.
Methods: We conducted an observational study from July to October 2020 at St. John's Medical College and Research Institute, Bengaluru, India. 24 asymptomatic healthcare workers taking HCQ prophylaxis for COVID-19 infection, as advised by their treating physicians were included. The pre-dose (0.00 hour) blood sample (2 mL each) was collected prior to dosing on day 1 (morning dose) and prior to each weekly dose for the next seven weeks. The post-dose samples (2 mL each) were collected 4.00 hours after the first dose on day 1 and also on 3rd (340 h) and 5th week (676 h). Altogether 11 blood samples were collected from each volunteer. HCQ in blood was determined using a validated LCMS/MS method. Amodiaquine was used as internal standard. The minimum HCQ trough concentration to inhibit 50% of viral infection (EC_{50}) is 0.72µM.
Results: Mean pre dose trough concentration (± SD) in ug/mL on day 7, 14, 21, 28 and 35 was 0.0554 (0.0406); 0.0770 (0.0340); 0.0835 (0.0368); 0.0869 (0.0372) and 0.1072 (0.0639) respectively. A gradual increase in trough concentration of HCQ was noticed till day 35. This concentration remained the same on day 42 and day 49. HCQ was tolerated well by the volunteers and all of them completed the study without being reported as COVID-19 positive.
Conclusion: In our study, HCQ at the 800 mg loading dose followed by 400 mg once a week did not attain the minimum HCQ trough concentration required to inhibit 50% of viral infection (EC_{50}).

INTRODUCTION

The positive sense RNA virus, SARS-CoV-2 belongs to the family coronaviridae and is the etiological agent responsible for the novel pneumonia (COVID-19). An outbreak of COVID-19 occurred towards the end of 2019 in Wuhan, China.[1]After that, the disease has spread in an unprecedented manner across the world affecting more than 90 million people as on 18th January, 2021.[2]On March 11, 2020, WHO declared COVID-19 as a 'global pandemic'. This rapid progression of COVID-19 pandemic led to overwhelming interest in treatment and prevention therapeutics. At present there is no approved therapy for COVID-19. A number of trials have been conducted, including hydroxychloroquine, dexamethasone, remdesivir, tocilizumab, intravenous immunoglobulin, and convalescent plasma. [3][4]Hydroxychloroquine (HCQ), a safer derivative of anti-malarial drug chloroquine, has shown a promising antiviral effect in various studies. It's possible mechanism of actions includes inhibition of viral attachment, entry into the host cell, new viral particle maturation and spread.HCQ is a potent inhibitor of COVID-19 as indicated by *in vitro* data. [5]

However, a prospective, placebo-controlled study enrolled 456 participants (152 in each of three groups: placebo, oral HCQ (600 mg daily for one week), or oral HCQ plus oral azithromycin (500 mg day one, 250 mg daily on days two through five) showed that HCQ+ azithromycin did not facilitate virologic cure in patients with mild or asymptomatic COVID-19.[6]

Similarly, interim results from the Solidarity Therapeutics Trial, coordinated by the World Health Organization, indicate that remdesivir, HCQ, lopinavir/ritonavir and interferon regimens appeared to have little or no effect on 28-day mortality or the in-hospital course of COVID-19 among hospitalized patients.[7]

On the other hand, consumption of four or more maintenance doses of HCQ was associated with a significant decline in the odds of getting infected (AOR: 0.44; 95% CI: 0.22-0.88); a dose-response relationship existed between frequency of exposure to HCQ and such reductions ($\chi^{[2]}$ for trend=48.88; P <0.001).[8]

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Another study showed that for once weekly HCQ prophylaxis, the hazard ratio was 0.72 (95%CI 0.44 to 1.16; P=0.18) and for twice weekly was 0.74 (95%CI 0.46 to 1.19; P=0.22) as compared with placebo. Median HCQ concentrations in whole blood were 98 ng/mL (IQR, 82-120) with once-weekly and 200 ng/mL (IQR, 159-258) with twice-weekly dosing. HCQconcentrations did not differ between participants who developed COVID-19 (154 ng/mL) versus participants without COVID-19 (133 ng/mL; P=0.08).[9]

There is an increased risk of contracting the disease for healthcare workers. Infections among healthcare workers will lead to catastrophic hospital outbreaks and pose grave risks to admitted patients. It is reported from various countries that about 3·5 – 20% of healthcare workers have acquired the disease.[10] HCQ has favourable pharmacokinetics like long half-life, high concentration in the lung tissue and acceptable safety profile. Due to this HCQ becomes one of the most preferreddrugs for study as a chemoprophylactic agent for COVID-19. *In vitro* studies showed that HCQ was effective against the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) virus.[5]However, the half maximal effective concentration (EC_{50}) for SARS-CoV-2 virus is different than for malaria with a > 20-fold higher *in vitro* EC_{50} of HCQ for SARS-CoV-2 vs. malaria. EC_{50} values for SARS-CoV-2 virus in the literature have ranged from 0.72–17.31 μM. [5][11]

It was not approved in any country for SARS-CoV-2 prevention till date. HCQ is also proposed as a prophylactic agentin those at high risk, such as healthcare workers, the immune-compromised and household contacts of infected individuals. However, there are no scientifically established doses for SARS-CoV-2. Several groups used pharmacometric modelling and simulation to propose potential regimens. No models have specifically evaluated regimens in the context of prophylaxis. Using a simulated modified dosing scenario, Al-Kofahi *et al*.[12] found that for pre-exposure prophylaxis, an 800 mg loading dose, followed by a 400 mg dose given 2 or 3 times weekly maintains weekly troughs above EC_{50} in 49–75% of the subjects after reaching steady-state. It seems a loading dose is a critical factor in rapid attainment of concentrations above the EC_{50}. Indian health authorities on 22nd March 2020 recommended using HCQ for pre-exposure prophylaxis in selected high-risk groups including healthcare workers at a dose of 800 mg loading followed by 400 mg weekly once for a total of 8 weeks.[13]

Knowledge about the pharmacokinetics of HCQ comes from its use in indications outside of COVID-19, such as malaria, rheumatoid arthritis, and systemic lupus erythematosus. Moreover, there is a dearth of pharmacokinetic data on HCQ for pre-exposure prophylaxis among Indian population. Objective of this study was to estimate the peak and trough values achieved in blood over the course of the prophylactic drug (HCQ) administration among Indian population. These parameters will help in determining the prophylactic dose required for Indian population for COVID-19.

MATERIALS & METHODS

This was an observational study which was conducted during the period 03July, 2020 – 19 October, 2020.

Overall Study Design and Plan

This study was conducted in the Division of Clinical Research and Training, St. John's Medical College and Research Institute, Koramangala, Bengaluru, Karnataka. This study was undertaken in accordance with the National Ethical Guidelines for Biomedical and Health Research Involving Human Participants, ICMR (2017), ICH E6 (R2) 'Guideline for GCP, the Declaration of Helsinki (Fortaleza, Brazil, October 2013) and applicable principles of GLP.

The study protocol, informed consent form, case report form and subject's diary were approved by the Institutional Ethics Committee (IEC Study Ref. No.146/ 2020), St. John's Medical College and Research Institute, Bengaluru.

24 asymptomatic healthcare workers who were already taking or going to take the ICMR advised HCQ prophylaxis for COVID-19 infection, as advised by their treating physicians were included in the study. Subjects who provided voluntary informed consent for collection of blood samples and relevant data for analysis and publication were enrolled. Volunteer number 04 did not report for week 03 and withdrew consent on 24 JUL 20. Volunteer number 20 did not report to the site from week 06 (23 SEP 20) onwards. Hence, total number of volunteers completed the study was 22.

Dose administration

As per the ICMR recommendation, HCQ sulphate 400 mg tablet was taken by each volunteer with snacks twice a day on day 1, followed by 400 mg once weekly for next seven weeks. Volunteers brought their own tablets and details of the tablet (Brand name, Lot/Batch number, expiry date and manufacturer's name) were documented.

After consumption of snacks, the volunteers took the drug in front of the clinical investigator or designee as advised by the treating physician. The time of intake of the tablet was noted for pharmacokinetic estimation. Nine HCQ tablets (400 mg each) were totally consumed by each volunteer during the study.

During week 01, volunteer numbers 10 and 11 did not consume the snacks before taking the drug in front of the clinical investigator/designee. But these volunteers had breakfast just before visiting the facility.

During week 07, volunteer number 05 did not take the drug in front of the clinical investigator or designee. However, the volunteer confirmed telephonically that she consumed tablet HCQ 400 mg along with snacks.

Blood collection

The pre-dose (0.00 hour) blood sample (2 mL each) was collected through direct venipuncture in pre-labeledvacutainers containing K_2EDTA as an anti-coagulant prior to dosing on day 1 (morning dose) and prior to each weekly dose for the next seven weeks. The post-dose blood samples (2 mL each) were collected at 4.00 hours after the first dose on day 1 and also on 3rd week (at 340 h) and 5th week (at 676 h). Altogether 11 blood samples were collected from each volunteer amounting to 22 mL total volume of blood collected. The blood samples were shifted in dry ice to bio-analytical facility at Norwich Clinical Services, Bangalore and stored at -70ºC ± 10ºC, until completion of analysis.

Estimation of HCQ in whole blood

A sensitive and selective LCMS/MS method was developed to estimate HCQ in K_2EDTA (anticoagulant) human blood over

the concentration range 0.0509µg/ml to 10.0561µg/ml using amodiaquine as an internal standard. HCQ was isolated from 100µl blood by using liquid-liquid extraction method by adding 200µl of 100mM sodium carbonate and 2.5 ml of TBME. It was followed by back extraction of the sample into aqueous phase by adding 0.5 ml of 0.5% glacial acetic acid to 0.5 ml of supernatant. Samples were separated by using Synergy ™ 4µ Polar-RP column (80 Å, 100 x 4.6 mm).

50°C. 10mM ammonium acetate in 0.1% Formic acid: Methanol: 70:30 (v/v) was used as a mobile phase.'

Atmospheric pressure ionization (API) interface operated in positive ionization mode was used for the multiple reaction monitoring (MRM). MRM transitions were monitored for HCQ as m/z 336.2 →247.1 and 356.2 →283.0 for amodiaquine. The operational conditions were optimized by infusing diluted stock solution of analyte and internal standard

Table 1 Mass Spectroscopy parameters optimized for analysis

Analyte/ IS	Declustering Potential (DP) (V)	Entrance Potential (EP) (V)	Collision Energy (CE) (V)	Collision Cell Exit Potential (CXP) (V)	Source parameters			
					Collision activated dissociation (CAD) (psi)	Dwell Time (ms) Ion	source voltage (V) Curtain	gas flow (CUR) (psi)
HCQ (Analyte)	95.000	10.000	30.000	15.000	8	300	5500	30
Amodiaquine (IS)	60.000	10.000	24.000	6.000				

IS: internal standard

Inter- and intra-batch precision and accuracy values were determined across six precision and accuracy batches by analyzing six replicates of Quality Control samples at lower limit of quantification (LOQQC), low (LQC), middle (MQC) and high (HQC) quality control samples in each batch. Mean recovery for HCQ was 88.76% and for internal standard amodiaquine was 76.91%. The samples were processed and analyzed under yellow monochromatic light.Linear $1/X^2$ weighting factor was used for calculation and data was processed using analyst software version 1.6.3.The method was validated as per FDA guidelines.[14]

Pharmacokinetic and Statistical analysis

Pharmacokinetic and statistical analysis was performed using Phoenix®WinNonlin® version 8.2. Protocol deviation was not observed in pharmacokinetic and statistical analysis. Actual sampling time point of blood collection was used for pharmacokinetic and statistical analyses.

RESULTS

24 volunteers who were asymptomatic to COVID-19 infection were recruited. However, 22 volunteers completed the study. Volunteer number 04 did not report for week 03 and withdrew consent on 24 JUL 20.Volunteer number 20 did not report from week 06 (23 SEP 20). Mentioned in the methods.

Mean age of volunteers was 30.04 ± 7.33 years (21- 49 years). Mean height and weight were 165.81 ± 6.49 cm and 66.36 ± 9.12, kilograms respectively. Out of 24 volunteers, 4 were females.

Safety assessment

Safety was assessed for all the 24 volunteers. No death or serious adverse event was reported in this study. Only 2 adverse events were reported – one was urticaria reported by volunteer number 04 on 14th July, 2020 with mild itching and further progressed to rashes on 16th July, 2020. This was resolved on 21st July, 2020 after providing treatment with tab. fexofenadine 180 mg BD. The other adverse effect was reported by volunteer number 05 on 20th July, 2020 as 'headache' which was resolved on the same day on treatment with Tab. Naproxen.

10 µl of sample was injected and flow rate was maintained at 1.0ml/min with splitter. Column oven temperature was kept at

Naproxen. None of these participants presented with COVID-19 infection symptoms during the course of study.HCQ was thus well tolerated at the ICMR recommended dose for prophylaxis of COVID-19.

HCQ analysis

The method was selective and specific for HCQ. For specificity and selectivity determination, six individual human plasma lots spiked with LLOQ and intended concentrations of internal standard were processed for determination of specificity and selectivity. No interferences were observed at the retention times of analyte and internal standard when peak responses in blanklots were compared against the response of spiked LLOQ containing IS mixtures

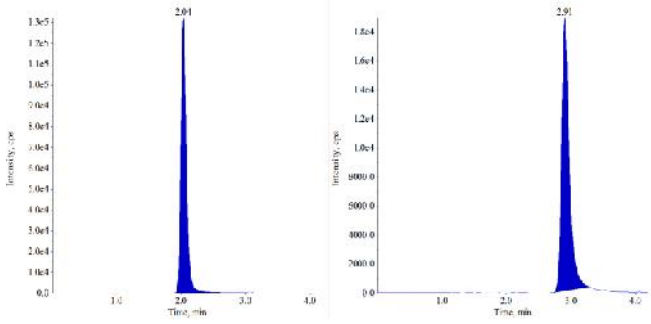

Figure 1 Representative chromatograms of analyte and internal standard.

A linear calibration curve in the concentration ranges 0.0509 - 10.0561µg/ml was prepared (Figure 2).

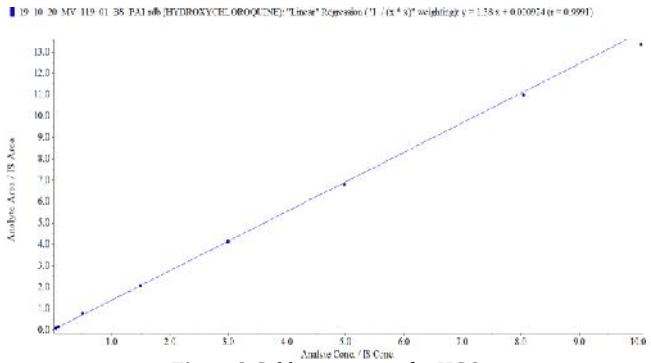

Figure 2 Calibration curve for HCQ

Precision and accuracy were determined by injecting a set of calibration curve samples and quality control samples. The

correlation coefficient of calibration curve was more than 0.99(r) as required by FDA guidelines. The accuracy and precision were 98.08 – 101.51% and <3.8 respectively, which were within acceptable limits.

Control (LOQQC), Low Quality Control (LQC), Middle Quality Control (MQC) and High Quality Control (HQC), were used to determine precision and accuracy for intra- and inter-day batches for all analytes. Results of precision and accuracy of quality control samples were presented in Table 2.

Table 2 Intra-day and inter-day accuracy and precision for the determination of HCQ in human blood

Sample ID	LOQQC (Nominal Conc. 0.0514 µg/ml)			LQC (Nominal Conc 0.1354µg/ml)			MQC (Nominal Conc 3.5250µg/ml)			HQC (Nominal Conc7.6630 µg/ml)		
	Mean calculated Conc (µg/ml)	Mean accuracy (%)	% CV	Mean calculated Conc (µg/ml)	Mean accuracy (%)	% CV	Mean calculated Conc (µg/ml)	Mean accuracy (%)	% CV	Mean calculated Conc (µg/ml)	Mean accuracy (%)	% CV
PA - 1	0.0481	93.64	2.13	0.1360	100.42	0.93	3.6291	102.95	3.32	7.5927	99.08	14.13
PA – 2	0.0486	94.52	4.23	0.1336	98.66	2.24	3.3962	96.35	1.01	7.4085	96.68	2.22
PA – 3	0.0500	97.18	3.26	0.1344	99.29	2.45	3.4677	98.37	2.60	7.6486	99.81	2.19
PA – 4	0.0507	98.57	3.96	0.1294	95.54	2.90	3.3019	93.67	2.25	6.9808	91.10	3.07
PA – 5	0.0513	99.77	1.35	0.1333	98.42	1.87	3.5781	101.50	2.02	7.4242	96.88	1.57
Inter-day	0.0497	96.74	3.77	0.1333	98.47	2.57	3.4746	98.57	4.06	7.4109	96.71	6.96

PK analysis

A gradual increase in trough concentration of HCQ was noticed till day 35. This concentration remained the same on day 42 and day 49. Mean trough concentration was 0.1072 ug/mL on day 35. These changes in trough concentrations were shown in Table 3.

Table 3 Trough (pre-dose) Concentrations of HCQ from day 0 to day 49

Sl. No.	Days	Concentration (± SD)(ug/mL)
1.	0	0.000
2.	7	0.0554 (0.0406)
3.	14	0.0770 (0.0340)
4.	21	0.0835 (0.0368)
5.	28	0.0869 (0.0372)
6.	35	0.1072 (0.0639)
7.	42	0.1002 (0.0345)
8.	49	0.0955 (0.0276)

Compared to day 7, the change in trough concentration on any other days were significantly more. However, when compared to day 14, the increase was not statistically significant on day 21.

Similarly, the 4h post dose concentrations on 3rd week (at 340h) (0.6696 ug/mL)(1.99 nmol/mL) and 5th week(at 676 h) (0.6340 ug/mL)(1.88 nmol/mL) were significantly higher compared to 4h concentration on day 1 (0.492 ug/mL) (1.46 nmol/mL). Although we had not determined the blood HCQ concentration at different time intervals to determine C_{max}, the concentration at 4h post-dose concentration on day 1 i.e. 1.46 nmol/mL matched well to the reported C_{max} (1.22 ± 0.40nmol/mL).[13]

DISCUSSION

In this observational study with 24 (± 10%) volunteers deemed sufficient to obtain good pharmacokinetic estimation (95% C.I) of mean HCQ levels in blood from week 1 to week 8 for the heterogeneous sample population. 24 volunteers were recruited. Due to drop out of 2 volunteers during the study only 22 volunteers had completed the study which is still sufficient to obtain a good pharmacokinetic result. No death or any serious adverse effects occurred during the study. Only 2 adverse effects – urticaria and headache were reported in the study and were also resolved successfully. This indicates that Six replicate analyses of QC samples (n=6) at four different concentrations– Lower Limit Of Quantification Quality

HCQ was well tolerated among the volunteers at the ICMR recommended dose. The loading dose was 800 mg followed by 400 mg once weekly for next seven weeks. Similar dosage was also used in several other clinical trials.[16] Moreover, none of these HCW spresented with COVID-19 symptoms till the end of the study and there were no serious adverse events in our study. Other studies among Indian populations also indicated that voluntary consumption of HCQ as prophylaxis among high risk individuals was associated with a significantly reduced risk of testing positive for COVID-19 as compared to individuals who did not volunteer to take. With pre-exposure HCQ prophylaxis 4 out of 54 participants were tested to be COVID19 positive compared to 20 out of 52 participants who were not taking HCQ prophylaxis. Univariate analysis of distribution of HCQ takers and non-HCQ takers across outcome of COVID19 test indicated the association of risk (Relative Risk = 0.193; 95% CI = 0.071-0.526; p = 0.001) of SARS-CoV-2 infection with lack of pre-exposure HCQ prophylaxis. Thus, taking pre-exposure HCQ prophylaxis was associated with an 80.7% reduction in the risk of acquiring a SARS-CoV-2 infection.[17][18]

There is no validated therapeutic concentration target forCOVID-19 prevention. Our study aims to find out whether HCQ prophylactic regimen recommended by the Indian government is enough to optimize exposures above the *in vitro* generated EC_{50}. EC_{50} values for SARS-CoV-2 virus in the literature have ranged from 0.72–17.31 µM. Since the volunteers who took part in our study were healthcare workers without COVID-19 infection, we consider lower limit for comparing our study results trough concentration.

Although in the simulation study reported by Al-Kofahi *et al*,[11]only 15% of the subjects had HCQ trough concentration above 0.72 µM when 800 mg HCQ was given as loading dose followed by 400 mg once a week which is incidentally the recommended dose of Indian health authorities. However, in our study involving healthcare workers none of them could attain the desired HCQ trough concentration when given the above recommended dose. The trough concentration was 0.1072µg/mL on day 35 and thereafter, it remains constant. This is not unexpected as *in vitro* to *in vivo* extrapolations could either underestimate or overestimate actual drug requirements. Simulation study of Al-Kofahi *et al*.[112] showed that to maintain weekly troughs above EC_{50} in > 50% of

subjects at steady-state in a pre-exposure prophylaxis setting, an 800 mg loading dose followed by 400 mg twice or 3 times weekly is required.

CONCLUSION

This study showed that Indian health authority recommended dose of 800 mg of HCQ loading dose followed by 400 mg of HCQ weekly once for a total of 8 weeks for pre-exposure prophylaxis in selected high-risk group of healthcare workers for COVID-19, the trough concentration achieved is only 0.1072 µg/mL. However, HCQ concentration in the blood achieved in this study matches well with the C_{max} values reported earlier. A study with more number of subjects and time points may help in determining the pharmacokinetic parameters of HCQ which will help in assessing the correct dosage for its prophylactic effect against COVID-19.

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