Pharmacy Education (2024) 24(6) 116 - 123
https://doi.org/10.46542/pe.2024.246.116123

ICOPMAP SPECIAL EDITION

RESEARCH ARTICLE

Pharmacokinetic profile and incurred sample stability of hydroxychloroquine in Volumetric Absorptive Microsampling (VAMS) using high-performance liquid chromatography-photodiode array

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Keywords
Hydroxychloroquine
Incurred sample stability
PDA
Systemic lupus erythematosus
Volumetric absorptive microsampling

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Abstract

Background: Hydroxychloroquine (HCQ) is a quinoline compound derived from chloroquine used to treat SLE. An in vitro stability evaluation was conducted to develop and validate the hydroxychloroquine analysis method. However, an assessment of the incurred sample stability is needed to ensure drug effectiveness, as in vitro evaluation doesn't reflect drug metabolism processes in the body. **Objective:** This research aims to obtain a pharmacokinetic profile of hydroxychloroquine using Volumetric Absorptive Microsampling (VAMS) as a safe sampling technique to use during the COVID-19 pandemic and evaluate the incurred sample stability of hydroxychloroquine samples. **Methods:** The chromatographic conditions used were column C_{18} (Waters, XBridge; 250 × 4.6 mm; 5µm); mobile phase acetonitrile-1% diethylamine (65:35); flow rate of 0.8 mL/min; column temperature 45°C; PDA detector analysis wavelength of 332 nm; and chloroquine as internal standard. **Results:** The pharmacokinetic profile of hydroxychloroquine in VAMS samples gave results that the C_{max} ranged from 322.61-505.32 ng/mL with an average of 425.33 ± 65.90 ng/mL; t_{max} was 4 hours; mean $t_{1/2}$ was 23.32 ± 9.65 hours; mean AUC_{0-72} was 5103.63 ± 1419.66 ng.h/mL; mean $AUC_{0-\infty}$ was 5763.97 ± 2155.26 ng.h/mL, and the AUC ratio was above 80%. **Conclusion:** The incurred sample stability of hydroxychloroquine in VAMS met the 2011 EMEA Bioanalytical Guideline requirements up to day 30.

Introduction

Hydroxychloroquine (HCQ) is a quinoline-group compound derivative of chloroquine. It is used to treat Systemic Lupus Erythematosus (SLE) in Indonesia. Hydroxychloroquine is formulated as coated tablets, 200 mg of which is consumed daily (Perhimpunan Reumatologi Indonesia, 2019).

In validating the hydroxychloroquine bioanalysis method, long-term stability evaluation in vitro was carried out using blood samples spiked with standard analytes and internals (Harahap *et al.*, 2021). However, stability evaluation in vitro validation using calibration standards and quality control samples does not reflect drug metabolism processes in the body (in vivo) (European Medicines Agency, 2011). So, evaluating the Incurred Sample Stability (ISS) in samples containing hydroxychloroquine is required to determine the stability and reproducibility of the concentration of hydroxychloroquine in the sample at the specified storage time and conditions (Lowes *et al.*, 2014). Evaluating Incurred sample stability requires several recommended samples around the maximum analyte concentration (Cmax) and the analyte elimination phase (European Medicines Agency, 2011; Lowes *et al.*, 2014). Therefore, it is necessary to test the

pharmacokinetic profile before evaluating the Incurred sample stability to obtain samples with concentrations around the maximum analyte concentration and analyte elimination phase.

The implementation of the microsampling technique has been in demand in recent decades. The microsampling technique produces many advantages compared to venipuncture, one of which is simpler sampling so that sampling can be done without the need for experts (Kok & Fillet, 2018). The existing microsampling technique is the Dried Blood Spot (DBS) technique. However, the DBS technique is deficient in the form of a hematocrit effect, which causes the spotting volume on Dried Blood Spot paper to be unstable or not uniform (De Kesel *et al*., 2014). Volumetric Absorptive Microsampling (VAMS) is a new microsampling technique using an absorbent that can absorb blood in a fixed volume. VAMS has the advantage of being minimally invasive because the sampling is done by finger prick and minimises problems related to hematocrit and homogeneity compared to the DBS method (Kip *et al*., 2017; Protti *et al*., 2019). The advantage of being easy to use VAMS has another positive impact: blood sampling is carried out by implementing the COVID-19 health protocol during the COVID-19 pandemic (Harahap *et al*., 2020).

Testing the pharmacokinetic profile of hydroxychloroquine using the VAMS biosampling method using High Performance Liquid Chromatography-Photodiode Array (HPLC-PDA) as well as evaluating the Incurred Sample Stability of hydroxychloroquine has never been done. Therefore, this study aims to obtain a pharmacokinetic profile of hydroxychloroquine in VAMS using HPLC-PDA with a method that has been optimized and fully validated (Harahap *et al*., 2021) . In addition, an evaluation of Incurred Sample Stability was carried out to determine the stability and reproducibility of analyte concentrations in six healthy subjects in VAMS 7, 14, and 30 days after the samples were stored under in vitro validated storage conditions. It is hoped that this study will be useful to ensure the stability of hydroxychloroquine in VAMS in in vivo studies.

Methods

Chemicals and reagents

Farneltik 200 mg hydroxychloroquine sulfate film-coated tablets were purchased from PT. Fahrenheit (Jakarta, Indonesia). VAMS was purchased from Neoteryx (USA). Hydroxychloroquine sulfate and chloroquine phosphate were purchased from Sigma-Aldrich Pte. Ltd (USA). The mobile chromatography phases contained chromatographic grade acetonitrile and diethylamine purchased from Merck KGaA (Darmstadt, Germany). Reagents such as n-hexane, ethyl acetate, and ammonia were purchased from Merck KGaA (Darmstadt, Germany). Aquabidest was obtained from PT. Ikapharmindo Putramas (Jakarta, Indonesia).

Calibration standards and quality controls

Stock solutions of hydroxychloroquine sulfate and chloroquine phosphate were prepared at 1000 µg/mL concentrations. Calibration curves were prepared by spiking with an appropriate volume of aquabidest for producing various concentrations of 2, 5, 10, 50, 100, 500, 1000, 3000, and 6500 ng/mL. Quality Control (QC) samples were prepared at low, medium, and high hydroxychloroquine concentrations of 6, 2600, and 4875 ng/mL.

Verification and validation

This study was validated using High-Performance Liquid Chromatography (HPLC) with a photodiode array (PDA) detector set at a wavelength of 300 nm. Separation was conducted on a C18 column (Waters, XBridge; 250 × 4.6 mm; 5µm). The analysis used an isocratic separation with acetonitrile-1% diethylamine (65:35% v/v), a column temperature of 45°C and a flow rate of 0.8 mL/min for 12 min. This method had been previously optimised and fully validated in the same laboratory (Harahap *et al*., 2021).

Verification and partial validation were performed using this method. A system suitability test was conducted using a solution containing hydroxychloroquine sulfate and chloroquine phosphate with a 1 µg/mL concentration, respectively. A hundred microliters of the solution were injected into the column and retention time, peak area, theoretical plates (N value), and tailing factor (Tf) were calculated. The Coefficient of Variation (CV) % determined precision from six repeat injections. Partial validations (intra-run accuracy, precision and linearity of the calibration curve) were determined using the requirements from the European Medicines Agency (EMEA) Bioanlytical Guidelines (European Medicines Agency, 2011).

Collecting samples

The test used blood as the matrix obtained from six selected healthy subjects administered 200 mg of hydroxychloroquine sulfate film-coated tablet (Farneltik). Blood samples were collected 13 times from six healthy subjects 30 mins before drug administration (pre-dose) and 0.5, 1, 2, 3, 3.5, 4, 5, 8, 12, 24, 48, and

72 hours after administration of a 200 mg of hydroxychloroquine sulfate film-coated tablet. Blood was collected by a trained phlebotomist using the finger prick technique and collected using VAMS. The VAMS samples were dried at room temperature (25°C) for 2 hours.

The preparation method of hydroxychloroquine samples was following the previous study (Harahap *et al*., 2021) . Dried VAMS samples were placed in a sample tube and 30 µL of 1 µg/mL chloroquine and 500 µL of 1% ammonia were added. The samples were shaken by vortex for 15 s and sonicated for 5 mins. 500 µL of n-hexane-ethyl acetat (50:50 v/v) were added to the samples and centrifuged at 10000 rpm. The supernatants were transferred to a new sample cup.

The supernatants were evaporated under a stream of nitrogen gas at 25°C, and the residue dissolved in a 150 µL mixture of chromatographic mobile phases. The aliquots were shaken by vortex for 15 s, sonicated for 1 min and analysed using HPLC.

Pharmacokinetic profiles were obtained by calculating the mean of the subjects' Cmax, tmax, t1/2, AUC0-t, and AUC0-∞ .

ISS analysis was performed on subjects' dried VAMS samples after they were stored at 25°C on days 7, 14, and 30 and prepared as described previously. For each subject, ISS samples were analysed at two concentrations in the Cmax and one concentration in the elimination phase.

Results

Figure 1 shows the representative chromatogram for system suitability tests. The calibration curve is shown in Figure 2. Figure 3 displays the mean pharmacokinetic profile of six subjects. The ISS trends for six subjects are shown in Figure 4.

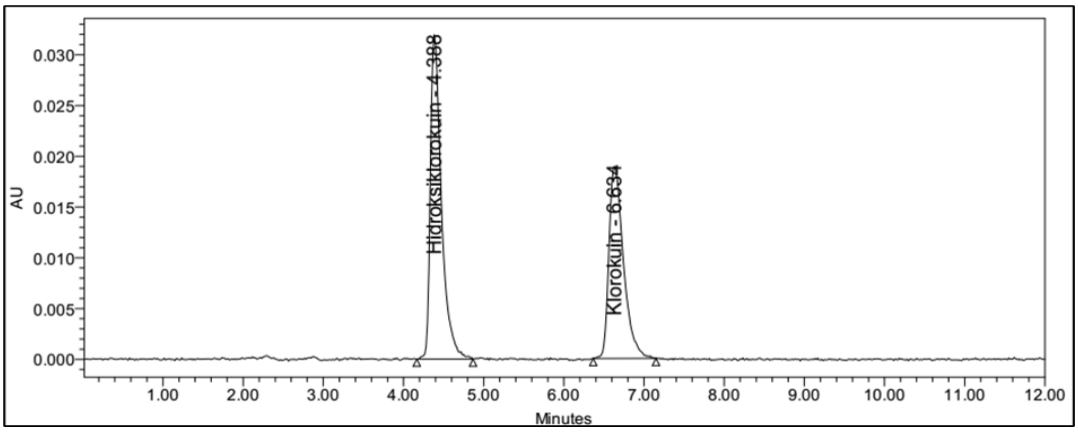
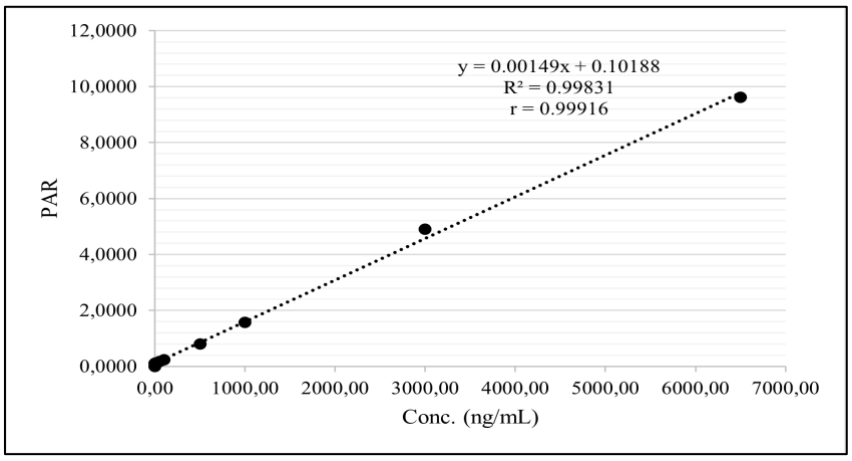


Figure 1: Representative chromatogram for system suitability tests



PAR: Peak Area Ratio

Figure 2: Calibration curve

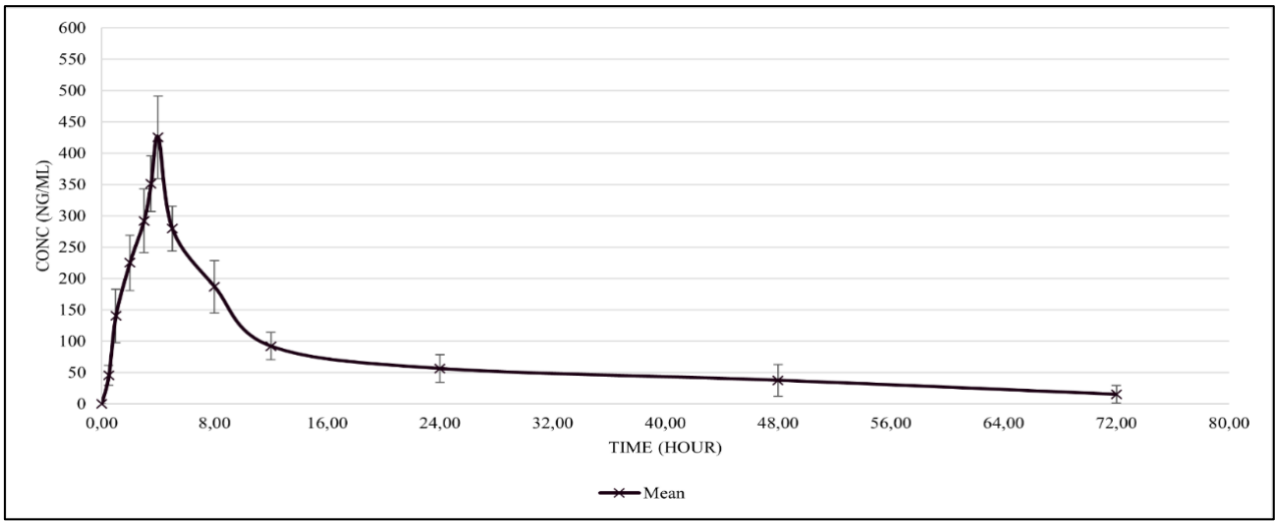


Figure 3: Mean pharmacokinetic profile of six subjects

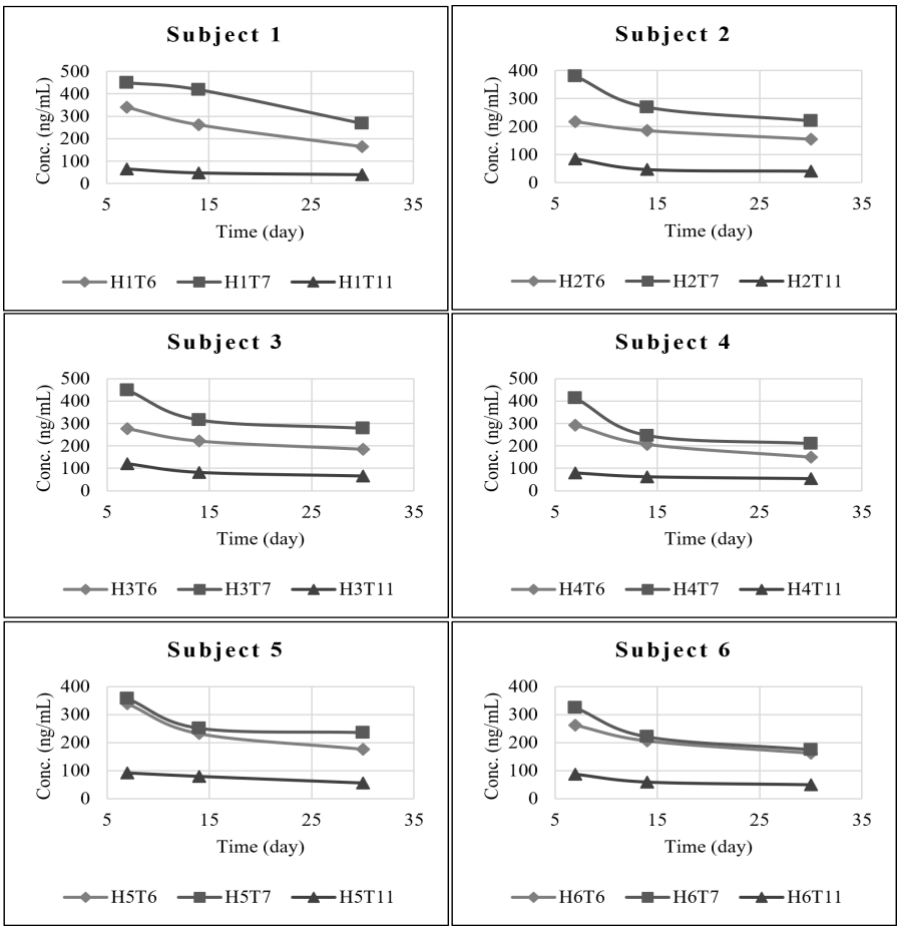


Figure 4: ISS Trends for each subject

Table I shows system suitability test results. Table II presents the calibration curve concentration results from the previous study. Meanwhile, Table III shows the calibration curve concentration results from this study. Table IV depicts intraday accuracy and precision. Table V presents the individual subjects' pharmacokinetic parameters. Table VI shows mean ISS results.

Table I: System suitability test results

No	Tf		N (plate)		HETP		Resolution
	HCQ	IS	HCQ	IS	HCQ	IS	
1	1.721806	1.547983	5588.04	7092.47	0.044738	0.035249	7.769518
2	1.694948	1.526574	5532.57	7044.79	0.045187	0.035487	7.841679
3	1.714832	1.516338	5456.03	6881.96	0.045821	0.036327	7.754228
4	1.716818	1.538451	5565.94	6835.05	0.044916	0.036576	7.723034
5	1.743731	1.543294	5470.08	6871.66	0.045703	0.036381	7.829780
6	1.710104	1.554505	5473.41	6873.23	0.045675	0.036373	7.837770
Mean	1.717040	1.537858	5514.35	6933.19	0.045340	0.036066	7.792668
SD	0.02	0.01	55.61	107.20	0.00	0.00	0.05
CV (%)	0.93	0.92	1.01	1.55	1.01	1.53	0.65

Table II: Calibration curve concentration results from previous study [2]

Cons. (ng/mL)	Area (µV/s)		PAR	Measured cons. (ng/mL)	% diff
	HCQ	IS			
0.00	0	40203	0.0000	0	0.00
2.00	2906	41121	0.07067	2.14	7.15
10.00	3480	42951	0.08102	9.45	-5.50
50.00	6019	42698	0.14097	51.76	3.52
100.00	8558	37943	0.22555	111.46	11.46
500.00	24315	28174	0.86303	561.40	12.28
1000.00	38079	27954	1.36220	913.73	-8.63
3000.00	133035	29873	4.45335	3095.51	3.18
6500.00	288319	31250	9.22621	6464.28	-0.55
Slope (b)	**Intercept (a)**		**r**	**R^2**	
0.00142	0.06763		0.99969	0.99938	

Table III: Calibration curve concentration results from this study

Cons. (ng/mL)	Area (µV/s)		PAR	Measured cons. (ng/mL)	% diff
	HCQ	IS			
0.00	0	30017	0.0000	0	0.00
2.00	3371	32039	0.1052	2.25	12.27
10.00	3547	30520	0.1162	9.64	-3.57
50.00	7662	44326	0.1729	47.72	-4.56
100.00	8451	34924	0.2420	94.19	-5.81
500.00	24572	31109	0.7899	462.53	-7.49
1000.00	39048	24807	1.5741	989.74	-1.03
3000.00	147277	30085	4.8954	3222.59	7.42
6500.00	283476	29454	9.6244	6401.83	-1.51
Slope (b)	**Intercept (a)**		**r**	**R^2**	
0.00149	0.10188		0.99916	0.99831	

Table IV: Intraday accuracy and precision

Conc. (ng/mL)	HCQ	IS	PAR	Meas. conc. (ng/mL)	Mean (ng/mL)	SD	CV (%)	% diff
LLOQ 2.00	3523	33438	0.1054	2.34				17.09
	3460	32933	0.1051	2.14				7.10
	3394	32439	0.1046	1.85	2.08	0.21	9.93	-7.51
	3074	29243	0.1051	2.18				9.03
	2964	28312	0.1047	1.89				-5.38
QCL 6.00	4083	37172	0.1098	5.35				-10.75
	3912	35102	0.1114	6.43				7.24
	4051	36711	0.1103	5.70	5.76	0.40	6.95	-5.07
	3988	36159	0.1103	5.66				-5.71
	3836	34776	0.1103	5.67				-5.54
QCM 2600.00	90131	24057	3.7466	2450.27				-5.76
	93454	26378	3.5429	2313.34				-11.03
	92090	26822	3.4334	2239.72	2430.83	182.70	7.52	-13.86
	112862	27224	4.1457	2718.59				4.56
	94809	25488	3.7198	2432.25				-6.45
QCH 4875.00	216901	27834	7.7927	5170.41				6.06
	169825	25185	6.7431	4464.80				-8.41
	228416	31807	7.1813	4759.40	4640.59	371.96	8.02	-2.37
	178685	28386	6.2948	4163.43				-14.60
	217355	31002	7.0110	4644.91				-4.72

Note: LLOQ: Lower Limit of Quantification; QCL: Quality Control Low; QCM: Quality Control Medium; QCH: Quality Control High

Table V: Individual subjects' pharmacokinetic parameters

Subject no.	C_{max} (ng/mL)	t_{max} (h)	$t_{1/2}$ (h)	AUC_{0-t} (ng.h/mL)	$AUC_{0-\infty}$ (ng.h/mL)	AUC_{0-t}/ $AUC_{0-\infty}$ (%)
H1	430.41	4.00	15.54	4285.11	4398.31	97.43
H2	384.68	4.00	25.48	4805.83	5302.01	90.64
H3	480.79	4.00	35.00	7484.90	9334.62	80.18
H4	428.19	4.00	13.28	4402.35	4464.77	98.60
H5	505.32	4.00	34.14	6046.01	7360.20	82.14
H6	322.61	4.00	16.50	3597.58	3723.91	96.61
Mean	425.33	4.00	23.32	5103.63	5763.97	90.93
SD	65.90	0.00	9.65	1419.66	2155.26	8.08
CV (%)	15.49	0.00	41.40	27.82	37.39	8.88

Table VI: Mean ISS results

ISS sample	% diff
Day 7	
1	-5.03
2	-1.71
3	10.62
Day 14	
1	-11.51
2	-9.92
3	2.79
Day 30	
1	-17.81
2	-14.76
3	-2.71

Discussion

System suitability

System suitability tests were conducted to determine the reproducibility and suitability of the selected methods. CV% passed the required criteria (CV ≤ 2%), and the results are presented in Table I with a representative chromatogram in Figure 1.

Linearity

Linearity was > 0.99, and accuracy (% diff) was ≤ ± 20% for the LLOQ and ≤ ± 15% for other concentrations. The linear equation for the calibration curve was y =

0.00149x + 0.10188, with x the hydroxychloroquine sulfate concentration (ng/mL) and y the PAR between hydroxychloroquine sulfate and the chloroquine phosphate internal standard. The calibration curve concentrations from previous and current studies are presented in Table II and Table III, respectively, and the calibration curve is presented in Figure 2.

Accuracy and precision

Accuracy is a measure of how close the calculated concentration of the analyte is to the actual concentration in the sample described as % diff, while precision is the relative similarity of repeated measurements that is described as the coefficient of variation (CV%). For determining the value of these parameters, blood containing hydroxychloroquine in VAMS was analysed at several concentrations, which are LLOQ, QC Low (QCL), QC Medium (QCM), and QC High (QCH) with five replicates, respectively. Accuracy and precision requirements were % diff, and CV% for LLOQ was ≤ ± 20% and ≤ ± 15% for other concentrations. The accuracy and precision results are presented in Table IV.

Pharmacokinetic profiles of subject's VAMS samples

Hydroxychloroquine concentrations were plotted to produce a pharmacokinetic profile for each subject to determine their pharmacokinetic parameters, namely, the maximum concentration (C_{max}), the maximum time (t_{max}), $t_{1/2}$, AUC_{0-t}, and $AUC_{0-\infty}$. The values of the pharmacokinetic parameters for each subject are presented in Table V., with a graph plotted in Figure 3. Based on EMEA Bioanalytical Guidelines (2011), incurred stability samples should include two concentrations in the C_{max} phase and one concentration in the elimination phase in each healthy subjects' VAMS samples. The ISS testing time points in each healthy subjects' VAMS samples were at the sixth and seventh time points (i.e. at or close to t_{max}) and the eleventh time point (i.e. elimination phase).

ISS

ISS testing was performed on days 7, 14, and 30 of VAMS sample storage and were counted from the day the pharmacokinetic profiles were created. Based on EMEA Bioanalytical Guidelines, the % diff requirements are ≤ ± 20% for at least 67% of reanalysed samples for ISS. The mean %diff of the ISS tests is presented in Table VI, and the concentration trends are in Figure 4.

Conclusion

The pharmacokinetic profiles of hydroxychloroquine in the VAMS samples of six healthy subjects exhibited a C_{max} range between 322.61-505.32 ng/mL with an average of 425 .33 ± 65.90 ng/mL; the t_{max} of 4 hours; the mean $t_{1/2}$ of 23.32 ± 9.65 hours; the mean AUC_{0-t} was 5103.63 ± 1419.66 ng.h/mL; the mean $AUC_{0-\infty}$ was 5763.97 ± 2155.26 ng hour/mL; and the AUC ratio is above 80%.

The ISS of hydroxychloroquine in VAMS meet the requirements up to 30 days with the % diff mean range of -17.81 to 10.62%. It is necessary to extend the sample storage time to know when hydroxychloroquine is unstable in VAMS in vivo.

Acknowledgement

This study was supported by the Directorate of Research and Community Services (DRPM), Universitas Indonesia, Depok, Indonesia.

Ethics approval and consent to participate

This study was approved by the Medical Research Ethics Committee of the Faculty of Medicine, Universitas Indonesia (KET-265/UN2.F1/ETIK/PPM.00.02/2021), protocol number 21-03-0236 and the subjects signed an informed consent form before participation.

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