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Application News

No. LCMSMS-453

Liquid Chromatograph Mass Spectrometer LCMS™-8050

Determination of Hydroxychloroquine Concentration in Human Plasma by LC-MS/MS Method

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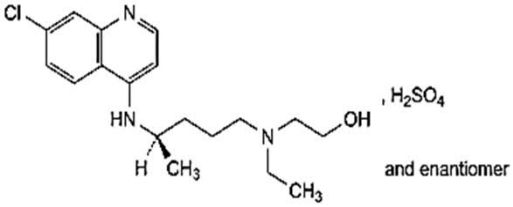
Abstract: This study provides a method for quantitative analysis of hydroxychloroquine (HCQ) in human plasma using Shimadzu ultrahigh performance liquid chromatography coupled with triple quadrupole mass spectrometry (UHPLC-MS/MS; Nexera™ with LCMS™-8050). The concentration of HCQ in human plasma can be accurately determined within 10 min after simple protein precipitation using acetonitrile. The method is evaluated in terms of specificity, linearity, limit of quantification (LOQ), repeatability and matrix effects. The results show that good linearity was obtained in a range from 0.5 ng/mL to 500 ng/mL with a correlation coefficient greater than 0.998. The LOQ was 0.5 ng/mL. The intra-day precision for low, medium and high concentrations was between 1.57% to 8.33% and the accuracy ranged from 97.91% to 106.02%. The matrix effects for low, medium and high concentrations of HCQ as well as the internal standard ranged from 98.31% to 108.17%. Shimadzu LCMS-8050 can be easily and quickly used for development and validation of critical drug candidates such has HCQ.

Keywords: LC-MS/MS, human plasma, hydroxychloroquine sulfate

❑ Introduction

Hydroxychloroquine sulfate (or hydroxychloroquine, HCQ), which is used to prevent and treat malaria, has been recommended worldwide for clinical treatment of novel coronavirus (COVID-19) since its outbreak. A research team from the Renmin Hospital of Wuhan University posted a preprint on medRxiv, aiming to evaluate the efficacy of HCQ in the treatment of patients with COVID-19. In the study, 31 patients received an additional 5-day oral HCQ (400 mg/d) treatment, besides the standard treatment (i.e. oxygen therapy, antiviral agents, antibacterial agents, and immunoglobulin, with or without corticosteroids). The results showed that the body temperature recovery time and the cough remission time of these patients were significantly shortened as compared with the patients without HCQ treatment. Moreover, more patients showed improvement in pneumonia symptoms in this HCQ group than the control group (i.e. 25 vs 17). Professor Didier Raoult from Aix Marseille Université with his collaborators published a clinical study on treatment of novel coronavirus with HCQ and azithromycin in the International Journal of Antimicrobial Agents. The results of the study, involving 36 novel coronavirus patients, showed that 100% of patients (6) who received treatment with HCQ and azithromycin combination were virologically cured. Twelve (12) of the patients who received only the HCQ treatment showed significant improvement too. It has been reported that the US FDA authorizated emergency using of HCQ sulfate in certain hospitalized patients on March 28th.

In this study, a method for quantitative determination of HCQ in human plasma was established on Shimadzu LCMS™-8050. The method has advantages of simplicity, high sensitivity, good repeatability and a wide linear range. It can be used to determine the concentration of HCQ in human plasma, providing a reference in clinical use.



Hydroxychloroquine sulfate
$C_{18}H_{28}ClN_3O_5S$
MW 433.95
CAS#: 747-36-4

❑ Experimental

1.1 Instrument

This study was conducted on a Shimadzu LCMS™-8050 system, consisting of LC-30AD (x2, pump), DGU-20A5 (online degasser), SIL-30AC (autosampler), CTO-20AC (column oven), CBM-20A (system controller), LCMS™-8050 triple quadrupole mass spectrometer, and LabSolutions™ Ver. 5.97.

1.2 Analytical conditions

LC conditions

Column: Shim-pack™ GIST C18-AQ, 100 mm x 2.1 mm I.D., 1.9 µm (P/N: 227-30807-02)
Mobile phase: (A) 0.1% Formic acid + 50 mM ammonium acetate in water;
(B) Methanol : Acetonitrile (1:1, v/v)
Flow rate: 0.50 mL/min
Column Temperature: 40 °C
Elution mode: Isocratic flow, 20% of mobile phase B

MS conditions

Interface: ESI, positive mode
Interface voltage: 4.5 kV
Nebulizer gas flow: 3.0 L/min, nitrogen
Heating gas flow: 10.0 L/min, dry air
DL temperature: 250 °C
Heat block temperature: 400 °C
Probe temperature: 300 °C
Drying gas flow: 10.0 L/min, nitrogen
MRM parameters: see Table 1

Table 1. MRM optimized parameters of HCQ sulfate and chloroquine diphosphate

Compound	CAS No.	Precursor ion $[M+H]^+$	Product ion (m/z)	Q1 Pre Bias (V)	CE (V)	Q3 Pre Bias (V)
hydroxychloroquine sulfate (HCQ)	747-36-4	336.20	247.10*	-12.0	-22.0	-28.0
			158.15	-12.0	-36.0	-18.0
Chloroquine diphosphate	50-63-5	320.20	247.10	-12.0	-22.0	-28.0

* Qualifier ion

1.3 Standards and reagents

Methanol, acetonitrile and ammonium acetate (chromatography grade) were purchased from Merck and were stored at room temperature. DMSO (analytical grade) was purchased from Chengdu Kelong Chemical Co., Ltd. and was stored at room temperature. Milli-Q® water was produced by a Millipore Milli-Q® Plus water purification system. Formic acid (chromatography grade, purity > 98%) was purchased from ANPEL Laboratory Technologies (Shanghai) Inc. and was stored at room temperature.

1.4 Preparation of reference standard and internal standard

HCQ sulfate stock solution: HCQ sulfate was accurately weighed and then dissolved in the mixture of methanol and water (1:1, v/v). The concentration of the stock solution was 10.0 µg/mL. The stock solution was stored at -20 ºC and was protected from light prior to use.

HCQ sulfate working solutions: The concentrations of HCQ sulfate working solutions were 5, 10, 20, 50, 100, 200, 500, 1000, 2000, and 5000 ng/mL, respectively, which were prepared by diluting the stock solution with the mixture of methanol and water (1:1, v/v). The working solutions were stored at -20 ℃ and were protected from light prior to use.

Chloroquine (CQ) diphosphate (internal standard) stock solution: The CQ diphosphate was accurately weighed and then dissolved in the mixture of methanol and water (1:1, v/v). The concentration of the stock solution was 10.0 µg/mL. The stock solution was stored at 4 ºC prior to use.

CQ diphosphate (internal standard) working solution: The concentration of CQ diphosphate working solution was 1000 ng/mL, which was prepared by diluting the stock solution with the mixture of methanol and water (1:1, v/v). The working solution was stored at -20 ºC prior to use.

1.5 Sample preparation

Calibration standards: each of HCQ sulfate working internal standard working solution (10 µL). The solution (5 µL) was added in a human plasma sample (95 µL), followed by adding obtained sample was vortexed for 30 seconds. Then, 400 µL of acetonitrile was added to the sample and the mixture was vortexed for 1 min before centrifuged at 14,000 rpm for 10 min. After centrifugation, 300 µL of the supernatant was transferred to a sample vial. A volume of 2 µL of the sample was injected to LC-MS/MS for analysis.

Quality control (QC) samples: each of the HCQ sulfate QC working solutions (5 µL) of 5, 200 and 4000 ng/mL were added to human a plasma sample (95 µL). The samples were prepared following the same procedure used for calibration standards.

Matrix effect (spiked human plasma) samples: Each of human plasma samples (95 µL) was mixed with acetonitrile (400 µL) by a vortex mixer for 1 min. Each of HCQ sulfate working solutions (5 µL) of 5, 200 and 4000 ng/mL was added to the plasma sample. Then, 10 µL of internal standard working solution was added into each sample. After that, the samples were prepared following the same procedure used for calibration standards.

Neat standard (spiked Milli-Q® water) samples: Each of HCQ sulfate working solution (5 µL) of 5, 200 and 4000 ng/mL was added into a different Milli-Q water sample. After that, the samples were prepared following the same procedure used for matrix-matched calibration standards.

$$Matrix\ effect\ (\%) = \frac{Peak\ area\ in\ spiked\ human\ plasma\ sample}{Peak\ area\ in\ spiked\ Milli-Q^{®}\ water\ sample} \times 100\%$$

❑ Results and Discussion

2.1 Selectivity

The blank human plasma sample (100 µL) was extracted and analysed following the abovementioned procedure. As shown in Figures 1 & 2, both HCQ and CQ were not detected in the blank sample. The results also showed that there was no obvious interference at the retention times of HCQ and CQ.

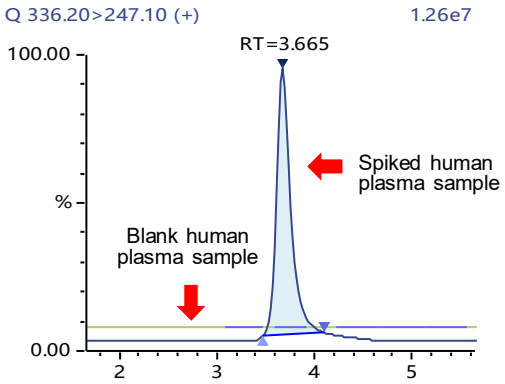


Figure 1. Chromatograms of HCQ in blank and spiked human plasma samples

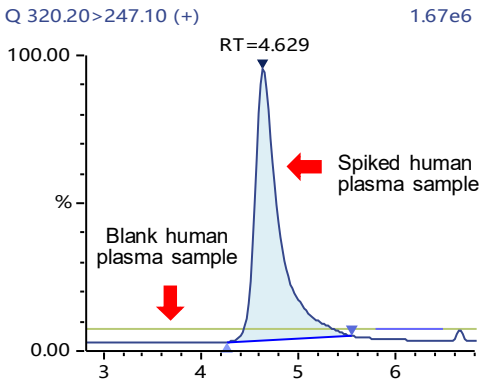


Figure 2. Chromatograms of CQ in blank and spiked human plasma samples

2.2 Linear range

The concentrations of the calibration standards prepared in human plasma were 0.5 ng/mL, 1 ng/mL, 2 ng/mL, 5 ng/mL, 10 ng/mL, 20 ng/mL, 50 ng/mL, 100 ng/mL, 200 ng/mL and 500 ng/mL, respectively. The calibration curve is shown in Figure 3. The linear equation and correlation coefficient (r) of the calibration curve are shown in Table 2, where Y represents the ratio of the peak area of HCQ to the peak area of CQ, and X represents the ratio of the concentration of HCQ to the concentration of CQ.

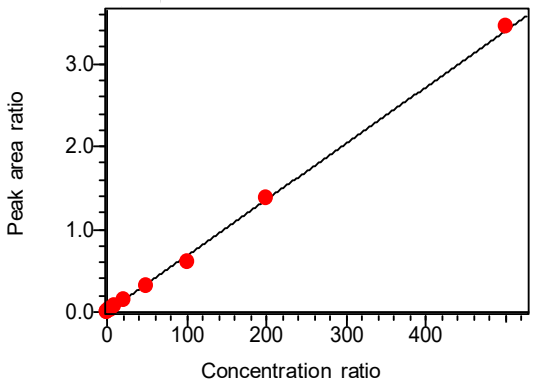


Figure 3. Matrix-matched calibration curve for HCQ with CS as Internal standard

Table 2. Matrix-matched calibration curve for HCQ (linear regression, weight: 1/C)

Compound	Calibration Equation	Linear range (ng/mL)	Accuracy (%)	Correlation coefficient, r
HCQ sulfate	Y = (0.00140144)X + 0.000398161	0.5 - 500	90.6 – 110.0	0.9988

2.3 Matrix effect

The result of the evaluation of the matrix effects is shown in Table 3.

Table 3. Matrix effects (n = 3)

Spiked level	Matrix effects (%)	
	Theoretical concentration (ng/mL)	HSQ sulfate
LOQ	0.5	108.17
MQC	20	105.52
HQC	400	98.31
Internal standard	105.72	

The result indicated that the matrix effects of HCQ and the internal standard (CQ) were in the range of 98.31% to 108.17%.

2.4 Precision and accuracy

The intra-day precision and accuracy of the analysis were evaluated with the quality control samples of three levels. The results are shown in Table 4.

Table 4. Precision and accuracy (n = 6)

Compound	Concentration (ng/mL)	Precision (RSD, %)	Accuracy (%)
HCQ sulfate	0.5	8.33	106.02
	20	5.19	101.83
	400	1.57	97.91

The results indicate that the intra-day precision of HCQ ranges from 1.57% to 8.33%, and the accuracy of HCQ ranges from 97.91% to 106.02%.

2.5 Stability in sample rack

The stability of HCQ sulfate in sample rack was evaluated by analyzing the quality control samples of three levels after 10 hours at 6℃ in the sample rack. The results are shown in Table 5.

Table 5. Stability of HCQ sulfate in sample rack

Compound	Concentration (ng/mL)	Precision (RSD, %)	Accuracy (%)
HCQ sulfate	0.5	7.33	94.72
	20	4.56	98.01
	400	1.76	96.30

The results indicate that HCQ was stable in sample rack at 6 ºC within for 10 hours.

❑ Conclusion

An LC-MS/MS method has been developed and evaluated for quantitative analysis of hydroxychloroquine (HCQ) sulfate in human plasma samples. Good linearity is obtained from 0.5 ng/mL to 500 ng/mL in human plasma spiked samples, with a correlation coefficient of 0.998. The LOQ of the method is 0.5 ng/mL in human plasma. The intra-day precision (RSD%) at low, medium and high concentration levels of HCQ falls in the range from 1.57% to 8.33%, and the accuracy ranges from 97.91% to 106.02%. The matrix effects evaluated at low, medium and high levels of HCQ as well as the internal standard CQ are from 98.31% to 108.17%. In summary, with Shimadzu LCMS™-8050, fast, sensitive and robust quantitative analysis of HCQ in human plasma samples can be carried out readily.

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