

# Analysis of Hydroxychloroquine and Metabolites in Human Serum and Plasma Using the Agilent Captiva EMR—Lipid by LC-QQQ

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## Abstract

A robust, specific, and sensitive method was developed and validated for the quantitative analysis of hydroxychloroquine (HCQ) and its three metabolites in human serum and plasma. Human serum or plasma samples (100  $\mu$ L) were prepared by protein precipitation followed with Agilent Captiva EMR—Lipid cleanup, then analyzed by LC/MS/MS. The assay exhibited a linear dynamic range of 0.4 to 100 ng/mL with correlation coefficients  $R^2 > 0.99$  for all four analytes. Method quantitation was validated with three spiking level QC samples at 1, 10, and 100 ng/mL, providing accuracy within  $100 \pm 15\%$  and precision CV  $< 15\%$  in both human serum and plasma. The method provided a reliable solution for the emerging application of quantitative HCQ and metabolites in human blood matrices.

## Introduction

Hydroxychloroquine (HCQ) is the hydroxylated form of the antimalarial drug chloroquine. This drug was discovered to be as effective as, but less toxic than chloroquine<sup>1</sup>, and became one of the most widely prescribed drugs for the prevention and treatment of malaria and other diseases. HCQ is commonly administered orally, and is metabolized in the liver into three active metabolites: desethylchloroquine (DCQ), desethylhydroxychloroquine (DHCQ), and bisdesethylchloroquine (BDCQ).

Most assays for HCQ and metabolites are based on high-performance liquid chromatography (HPLC) coupled with fluorescence (FL) detection. However, these assays are generally nonselective and require high sample volumes. LC/MS/MS has become the preferred detection tool for bio-analysis, as it provides improved sensitivity and selectivity compared to conventional HPLC-FL detection.<sup>2-4</sup> Common sample preparation techniques used in these assays include simple protein precipitation (PPT) or liquid-liquid extraction (LLE). However, these sample preparation techniques can be time-consuming and labor-intensive, and can result in more complex matrix interferences, causing ion suppression on LC/MS/MS.

Agilent Captiva EMR–Lipid cartridges and plates use the novel EMR–Lipid sorbent, which selectively removes major lipid classes from biological sample matrices. The mechanism is based on a combination of size exclusion and hydrophobic interaction between lipid compounds and the EMR–Lipid sorbent, and thus, does not cause unwanted analyte loss. The common protein precipitation process, followed by Captiva EMR–Lipid cleanup, provides highly efficient and selective blood matrix cleanup for proteins and phospholipids. Previous studies have demonstrated that >99% of phospholipids from biological blood matrices are removed.<sup>5</sup> The Captiva EMR–Lipid 96-well plate format allows

for efficient, high-throughput sample preparation using simplified in-well PPT, followed with passing-through cleanup. This saves time and effort on multiple sample transferring, reducing operation errors and potential analyte loss. This methodology was demonstrated in multiple clinical research applications for biological blood sample analysis<sup>6,7</sup>, including the quantitation of HCQ and metabolites in human whole blood.<sup>8</sup> This study extended the method application to human plasma and serum to complete the quantitative analysis of HCQ and metabolites in different human blood matrices. Table 1 shows analyte information for four targets (HCQ, DCQ, DHCQ, and BDCQ) and the internal standard HCQ-d<sub>4</sub>.

**Table 1.** Molecules of interest.

Molecule	Structure	Chemical Formula	[M+H] <sup>+</sup> (m/z)	pKa
Hydroxychloroquine (HCQ)		C <sub>18</sub> H <sub>26</sub> ClN <sub>3</sub> O	336.1837	8.3 and 9.7 [9a]
Hydroxychloroquine-d <sub>4</sub> (HCQ-d <sub>4</sub> )		C <sub>18</sub> H <sub>22</sub> D <sub>4</sub> ClN <sub>3</sub> O	340.2088	N/A
Desethylhydroxychloroquine (DHCQ)		C <sub>16</sub> H <sub>22</sub> ClN <sub>3</sub> O	308.1524	10 and 15.6 [9b]
Desethylchloroquine (DCQ)		C <sub>16</sub> H <sub>22</sub> ClN <sub>3</sub>	292.1575	8.4 and 10.9 [9a]
Bisdesethylchloroquine (BDCQ)		C <sub>14</sub> H <sub>18</sub> ClN <sub>3</sub>	264.1262	10.2 [9b]

## Experimental

### Reagents and chemicals

All reagents and solvents were LC/MS grade. OmniSolv Acetonitrile (ACN), methanol (MeOH) and LiChropur formic acid (FA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Reagent-grade ammonium hydroxide (NH<sub>4</sub>OH) was obtained from Ward's Science (Rochester, NY, USA). Analyte standards and internal standards (IS) were obtained from Toronto Research Chemical (North York, ON, Canada). Human serum and plasma K<sub>2</sub>EDTA were obtained from BioIVT (Westbury, NY, USA).

### Standards and solutions

Individual stock solutions of 1 mg/mL of HCQ and internal standard (HCQ-d<sub>4</sub>) were prepared in water. Individual stock solutions of BDCQ, DHCQ, and DCQ at 1 mg/mL were prepared in methanol (MeOH). All stock and working solutions were stored at -20 °C. Intermediate dilutions were prepared using MeOH:water (1:1, v:v).

A combination of four-analyte standard spiking solution was prepared in water at 10 µg/mL. The IS spiking solution was prepared in water at 1 µg/mL and was used to spike into samples directly.

Mobile phase A (water with 0.1% FA) was prepared by adding 1 mL of FA in to 1 L of Milli-Q water. Mobile phase B (ACN with 0.1% FA) was prepared by adding 1 mL of FA into 1 L of ACN. Crashing solvent (95/5 ACN/MeOH) was prepared by mixing 190 mL of ACN and 10 mL of MeOH. This was stored at -20 °C. Additional elution solvent (80/20 ACN/water with 5.8 % NH<sub>4</sub>OH) was prepared by combining 160 mL of ACN and 40 mL of Milli-Q water, followed by 50 mL of concentrated NH<sub>4</sub>OH (29% ammonia solution). Reconstitution solution (95/5 ACN/water with 0.1% FA) was prepared by combining 95 mL of

ACN, 5 mL of water, and 100 µL of FA. All reagents were stored and used at room temperature.

### Sample preparation equipment and consumables

- Agilent Captiva EMR—Lipid, 96-well plate, 40 mg (part number 5190-1000)
- Agilent positive pressure manifold 96 processor (PPM-96) (part number 5191-4116)

- Agilent square 96-well 2 mL collection plate (part number 5133009)
- Agilent square 96-well sealing caps (part number 5133005)
- Vortexer, VWR
- Shaker, ThermoScientific
- Evaporator, CentriVap Complete, Labconco
- Centrifuge 5424 R, Eppendorf
- Pipettes

### Instrument method

The samples were run on an LC-QQQ system under the following conditions:

HPLC Conditions			
Column	Agilent ZORBAX Eclipse XDB-C8, 2.1 × 50 mm, 3.5 µm (p/n 971700-906)		
Flow Rate	0.3 mL/min		
Column Temperature	40 °C		
Injection Volume	10 µL		
Mobile Phase	A) water with 0.1 % formic acid B) ACN with 0.1 % formic acid		
Gradient	Time (min)	%B	Flow rate (mL/min)
	0	5	0.3
	1.0	5	0.3
	2.0	8	0.3
	5.0	10	0.3
	5.1	70	0.3
7.0	70	0.3	
Post Time	3.0 min		
QQQ Conditions			
Gas Temperature	350 °C		
Gas Flow	7 L/min		
Nebulizer	35 psi		
Sheath Gas Heater	350 °C		
Sheath Gas Flow	11 L/min		
Capillary	3,500 V (POS)		
Data Acquisition	MRM as shown in Table 2		

Table 2. Target analyte MRM conditions.

Analyte	Precursor Ion (m/z)	Product Ion (m/z)	CE (V)	RT (min)
HCQ	336.2	247.0	19	3.17
		158.2	19	
HCQ-d <sub>4</sub> (IS)	340.2	251.0	19	3.16
		162.2	19	
BDCQ	264.1	179.0	20	2.55
		247.0	15	
DHCQ	308.1	179.0	20	2.70
		130.2	17	
DCQ	292.2	179.0	18	3.10
		114.3	16	

### Calibration standards and quality control (QC) sample preparation

The dynamic range for the calibration curve ranged from 0.4 to 100 ng/mL, including 0.4, 1, 5, 10, 50, 80, and 100 ng/mL in matrix. These standards were prepared by spiking an appropriate volume of standard spiking solution into the plasma or serum blank, and vortexing. Three levels of QC samples were run for method verification tests, including low QC of 1 ng/mL, mid QC of 10 ng/mL, and high QC of 100 ng/mL. These QC samples were prepared by spiking an appropriate volume of spiking solution into the matrix blank. An appropriate volume of IS spiking solution was then spiked into calibration standards and QC samples to generate the final IS concentration of 50 ng/mL in matrix. All samples were vortexed gently, completing the sample preparation process. The matrix-matched QCs were prepared by reconstituting the dried matrix blank residue with corresponding neat standard solution at 10 ng/mL and 100 ng/mL. Neat standard solutions were prepared by appropriately diluting the standard spiking solution with reconstitution solution to 10 and 100 ng/mL.

### Sample preparation

The sample preparation procedure is described in Figure 1. The method follows the same steps as the sample preparation method for human whole blood<sup>9</sup>, with the exception that dilution with water is not required after IS spiking into samples for both serum and plasma. Water was added to lyse the red blood cells by osmotic breakdown in the whole blood sample to account for any analytes internalized into the erythrocytes. This step, however, is not necessary for the preparation of serum or plasma.

### Method verification

The developed method was verified through accuracy and precision (A&P) runs. Two sets of calibration standards, six replicates of the three levels of QC samples and matrix blanks were prepared appropriately. Two sets of calibration standards were run at the beginning and end of sequence, bracketing the three levels of QC samples from low to high. Matrix blank samples were run for method selectivity and carry over evaluation.

### Analyte absolute recovery and matrix effect

Analyte absolute recoveries were evaluated based on analyte peak area comparison between prespiked QCs and matrix-matched QCs at mid-level (10 ng/mL) and high level (100 ng/mL). Matrix evaluation was based on analyte peak area comparison between matrix-matched QCs and neat standards at equivalent concentrations of 10 and 100 ng/mL.

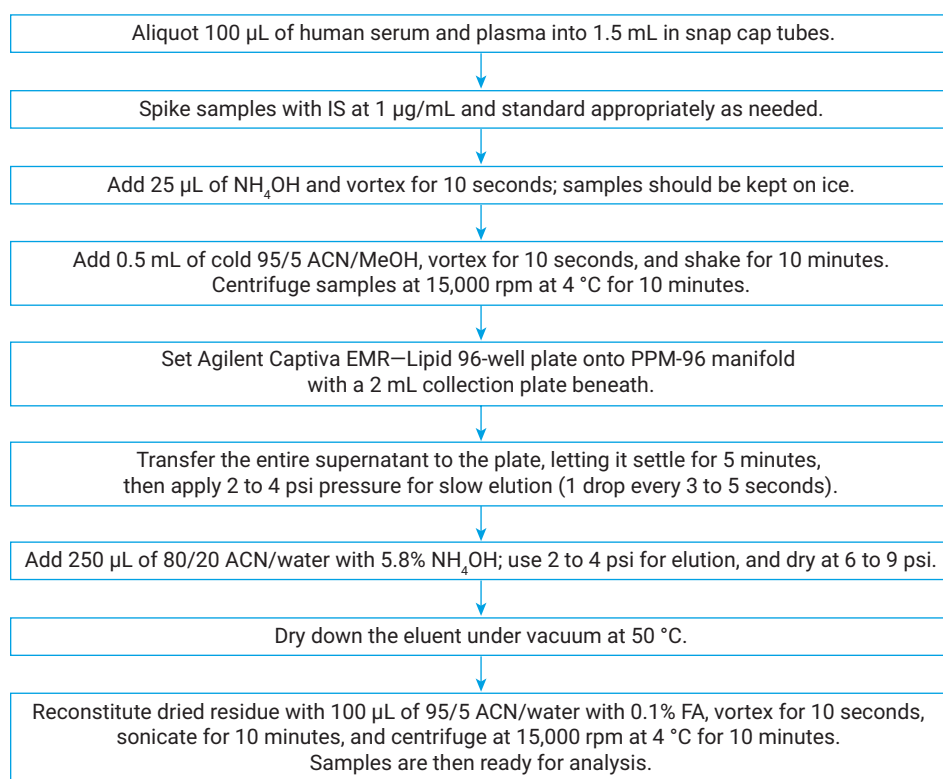


Figure 1. Sample preparation procedure scheme.

## Results and discussion

### Sample preparation method

The protein precipitation method using organic crashing solvents (such as ACN or MeOH) was used for HCQ and metabolite extraction in biological blood matrices.<sup>10</sup> Basic crashing solvent with 1% ammonium hydroxide (NH<sub>4</sub>OH) was also used for basic drugs.<sup>11</sup> As HCQ and metabolites are basic compounds, increasing the matrix pH with ammonium hydroxide should improve the extraction yield (Table 1). At physiological pH in blood matrices, the major form of HCQ and its metabolites is the di-cation form. It was reported that a crashing solvent of 5.8% NH<sub>4</sub>OH (v:v) enabled complete neutralization of the target analytes in matrix, and thus increased the extraction yield.<sup>12</sup> After testing different crashing solvents/solvent mixtures in the three blood matrices, a mixture of 95/5 ACN/MeOH with 5.8% NH<sub>4</sub>OH was optimized as the crashing solvent for protein precipitation extraction. Additionally, a washing step using 80/20 ACN/water with 5.8% NH<sub>4</sub>OH after the elution improved recovery.

### Analyte recovery and matrix effect

Analyte recovery evaluation results are shown in Figure 2. All targets showed absolute recoveries within the range of 60 to 120% with <15% RSD, in both human plasma and serum matrices. Analyte recoveries at low and high levels aligned well with each other, indicating consistent extraction efficiency provided by the sample preparation method at different levels.

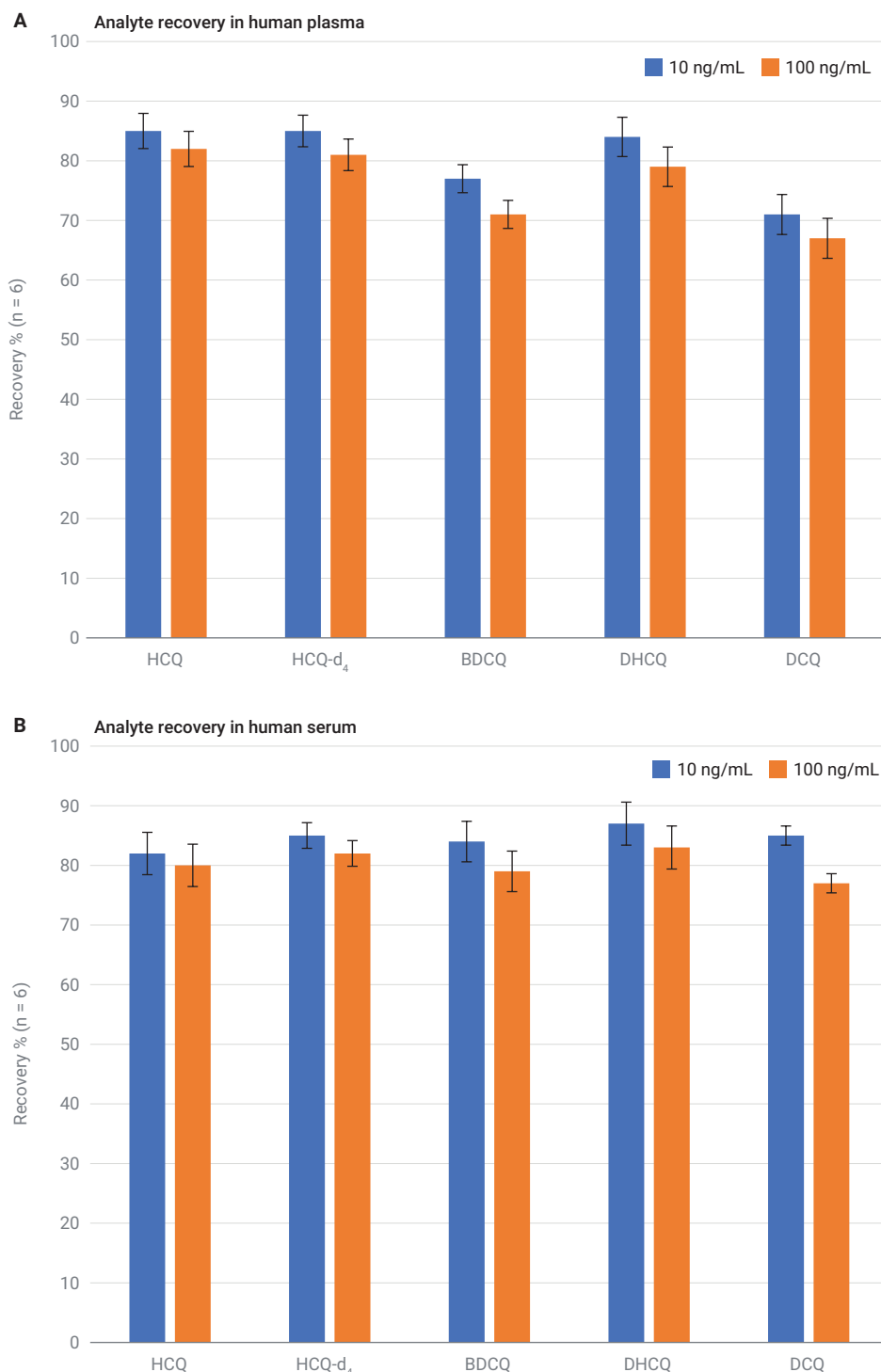
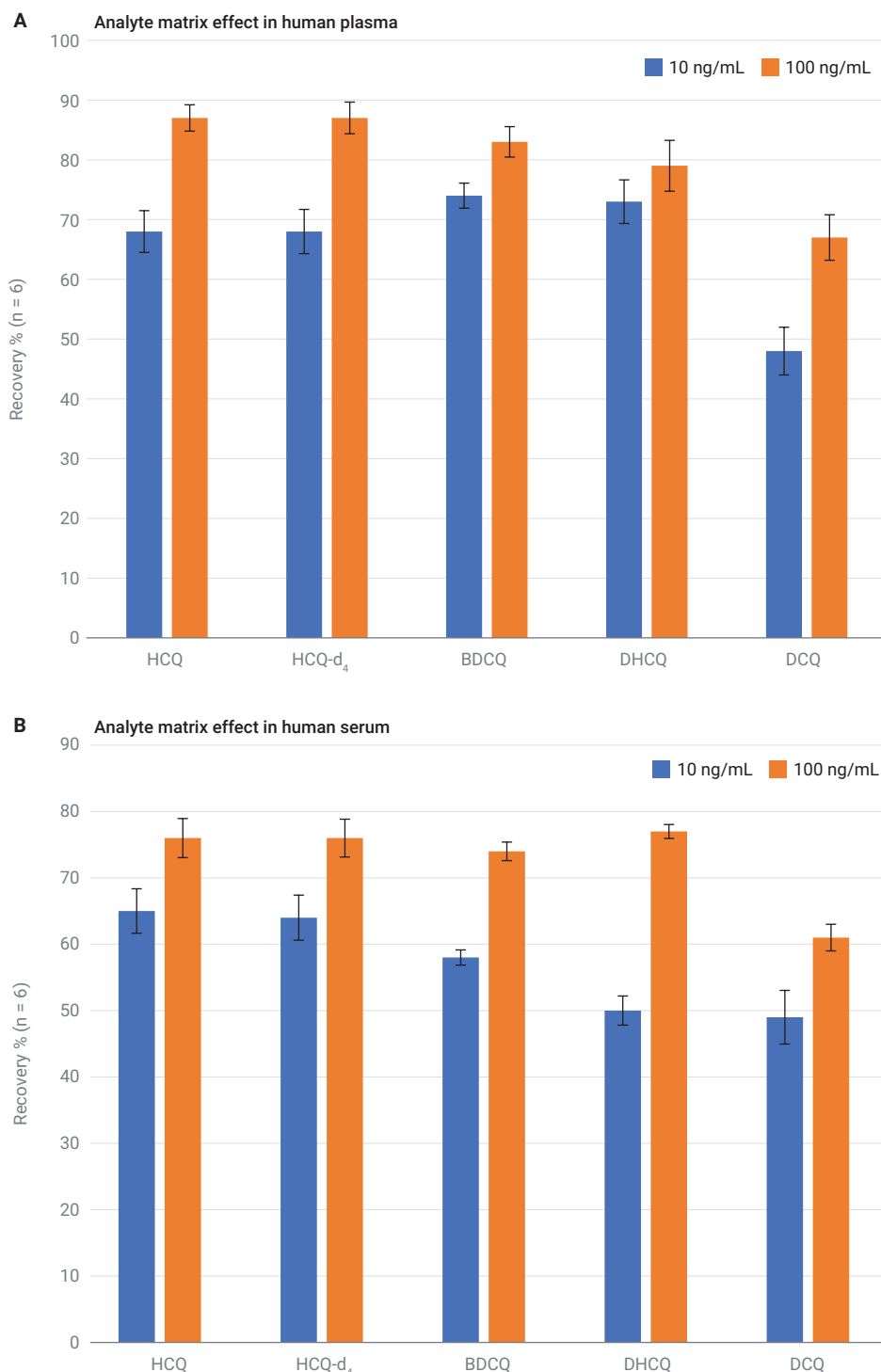


Figure 2. Analyte recovery, 10 ng/mL and 100 ng/mL in human plasma (A) and human serum (B).

Analyte matrix effects evaluation results are shown in Figure 3. All targets showed a matrix effect of >50% (except low-level DCQ) in both human plasma and serum matrices. In general, the low-level samples showed lower matrix effect values in both plasma and serum, indicating a greater matrix impact on lower concentration samples. Even though matrix effect is a good parameter for analytical method evaluation, no specific acceptance criteria exists as long as the quantitation method is reliable.

### Method sensitivity and selectivity

As part of method verification, method sensitivity was assessed based on the signal-to-noise ratio (S/N) at the LOQ level, and method selectivity was assessed based on the comparison of matrix blank contribution to the corresponding analyte peak area at the LOQ level. Figures 4 and 5 show the chromatograms of matrix blank and limit of quantitation (LOQ) for each analyte, in human serum and plasma, respectively. At the defined LOQ of 0.4 ng/mL, the analyte S/N was  $\geq 10$ . The matrix blank contribution was less than 20% of analyte responses at the LOQ level. The results clearly demonstrate method sensitivity and selectivity, confirming quantitation method reliability.



**Figure 3.** Analyte matrix effect, 10 ng/mL and 100 ng/mL in human plasma (A) and human serum (B).

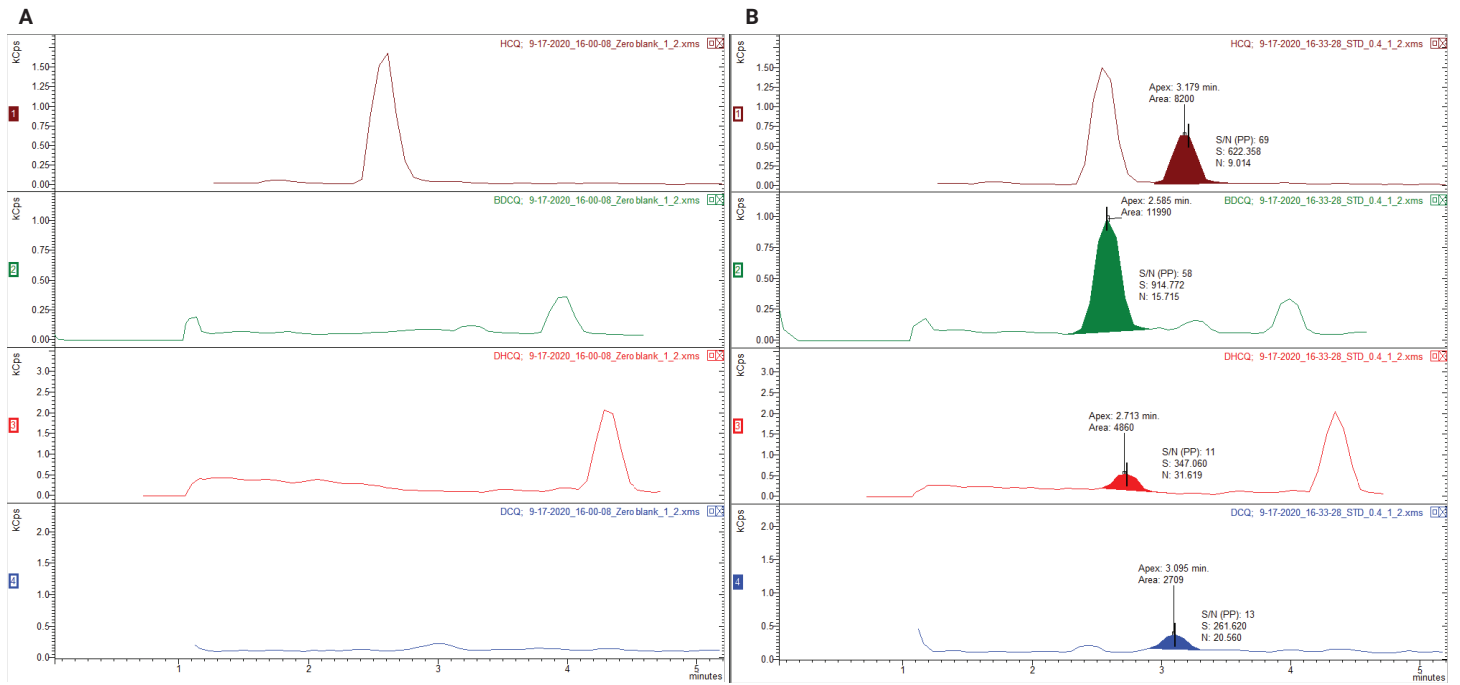


Figure 4. LC/MS/MS MRM chromatograms of human serum matrix blank (A) and LOQ (B) for the four targeted analytes.

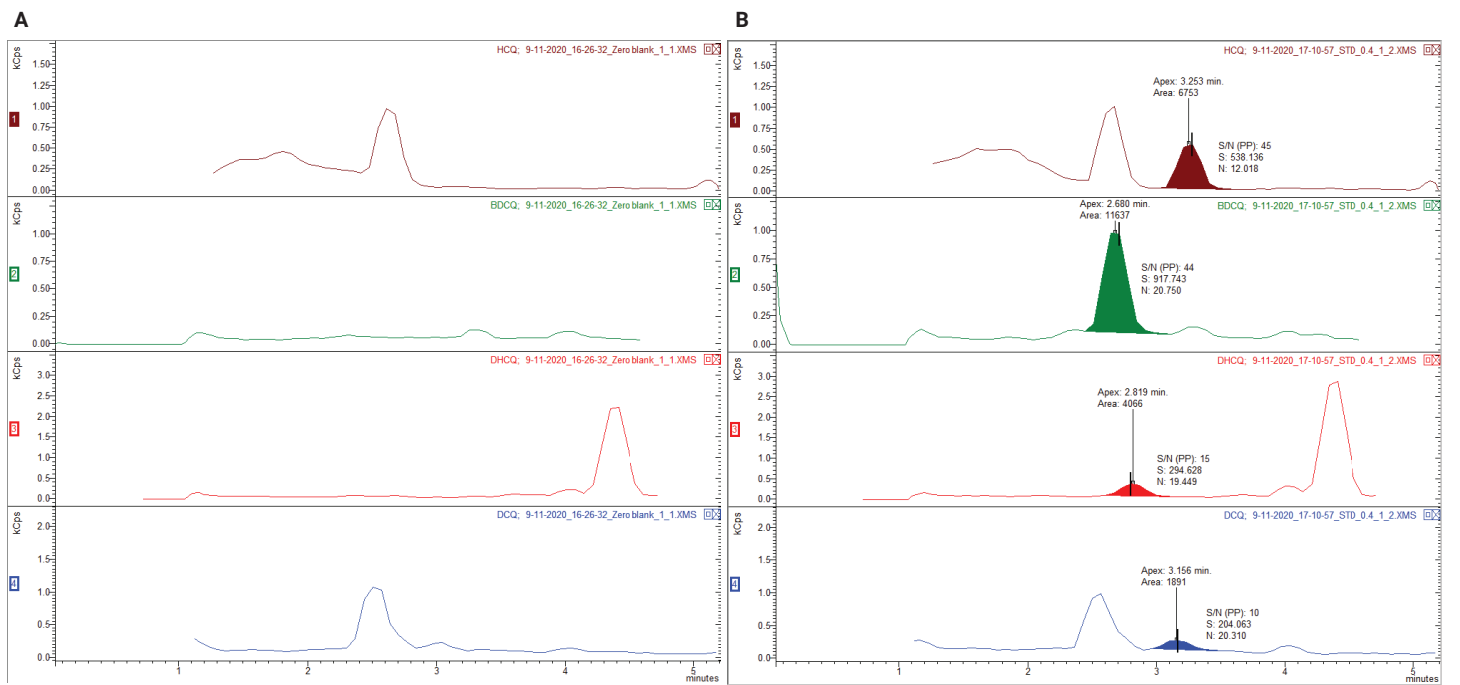


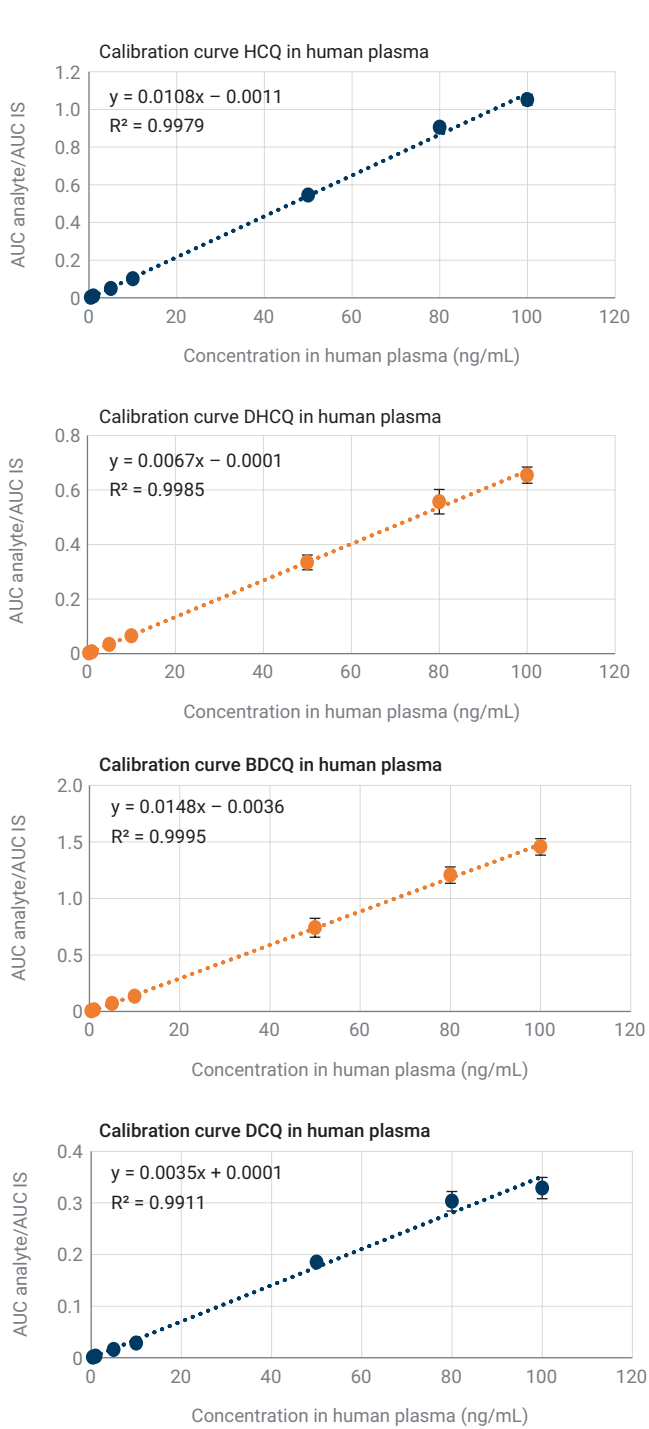
Figure 5. LC/MS/MS MRM chromatograms of human plasma matrix blank (A) and LOQ (B) for the four targeted analytes.

### Calibration curve linearity

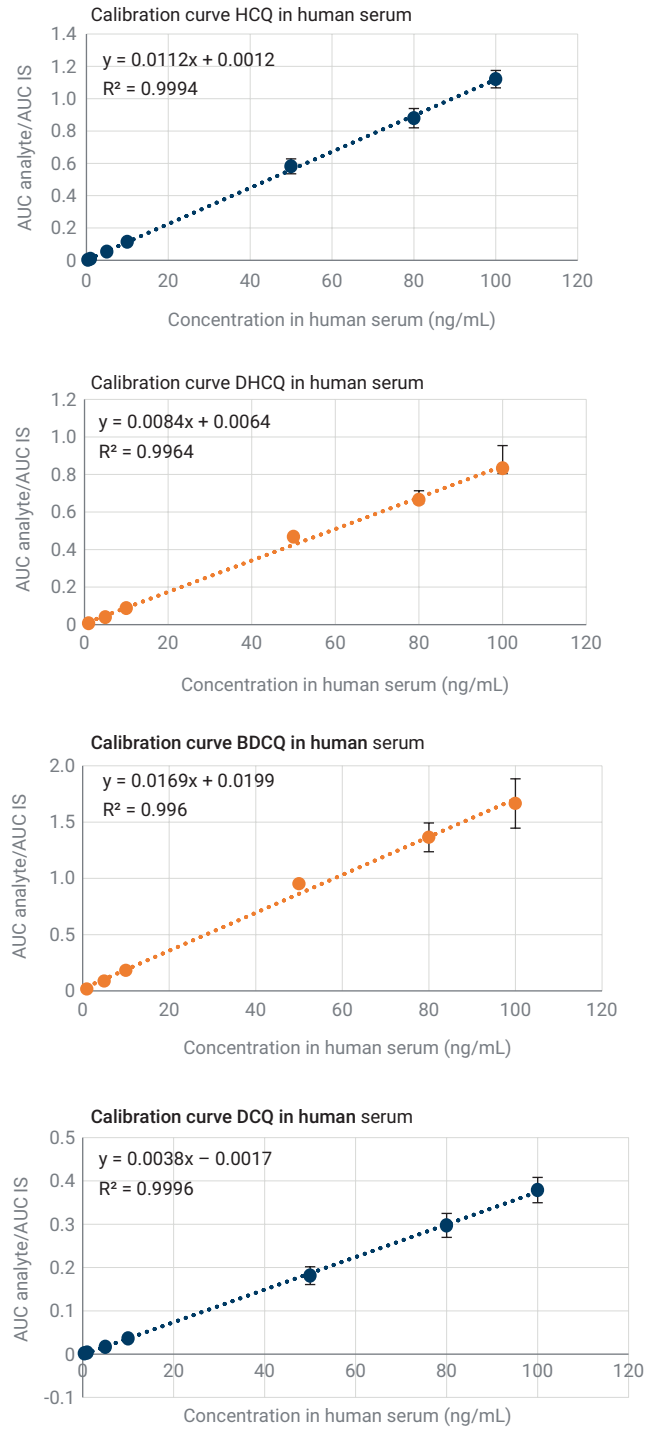
Method linearity was demonstrated in the dynamic range of 0.4 to 100 ng/mL in matrix. The calibration curves were

regressed using linear regression fit, with a weight of 1/x. The calibration curves for each analyte in human serum are shown in Figure 6, and the corresponding

curves for plasma in Figure 7. All curves showed excellent linearity over the calibration range, with  $R^2 > 0.99$ .



**Figure 6.** Calibration curves of four targeted analytes in human serum for the range of 0.4 to 100 ng/mL.



**Figure 7.** Calibration curves of four targeted analytes in human plasma for the range of 0.4 to 100 ng/mL.



### Quantitation accuracy and precision

The developed method was verified by accuracy and precision (A&P) runs to collect the complete quantitation results. The results shown in Table 3 include the accuracy and CV for all analytes at three levels in two matrices. Quantitation results from the A&P run demonstrated excellent method accuracy and precision results, meeting the typical acceptance criteria for clinical research testing (defined as an accuracy of  $100 \pm 15\%$  and  $CV \leq 15\%$ ).

### Conclusion

A robust method using protein precipitation followed with Captiva EMR–Lipid cleanup was established for fast and reliable analysis of hydroxychloroquine and metabolites in human serum and plasma using LC-QQQ. The method provided excellent quantitation results for analyte accuracy and precision, calibration curve linearity, sensitivity and selectivity, efficient recovery, and matrix removal, and provided a simplified workflow.

**Table 3.** Method accuracy and precision results for HCQ and three metabolites in human serum and plasma.

Sample Matrix	Spiking Concentration	HCQ	DHCQ	DCQ	BDCQ	
Human Plasma	1 ng/mL (n = 6)	Calc. conc. (ng/mL)	1.06	1.09	1.09	1.09
		Accuracy %	106	109	109	109
		CV %	1.9	4.1	2.5	7.3
	10 ng/mL (n = 6)	Calc. conc. (ng/mL)	10.27	10.48	10.90	10.50
		Accuracy %	103	105	109	105
		CV %	3.6	2.3	6.5	5.0
	100 ng/mL (n = 6)	Calc. conc. (ng/mL)	105.64	104.70	111.93	109.95
		Accuracy %	106	105	112	110
		CV %	2.1	5.6	5.0	6.3
Human Serum	1 ng/mL (n = 6)	Calc. conc. (ng/mL)	1.04	1.15	1.00	1.15
		Accuracy %	104	115	100	115
		CV %	3.5	5.5	12.6	3.1
	10 ng/mL (n = 6)	Calc. conc. (ng/mL)	10.39	11.51	11.28	11.52
		Accuracy %	104	115	113	115
		CV %	4.2	2.5	5.4	3.5
	100 ng/mL (n = 6)	Calc. conc. (ng/mL)	102.56	103.31	114.82	98.47
		Accuracy %	103	103	115	98
		CV %	1.7	4.2	2.7	5.4

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