Chapter 19

Quantification of Hydroxychloroquine in Blood Using Turbulent Flow Liquid Chromatography-Tandem Mass Spectrometry (TFLC-MS/MS)

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Abstract

Hydroxychloroquine (HQ) is used routinely in the treatment of autoimmune disorders such as rheumatoid arthritis and lupus erythematosus. Issues such as marked pharmacokinetic variability and patient non-compliance make therapeutic drug monitoring of HQ a useful tool for management of patients taking this drug. Quantitative measurements of HQ may aid in identifying poor efficacy as well as provide reliable information to distinguish patient non-compliance from refractory disease. We describe a rapid 7-min assay for the accurate and precise measurement of HQ concentrations in 100 µL samples of human blood using turbulent flow liquid chromatography coupled to tandem mass spectrometry. HQ is isolated from EDTA whole blood after a simple extraction with its deuterated analog, hydroxychloroquine-d4, in 0.33 M perchloric acid. Samples are then centrifuged and injected onto the TFLC-MS/MS system. Quantification is performed using a nine-point calibration curve that is linear over a wide range (15.7–4000 ng/mL) with precisions of <5 %.

Key words Hydroxychloroquine, Therapeutic drug monitoring, Turbulent flow liquid chromatography, Tandem mass spectrometry, Blood, Quantification

1 Introduction

Hydroxychloroquine (HQ), 2-[4-[(7-chloroquinolin-4-yl)amino] pentyl-ethylamino]ethanol, is a hydroxylated form of chloroquine, an aminoquinoline first synthesized in the 1930s for the treatment of malaria. Since then, HQ has also shown effectiveness in the treatment of several autoimmune and inflammatory disorders including lupus erythematosus, rheumatoid arthritis, chronic Q fever, Sjögren's syndrome, and various skin diseases [1].

HQ is commonly prescribed in oral doses of 200 or 400 mg per day. However, HQ is characterized by a long delay in onset of action and demonstrates wide pharmacokinetic variability among patients. Elimination half-life is estimated at up to 40 days [2]. Several studies indicate that low blood HQ concentrations may

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predict disease exacerbation [3, 4]. Additionally, measurement of blood HQ concentrations can identify patients considered non-compliant to treatment regimens and may improve management of refractory disease [5].

Previous methods of quantifying HQ blood levels, which included HPLC with fluorescence detection, consisted of extensive preparatory steps and run times of at least 15 min [6, 7]. Liquid chromatography paired with mass spectrometry may offer a more efficient method for HQ analysis [8]. The following chapter describes a simple, rapid (7 min), accurate and precise method to measure HQ in whole blood samples using turbulent flow liquid chromatography coupled to electrospray-positive ionization tandem mass spectrometry (TFLC-MS/MS) [9]. In this assay, HQ is isolated from 100 μL of EDTA whole blood after a simple extraction with an internal standard, hydroxychloroquine-d4, in perchloric acid solution. Samples are then centrifuged and ready to be injected onto the TFLC-MS/MS system.

2 Materials

2.1 Samples

Whole blood samples collected by standard venipuncture are required for analysis. Whole blood should be collected in EDTA tubes. Prior to analysis, specimens should be stored at 4 °C and analyzed within 2 weeks. Specimens may be stored at −20 °C for up to 6 months.

2.2 Solvents and Reagents

1. Perchloric acid, 70 % solution in water.
2. Mobile Phase A (10 mM ammonium formate and 0.1 % formic acid in water): Add 0.7 mL of ammonium hydroxide stock and 1.4 mL of formic acid to a 1 L volumetric flask filled with 990 mL of water. Bring to full volume with water. Stable at 25 °C for up to 1 month.
3. Mobile Phase B (10 mM ammonium formate and 0.1 % formic acid in methanol): Add 0.7 mL of ammonium hydroxide stock and 1.4 mL of formic acid to a 1 L volumetric flask filled with 990 mL of methanol. Bring to full volume with methanol. Stable at 25 °C for up to 1 month.
4. Mobile Phase C (40:40:20 isopropanol, acetonitrile, acetone): Fill a 4 L screw-cap glass bottle with 1.6 L of isopropanol. Add 1.6 L of acetonitrile and 800 mL of acetone. Close bottle and invert, allow to vent, then close and store. Stable at 25 °C for up to 1 week.
5. 0.33 M perchloric acid solution: Add 14.27 mL of 70 % perchloric acid to a 500 mL volumetric flask. Fill to full volume with water and invert to mix. Decant into glass bottle. Stable at 4 °C for up to 6 months.
6. Human drug-free pooled EDTA (lavender top) blood.

2.3 Internal Standards and Standards

1. Primary standard: Hydroxychloroquine sulfate.
2. Primary internal standard: Hydroxychloroquine-d4 sulfate.
3. Internal standard stock solution: Add 1 mL of water to 1 mg of hydroxychloroquine-d4 to make a 1 mg/mL solution. Stable at 4 °C for up to 6 months.
4. Internal Standard Working Solution: Add 10 μL of the 1 mg/mL hydroxychloroquine-d4 stock to 499.990 mL 0.33 M perchloric acid to make a 20 ng/mL solution. Stable at 4 °C for up to 6 months.

2.4 Calibrators and Controls

1. Calibrators: Prepare Calibrator 9 at 4000 ng/mL by spiking 80 μL of a 1 mg/mL solution of hydroxychloroquine into 20 mL of EDTA blood. Prepare Calibrators 1–8 by making serial dilutions of Calibrator 9 as described in Table 1. For each dilution step, add 10 mL of the previous spiked calibrator with 10 mL of drug-free blood and mix. The calibrators should be aliquoted to 500 μL and are stable at −80 °C for up to 1 year.
2. Controls: As described in Table 2, prepare a 1500 ng/mL "high" control by spiking 30 μL of a 1 mg/mL solution of hydroxychloroquine into 20 mL of EDTA blood. Prepare a 50 ng/mL "low" control by spiking 1 μL of a 1 mg/mL solution of hydroxychloroquine into 20 mL of EDTA blood. The controls should be aliquoted to 500 μL and are stable at −80 °C for up to 1 year.

Table 1
Preparation of hydroxychloroquine calibrators

Calibrator	Volume of previous standard (mL)	Drug-free whole blood (mL)	Final concentration (ng/mL)
9	0.08 (1 mg/mL stock solution)	20	4000
8	10 (#9)	10	2000
7	10 (#8)	10	1000
6	10 (#7)	10	500
5	10 (#6)	10	250
4	10 (#5)	10	125
3	10 (#4)	10	62.5
2	10 (#3)	10	31.25
1	10 (#2)	10	15.65

Table 2
Preparation of hydroxychloroquine quality controls

Control	Volume of 1 mg/mL stock solution (μL)	Drug-free whole blood (mL)	Final concentration (ng/mL)
High	30	20	1500
Low	1	20	50

Table 3
HPLC gradient for detection of hydroxychloroquine

			TFLC system Cyclone 50 × 0.5 mm							LX system HypersilGold C8 50 × 2.1 mm, 70 °C			
Step	Start time (min)	Duration (s)	Flow (mL/min)	Grad	%A[a]	%B[b]	%C[c]	TEE	Loop	Flow (mL/min)	Grad	%A[a]	%B[b]
1	0	30	1.50	Step	100.0	–	–	–	Out	0.70	Step	100.0	–
2	0.50	30	0.20	Step	100.0	–	–	TEE	In	0.70	Step	100.0	–
3	1.00	30	1.00	Step	–	–	100.00	–	In	0.70	Ramp	50.0	50.0
4	1.50	30	1.00	Step	–	100.0	–	–	In	0.70	Ramp	25.0	75.0
5	2.00	30	1.00	Step	–	–	100.00	–	In	0.70	Ramp	–	100.0
6	2.50	30	1.00	Step	–	100.0	–	–	In	0.70	Ramp	100.0	–
7	3.00	30	0.50	Step	100.0	–	–	–	In	0.70	Ramp	–	100.0
8	3.50	45	1.00	Step	65.0	35.0	–	–	In	0.70	Step	–	100.0
9	4.25	45	1.50	Step	100.0	–	–	–	Out	0.70	Step	100.0	–

[a]Mobile Phase A: 10 mM ammonium formate and 0.1 % formic acid in water
[b]Mobile Phase B: 10 mM ammonium formate and 0.1 % formic acid in methanol
[c]Mobile Phase C: 40:40:20 isopropanol, acetonitrile, acetone

2.5 Analytical Equipment and Supplies

1. ThermoFisher TSQ Vantage tandem mass spectrometer with HESI probe.
2. TFLC column: Cyclone, 50 × 0.5 mm.
3. Analytical column: Hypersil Gold C8, 3 μm, 50 × 2.1 mm.
4. 1.5 mL microcentrifuge tubes.
5. Assign instrumental operating parameters according to Tables 3 and 4. Parameters are instrumentation-specific.

Table 4
TSQ Vantage instrument parameters and ion transitions

Spray voltage (V)	4000
Sheath gas pressure	40
Ion sweep gas pressure	2.0
Aux gas pressure	20
Capillary temperature (°C)	200
Hydroxychloroquine	
Parent ion	336.2
Product ion	247.1
Collision energy	20
S-lens RF amplitude	133
Hydroxychloroquine-d4	
Parent ion	340.2
Product ion	251.1
Collision energy	21
S-lens RF amplitude	137

3 Methods

3.1 Stepwise Procedure

1. Pipet 100 µL of whole blood to a 1.5 mL microcentrifuge tube.
2. Add 1000 µL of Internal Standard Working Solution to the blood.
3. Cap and vortex the mixture for 30 s.
4. Centrifuge the sample at 13,400 × *g* for 5 min.
5. Transfer the supernatant to a glass vial for loading into the autosampler.
6. Inject 10 µL of sample onto the TFLC-MS/MS system.
7. Instrumental operating parameters are given in Tables 3 and 4. Include a 2 min wash step between samples (*see* **Note 1**).

3.2 Analysis

1. Data are analyzed using Thermo Scientific TraceFinder software (*see* **Note 2**). The program uses the values obtained for the calibrators to construct a calibration curve based on the ratio of each calibrator to the internal standard. This calibration curve is then used to quantitate the unknowns.
2. Liquid chromatography retention times for hydroxychloroquine and hydroxychloroquine-d4 are set at 2.08 ± 0.4 min (*see* Fig. 1).

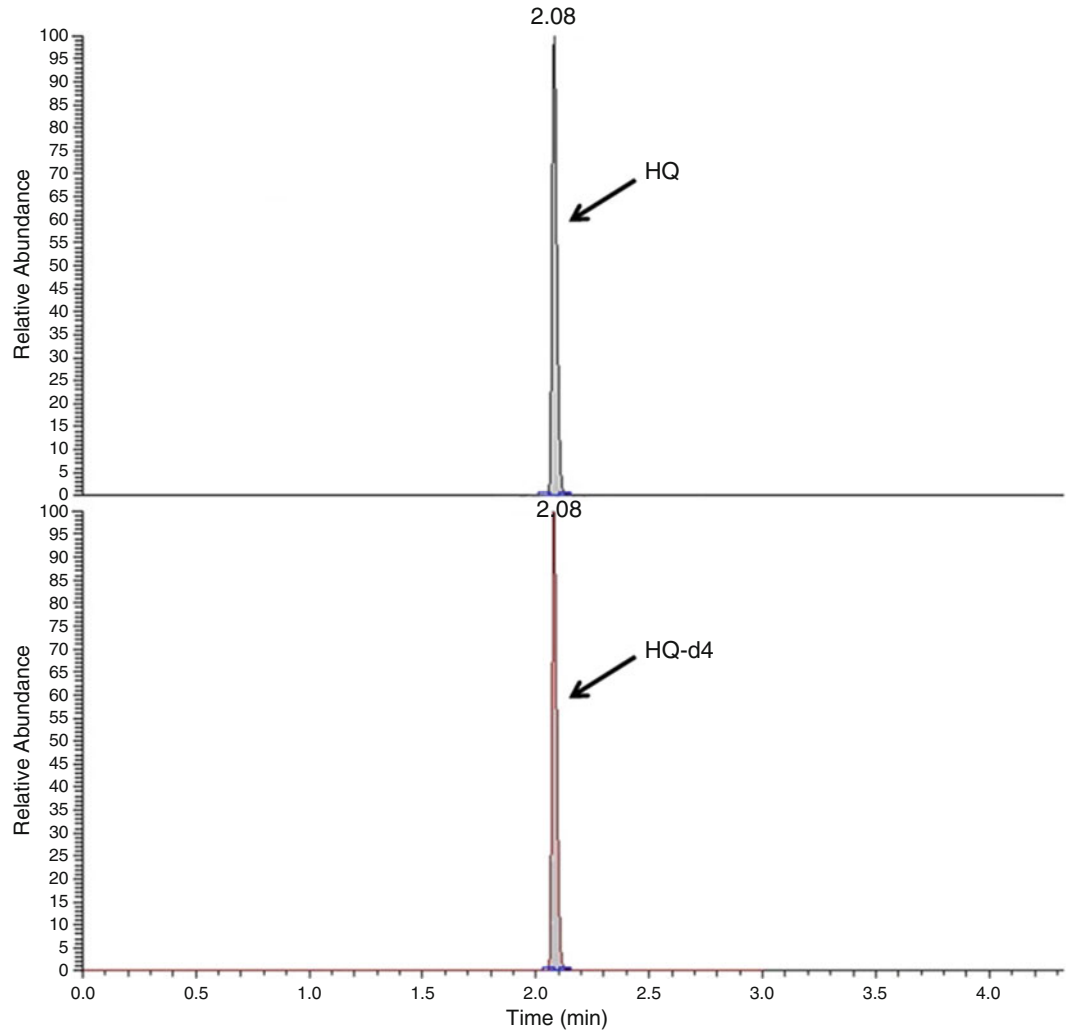

Fig. 1 TFLC-MS/MS ion chromatograms of hydroxychloroquine and hydroxychloroquine-d4

3. The linear range for the assay is the same as the calibration limits of 15.7–4000 ng/mL with precisions of <5 % over the entire range. The lowest concentration that resulted in a CV of 20 % was determined to be 6.0 ng/mL. However, specimens that are lower than the lowest calibrator are reported as less than that value ("<15.7 ng/mL"). *See* **Notes 3** and **4** for further information regarding linearity and accuracy, respectively.

4 Notes

1. A challenge in the development of this assay was significant carryover from one sample to the next. A rigorous validation of carryover was conducted as previously described by running

"high" (2000 ng/mL) and "low" (15.7 ng/mL) spiked controls in various sequences [9]. Inclusion of a 2 min wash step after elution of hydroxychloroquine significantly minimized carry-over, defined as the mean of low–low results subtracted from the mean of high–low results, to within <3 standard deviations of the low–low results.

2. It is important to note the shapes of the standard peaks to verify that the drug has eluted in well resolved symmetrical peaks. If this has not occurred, it is indicative of a problem with the run. Additionally, each sample peak should be evaluated for quality. If a peak is excessively jagged, noisy, or deviates from a bell shape, data for this injection must not be used and troubleshooting must be performed.
3. Though a majority of reported HQ concentrations fall between 50 and 1700 ng/mL, at least one reported level exceeded 2000 ng/mL [10]. For any specimens greater than the highest calibrator, a dilution should be made with drug-free whole blood. Add 100 µL of sample to 100 µL of drug-free EDTA blood, mix gently by inverting, and extract as normal. Dilutions of concentrations as high as 4000 ng/mL were validated with this assay and yielded a CV of within 3 % at each level. Therefore, we have added a 4000 ng/mL high-end calibrator to the assay to cover all anticipated result values.
4. Because a reference method for this assay does not exist, this assay was validated with side-by-side comparisons of samples both analyzed in-house and sent to a reference laboratory for analysis by LC-MS/MS as previously described [9]. Deming regression and statistical analysis yielded a Pearson correlation of 0.9974, indicating excellent correlation over the concentration range of 15–2000 ng/mL. Because no proficiency testing for hydroxychloroquine is available at this time, our laboratory participates in a twice yearly sample exchange with another laboratory performing hydroxychloroquine testing to establish acceptability criteria.

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