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Improved validated assay for the determination of mefloquine and its carboxy metabolite in plasma, serum and whole blood using solid-phase extraction and high-performance liquid chromatography

Michael D. Green[a,*], Yngve Bergqvist[b], Dwight L. Mount[a], Samantha Corbett[a], Martin J. D'Souza[c]

[a]*Division of Parasitic Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Public Health Service, 1600 Clifton Road, Mailstop F-12, Atlanta, GA 30333, USA*
[b]*Dalarna University College, S-781 88 Borlänge, Unit of Tropical Pharmacology, Division of Clinical Pharmacology, Karolinska Institute, Huddinge University Hospital, S-141 86 Huddinge, Sweden*
[c]*Mercer University, Southern School of Pharmacy, 3001 Mercer Drive, Atlanta, GA 30341, USA*

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Abstract

An improved high-performance liquid chromatography method using a low silanol activity octadecylsilica column and a solid-phase extraction technique is validated for the simultaneous analysis of mefloquine and its carboxy metabolite in whole blood, plasma and serum. An octadecylsilica column with high silanol activity is compared to a column of low activity in terms of pH dependent variability of chromatographic retention times for mefloquine and its carboxy metabolite. The low silanol activity column showed a relatively large mobile phase pH range where retention times for both components are consistent. The solid-phase extraction procedure consists of a simple protein precipitation step followed by sample concentration and extraction using a C_{18} membrane disk. The inter- and intra-assay variability for a therapeutic concentration of mefloquine (1000 ng/ml) is less than 2% in whole blood, plasma and serum while carboxymefloquine (1000 ng/ml) is 2.3% or less. At concentrations as low as 100 ng/ml the inter-assay variability is 6.2% or less for both analytes. This method shows a robust analytical procedure for the simultaneous analysis of mefloquine and its carboxy metabolite where precise measurements are useful in pharmacokinetic studies and in estimating drug compliance.

Keywords: Mefloquine; Carboxymefloquine

1. Introduction

Mefloquine (MQ) has proven to be an effective prophylactic drug against malaria and is highly recommended for non-immune individuals traveling to areas where chloroquine-resistant *Plasmodium falciparum* is present [1]. In patients who have reputedly used mefloquine prophylaxis and are infected with *Plasmodium falciparum* malaria, it is useful to monitor mefloquine levels in blood to assess whether such prophylaxis failures result from resistance or from inadequate drug levels.

*Corresponding author. Tel.: +1-770-488-4039; fax: +1-770-488-4108.
E-mail address: MDG4@CDC.GOV (M.D. Green)

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Todd et al. has showed that a consistent ratio of the carboxy metabolite of mefloquine (MMQ) to (MQ) is attained during steady state [2]. Therefore, this ratio should be considered when determining whether an individual has regularly complied with their prophylactic regimen. An accurate MMQ-to-MQ ratio requires a robust analytical method that can simultaneously determine blood levels of both components. The few high-performance liquid chromatographic methods available for determining both compounds simultaneously involve ion-pairing extraction methods or column-switching techniques [3–5].

Concurrent extraction and chromatography of MQ and MMQ are difficult due to their widely disparate ionization constants. Since MMQ is a weak acid with *a* pK_a 4.5 and MQ is a weak base with a pK_b of 8.5 (Fig. 1), consistent extraction efficiency and chromatographic separation are very sensitive to pH changes. The silanol effect associated with the octadecylsilica (C_{18}) high-performance liquid chromatography (HPLC) column matrix is also pH dependent and significantly influences chromatographic separation of ionic compounds. Therefore the aim of this study is to evaluate and compare the use of a low silanol activity column on the chromatographic separation of MQ and MMQ.

A solid-phase extraction (SPE) technique was developed to simultaneously extract MQ and MMQ from whole blood, plasma or serum. This extraction method is validated for preciseness, accuracy and robustness.

2. Experimental

2.1. Reagents[1] and calibrators

MQ and MMQ were provided by Hoffman-LaRoche (Basel, Switzerland). The internal standard (I.S.) used in the assay is WR184806 (Fig. 1) and was obtained from Walter Reed Army Institute of Research. The HPLC-grade acetonitrile was obtained from Burdick and Jackson (Muskegon, MI, USA).

[1]Use of trade names is for identification only and does not imply endorsement by the Public Health Service or by the US Department of Health and Human Services.

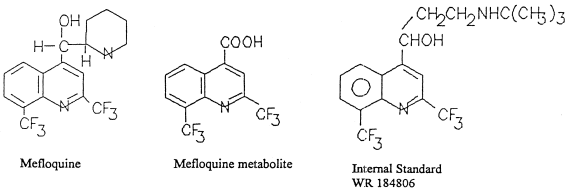

Fig. 1. Structure of mefloquine (MQ), carboxy metabolite of mefloquine (MMQ) and internal standard (I.S.), WR 184806.

All other chemicals were obtained from Sigma (St. Louis, MO, USA) and were of HPLC or analytical grade.

2.2. Apparatus and conditions

The HPLC system consisted of a ChromTech ISO-2000 (Apple Valley, MN, USA) pump with a LDC/Milton Roy spectroMonitor III (Riveria Beach, FL, USA) variable-wavelength detector set at a single wavelength of 222 nm. Components from the SPE evaluation were analyzed using a 250×4.6 mm SymmetryShield RP18 5μm column (Waters, Milford, MA, USA) with a mobile phase of acetonitrile–0.1 *M* KH_2PO_4, (40:60, v/v), adjusted to pH 6.0 with ammonium hydroxide. The mobile phase flow-rate through the column was 1.0 ml/min with a column temperature of 35°C. A Waters 250×4.6 mm (Spherisorb S5 ODS1) column (relatively high silanol activity [6]) was used to compare variations of component retention times with pH changes and acetonitrile concentrations of the mobile phase. Also the effects of KH_2PO_4 concentration on retention time and peak area were observed.

2.3. Calibration

A stock solution of MQ base was prepared by adding 10.10 mg of the hydrochloride salt to a 50-ml volumetric flask and filling to the mark with 0.1 *M* HCl. The working calibration solution (25 μg/ml MQ base) was prepared by transferring 1.36 ml of the stock solution to a 10-ml volumetric flask and diluting to the mark with deionized water.

A stock solution of MMQ was prepared by adding 1.56 mg to a 10-ml volumetric flask and filling to the mark with 0.1 *M* NaOH. The working calibration solution (25 μg/ml) was prepared by transferring

1.60 ml of the MMQ stock solution to a 10-ml volumetric flask and diluting to the mark with deionized water.

The molar absorptivities of the working solutions were recorded to assess accuracy and monitor stability. Human whole blood, plasma and serum were spiked with the working calibration solutions to yield final MQ and MMQ concentrations of 1000, 500, 250, 100 and 0 ng/ml.

2.4. Protein precipitation

Two hundred microliters of I.S. WR184806 (2 mg/ml in 0.01 *M* HCl) were added to 1.5-ml polypropylene microfuge tubes containing 200 μl of sample and mixed thoroughly. Fifty μl of $ZnSO_4$ (0.2 *M*) was added to each sample while vortexing. Then 550 μl of acetonitrile were added and the sample vortexed again until the sample appeared homogeneous. After standing at room temperature for 10 min, the samples were centrifuged at 10 000 *g* for 10 min. The supernatant was transferred to a 15-ml polypropylene centrifuge tube and 0.1 *M* phosphate buffer, pH 3, was added to yield a final eluate volume of 3 ml.

2.5. Solid-phase extraction

Empore, 7 mm diameter C_{18} SPE disks (Varian, Harbor City, CA, USA) were equilibrated by passing 0.5 ml of methanol followed by 0.5 ml of deionized water. The membranes were not allowed to dry. The samples (3 ml) were transferred to the SPE cartridges and allowed to pass through the membrane with the aid of a Vac-Elut extraction manifold (Analytichem, Harbor City, CA, USA). The SPE disks were eluted with 0.5 ml of methanol into a polypropylene tube and dried with nitrogen or air. The residue was dissolved in 200 μl of HPLC mobile phase and 50 or 100 μl was injected into the HPLC system for analysis.

2.6. Optimization and robustness of SPE

To obtain the eluate acetonitrile concentration for optimal sample recovery, various amounts of acetonitrile were added to 200 μl of spiked whole blood and treated as described in Section 2.4. The final acetonitrile concentrations of the sample eluates were 10, 13, 20 and 27%. The sample eluates were extracted as described in Section 2.5 and the recoveries for MQ and MMQ were determined. Previous experiments have shown the optimal eluate pH to be 3. To determine if variations of pH affect sample recovery, the pH of the eluate was adjusted 0.5 units above and below the optimum pH of 3 and extracted as described previously.

2.7. Comparison of SPE method with liquid–liquid extraction method (accuracy)

Whole blood samples from individuals receiving MQ prophylaxis were extracted using the described SPE method and analyzed by HPLC. The same set of samples were also analyzed independently (Dr. Bergqvist, Falun, Sweden) using a liquid–liquid ion-pair extraction HPLC method previously reported [5]. Briefly, plasma and whole blood are deproteinized with a combination of zinc and acetonitrile before extraction. MQ and MMQ are extracted simultaneously at pH 4 by methyl *tert*.-butyl ether, where MQ is extracted as an ion pair with heptanesulfonate. The levels of MQ and MMQ from each laboratory were compared.

2.8. Intra- and inter-assay precision and accuracy

Solid-phase extractions were performed on four replicates at each concentration of MQ and MMQ in whole blood, plasma and serum on each of four separate days. The concentrations were determined by HPLC using the SymmetryShield column. The calibration curve was prepared on the day of analysis and was constructed from the measurement of peak areas ratio of the analytes and internal standard. To assess precision, coefficients of variation (CVs) were determined for intra- and inter-assay variability. Accuracy was determined from the difference of the expected and calculated values relative to the expected values (% deviation).

2.9. Interferences

Retention times for widely used antimalarial drugs such as quinine, chloroquine, sulfadoxine, pyrimethamine and the desethyl metabolite of chloroquine

were assessed for possible chromatographic interferences.

3. Results and discussion

3.1. HPLC column evaluation

Fig. 2 illustrates the influence of mobile phase pH on the chromatographic retention characteristics of MQ, MMQ and I.S. for the Spherisorb ODS1 and the SymmetryShield RP-18 columns. Although both columns are very similar in their hydrophobic characteristics, their silanol activities are quite different [6]. The charge on the solid-phase matrix surface silanol groups changes with mobile phase pH. At pH 2, the charge is neutral; as the pH increases, a negative charge develops until the surface silanols are fully ionized at a pH of 7 [6]. The matrix then behaves as a cation exchanger, resulting in strong interactions with basic analytes. Fig. 2 clearly shows that a mobile phase pH below the ionization constant for the weakly acidic MMQ (pK_a=4.5), greatly influences the retention times for MMQ with both columns. The Spherisorb column showed a mobile phase pH effect (pH>4.5) on the retention times for MQ and I.S., demonstrating the influence of silanol charge on the chromatography characteristic for these basic compounds (Fig. 2a). The low silanol activity of the SymmetryShield matrix is attributed to an embedded polar group which reduces interactions with silanols [7], resulting in little change of MQ and I.S. retention as a function of mobile phase pH above 4.5 (Fig. 2b). The effects of mobile phase acetonitrile and KH_2PO_4 concentrations as well as pH (Figs. 2–4) are useful for adjusting the retention times of the analytes as well as any interfering peaks to achieve good separation. The decrease in retention time for the basic analytes with increased concentration of the KH_2PO_4 seems to be an ion-exchange mechanism where the K^+ ion is added on the negative silanol group. Fig. 4 shows the decrease in elution times of the positive charged analytes relative to the increased concentration of K^+.

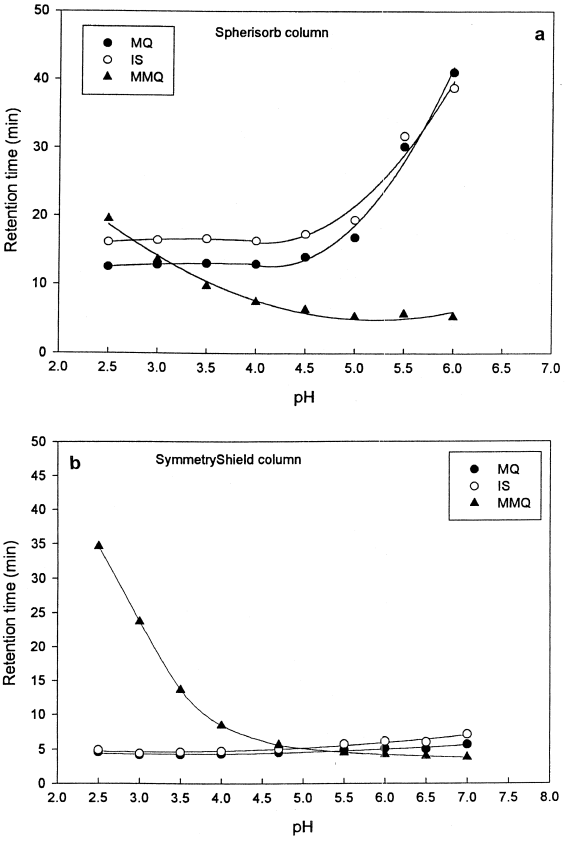

Fig. 2. Retention time variation due to the influence of mobile phase pH using: (a) Spherisorb column, and (b) SymmetryShield column. The mobile phase used for both columns consists of acetonitrile–0.1 *M* phosphate buffer (45:35). The flow-rate is 1 ml/min with the column temperature at 35°C.

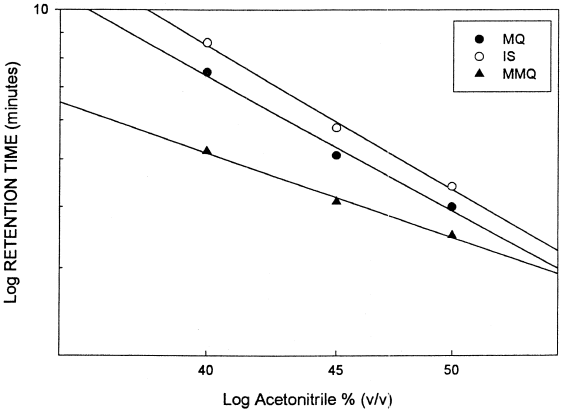

Fig. 3. Effects of acetonitrile concentration on retention times using the SymmetryShield column.

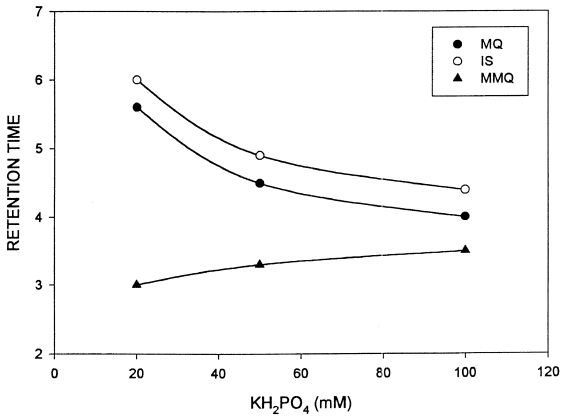

Fig. 4. Effects of KH_2PO_4 concentration (in the 55% aqueous portion of mobile phase) on retention times using the SymmetryShield column.

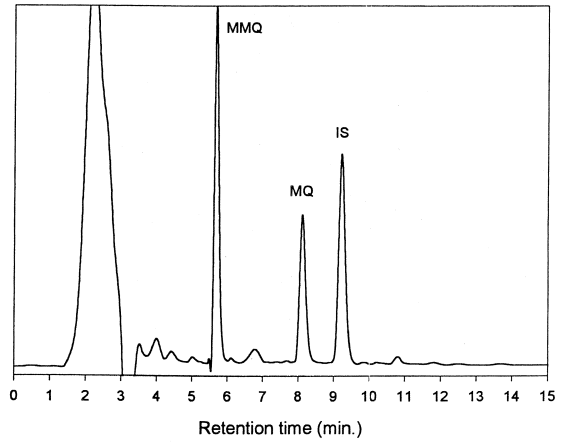

Fig. 5. Typical chromatogram of extracted spiked whole blood (MQ and MMQ concentration=1000 ng/ml using the SymmetryShield column.

3.2. Interferences

Other commonly used antimalarials such as quinine, chloroquine, desethyl metabolite of chloroquine, sulfadoxine and pyrimethamine showed no interference with MQ, MMQ or I.S.

3.3. SPE robustness evaluation

A chromatogram of spiked whole blood employing the SPE extraction method previously described is shown in Fig. 5. SPE optimization experiments reveal a sample eluate acetonitrile concentration of about 18% was sufficient to achieve maximum recovery of the analytes from the SPE cartridges. Acetonitrile concentrations between 16 and 20% did not significantly influence extraction recoveries of the analytes. Also, variations of the eluate pH ranging from 2.5 to 3.5 had negligible effect on analyte recovery.

3.4. SPE extraction recovery

The percent recovery of MQ and MMQ from each of the biological matrices is shown in Table 1. MQ extraction recovery incorporating the C_{18} membrane was not very different than recovery of MQ from the protein precipitation step indicating that most of the extraction loss occurs during the precipitation phase. A comparison could not be made with MMQ due to interfering peaks in the supernatant sample.

3.5. Precision and accuracy

Assay precision and accuracy are summarized in Tables 2 and 3. There were no significant differences

Table 1
Extraction recovery ($n=4$)

	Concentration (ng/ml)	Whole blood (mean±SD) (%)	Plasma (mean±SD) (%)	Serum (mean±SD) (%)
Mefloquine	100	83±4	67±8	84±4
	1000	70±7	77±2	76±5
Carboxy metabolite	100	93±6	94±4	99±5
	1000	87±10	97±2	95±5

Table 2
Intra- and inter-assay precision of MQ and MMQ

Sample	Concentration (ng/ml)	Whole blood CV (%)	Plasma CV (%)	Serum CV (%)
Mefloquine				
Intra-assay (n=16)	100	15	11	9.3
	250	6.4	5.5	3.3
	500	5.3	4.1	3.7
	1000	1.8	0.8	0.8
Inter-assay (n=4)	100	5.8	5.4	3.3
	250	4.1	2.3	1.3
	500	4.6	2.7	0.8
	1000	0.8	0.5	0.2
Carboxy metabolite				
Intra-assay (n=16)	100	14	12	14
	250	7.5	2.5	3.2
	500	8.4	5.0	5.2
	1000	2.3	1.1	1.2
Inter-assay (n=4)	100	6.2	5.0	2.5
	250	7.0	1.4	1.7
	500	6.6	1.7	1.6
	1000	1.3	0.4	0.3

in precision between sample matrices (whole blood, plasma, or serum). The results of the inter-laboratory comparison of our method with an established method showed a good correlation (Fig. 6).

3.6. Limit of determination

The limit of determination for MQ using 200 μl of whole blood, plasma, or serum was estimated to be 80, 70 and 60 ng/ml, respectively, with an intra-assay CV of 20%. For MMQ, the limit of determination at a CV of 20% was 70, 50 and 60 ng/ml in 200 μl of whole blood, plasma and serum, respectively.

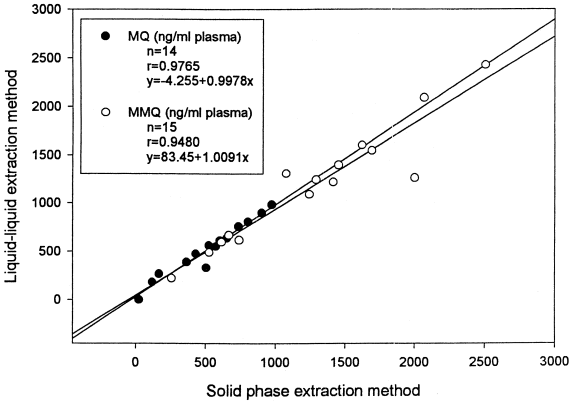

Fig. 6. Inter-laboratory comparison of extraction techniques.

4. Conclusions

Quantitation of MMQ as well as the parent drug at steady state conditions could provide information as to whether a patient complied with prophylactic medication. Therefore, a precise analytical method for the simultaneous determination of both MQ and MMQ is desirable.

The use of a chromatographic column matrix with low silanol activity with a simple SPE procedure has been shown to be very reliable for the simultaneous quantitation of MQ and MMQ. Relative chromatographic retention times for MQ and MMQ showed little dependence on mobile phase pH. Also, small changes in pH and acetonitrile concentration of the extraction eluate did not significantly affect extraction recovery. Inter- and intra-assay variability revealed a highly precise method for determining blood levels of MQ and MMQ concurrently. These

Table 3
Assay accuracy (n=16)

Sample	Concentration (ng/ml)	Whole blood CV (%)	Plasma CV (%)	Serum CV (%)
Mefloquine	100	6.3	16	2.1
	250	2.3	0.3	0.2
	500	0.5	5.8	0.9
	1000	0.5	1.3	0.2
Correlation coefficient		0.9999	0.9986	1.000
Regression equation		$y=0.00068x-0.00119$	$y=0.00076x-0.00119$	$y=0.00072x+0.02587$
Carboxy metabolite				
	100	1.6	9.8	4.6
	250	0.1	6.2	1.0
	500	1.5	1.2	2.3
	1000	0.3	0.0	0.5
Correlation coefficient		1.000	0.9996	0.9999
Regression equation		$y=0.00124x-0.01370$	$y=0.00087x-0.01370$	$y=0.00084x+0.01829$

observations show very good robustness and reliability of the SPE extraction technique and the HPLC method with the SymmetryShield column.

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