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Research Article

High-Performance Liquid Chromatography with Diode Array Detection for the Simultaneous Quantification of Piperaquine and Mefloquine in Human Plasma in Vietnamese Uncomplicated Malaria Patients

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ABSTRACT

A bioanalytical method using high-performance liquid chromatography with diode array detection was developed and validated for quantification of piperaquine and mefloquine in human plasma. After extraction from 200 µL of plasma by using solid phase extraction on MCX-96 well plate; piperaquine, mefloquine and quinine (internal standard) were separated using a mobile phase consisting of trichloroacetic acid 1 mM and trimethylamine 0.025%, pH 3.4-Acetonitrile applying gradient elution on a Poroshell 120, C8 end capped 100 × 4.6 mm, 2.7 µm column at a flow rate of 1.0 mL/min at 35°C. The peaks were monitored with a diode array detection set at a wavelength of 343 nm for piperaquine, quinine and 283 nm for mefloquine. The limit of quantification was 10 ng/mL, 50 ng/mL for piperaquine and mefloquine, respectively. The method was linear over the range of 10 to 2,000 ng/ml for piperaquine and 50 to 10,000 ng/ml for mefloquine in plasma. The intra, inter assay precision were less than 8% overall quality control ranges for these analytes. The accuracies varied between 99.85% and 104.6% at all quality control levels for piperaquine and mefloquine. The validated method was applied to measure the concentrations of piperaquine, mefloquine in samples collected from 12 uncomplicated malaria patients in Binh Phuoc province, Vietnam.

Keywords: *Antimalarial; High-performance liquid chromatography; Mefloquine; Piperaquine; Plasma*

Abbreviations: ANOVA: Analysis of variance; ACN: Acetonitrile; ACTs: Artemisinin combination therapies; CBA: Carboxylic acid sorbent; CCs: Calibration curve; DAD: Diode array detector; DHA-PPQ: Dihydroartemisinin–piperaquine; FA: Formic acid; FDA: Food and Drug Administration; H3PO4: Phosphoric acid; HCOOH: Formic acid; IS: Internal standard; LLE: Liquid-liquid extraction; LOD: Limit of detection; LOQ: limit of quantification; MCX: mixed mode cation exchange; MeOH: Methanol; MFQ: Mefloquine; NH4OH: Ammonium hydroxide; PE: Polyethylene; PPQ: Piperaquine; SPE: Solid phase extraction; QC: quality control; QCH: high level; QCL: Low level; QCM: Medium level; QN: Quinine; RSD: Relative standard deviation; TACTs: Triple artemisinin combination therapy; TCA: Trichloroacetic acid; TEA: Triethylamine; ULOQ: Upper limit of quantification.

INTRODUCTION

DHA-PPQ, one of the few ACTs is recommended worldwide for treatment of P. falciparum malaria. Several studies have reported and demonstrated the efficacy and safety of ACT against falciparum malaria in Asia. However, recent treatment failures with DHA-PPQ, the current first-line ACT in Cambodia, were reported, leading to the suggestion that piperaquine resistance may be emerging in this country [1,2]. A highly drug-resistant P. falciparum co-lineage is evolving and spreading. As a consequence, treatment failures after dihydroartemisinin-piperaquine are becoming more widespread across the eastern Greater Mekong subregion [3].

In Vietnam, the national malaria control programme is managed by the government and malaria is considered well-controlled. As a result, the clinical cases of malaria and deaths in Viet Nam decreased significantly from 1991 to 2015 [4]. Practically, DHA-

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PPQ has been the first-line treatment and the most extensively used anti-malarial drug for malaria treatment since 2005. However, a few studies reported that the proportion of patients with persistent parasites on day 3 after starting DHA-PPQ treatment has progressively increased from 38 to 57%, with a corresponding reduction in cure rates from 0 to 26% in Binh Phuoc province between 2012 and 2015 [5]. In addition, high treatment failure rates have been observed with Vietnam's first-line treatment, DHA-PPQ [6]. Data from Binh Phuoc province in the early of 2018 demonstrated the percentage of day 3 positivity was 73%, and the recurrence rate was 52% (unpublished data). This evidence has urgently prompted an evaluation of the efficacy of alternative treatments in areas where DHA-PPQ is failing and discussions of a change in Vietnam's current treatment policy. However, new antimalarial compounds will not be generally available within the next few years [7]. Therefore, TACTs, the combination of artemisinin derivatives with two existing long-acting partner drugs (piperaquine and mefloquine), was proposed as a potential strategy to control artemisinin resistance to the treatment of P. falciparum malaria [8]. With different modes of action, this combination will help to minimize the risk of drug resistance development [9]. So far, TACTs are under assessment for their tolerability, safety and efficacy. If TACTs are proved to be safe and well-tolerated, they can be used as a ready treatment to deal with high failure rate in the cure for P. falciparum malaria in Vietnam. Additionally, a recent study has reported the safety, tolerability, and efficacy of TACTs in the clinical setting [10].

PPQ and MFQ, the long acting partner drugs, act against P. falciparum. The concentrations of PPQ and MFQ in disease situation may play a vital role in confirming the status of P. falciparum true resistance. Several clinical studies have demonstrated that when following DHA-PPQ treatment, a day 7 PPQ plasma concentration of <30 ng/mL was associated with a higher risk of developing recurrent falciparum infections [11-13]. Therefore, the PPQ's concentration in plasma at day 7, which was ≥30 ng/mL, was used as the threshold value for determining adequate PPQ exposure [2]. The presence of parasites in plasma concentration at (or before) day 7 exceeding 30 ng/mL would indicate PPQ resistance. For MFQ, Simpson JA et al., reported that concentrations of above 500 µg/l at day 28 must be achieved to kill the parasite [14]. This level was considered as the cut off to determine MFQ resistance, consequently.

Several HPLC methods have been described for the measurement of PPQ [15-22], MFQ and its metabolites or some other drugs [23-27]. However, there are just a few HPLC methods that have been reported to determine PPQ and MFQ simultaneously. According to a study of Hodel et al., a LC-MS/MS was described for simultaneous determination of 14 anti-malarial drugs included PPQ and MFQ [28]. In addition to the HPLC methods, there are several commonly applied extraction procedures such as simple protein precipitation [18,28], SPE [17-19], and LLE, which was the alternative process for sample treatment [16,22,23]. Noticeably, the required volume of samples for PPQ and MFQ analysis varied from 250 to 1000 µL using UV detection [15-17], and in particular 50-200 µL with liquid chromatography tandem mass spectrometry system [18,19,28]. Additionally, the dried blood spot is the new approach to collect and store anti-malarial drugs with small sample volumes, leading to many HPLC methods that were established to analyze anti-malarial drugs, including PPQ [29] and MFQ [24,30].

Herein, we develop and validate a HPLC method with diode array detection for simultaneous quantification of PPQ and MFQ in 200 µL of human plasma. This method can be applied to measure PPQ and MFQ levels in plasma samples collected from uncomplicated P.falciparum patients, who were administered to ACT or TACT regimens for malarial treatment in regions are currently in circulation with malaria in Viet Nam. With hope, this method can be applicable for measuring PPQ and MFQ concentrations in plasma samples from P.falciparum patients in developing countries.

MATERIALS AND METHODS

Materials

PPQ, MFQ and QN were purchased from Sigma-Aldrich Singapore. TCA, TEA, H3PO4, HCOOH, ACN-gradient HPLC grade, and MeOH-HPLC grade were purchased from Merck. Water was provided by a Purelab UHQ system (ELGA, Marlow, UK). Blank plasma samples were provided by Blood Transfusion and Haematology Hospital in Ho Chi Minh City.

Instrumentation

The liquid chromatography system was a Lachrom Elite-Hitachi (Merck) consisting of an L-2200 autosampler, L-2130 (2 pumps), a L-2350 column Oven and a L-2455 DAD. Data acquisition was performed using EZchrom Elite version 3.18 HPLC System Manager Software (Merck-Hitachi Japan). The analytical compounds were analysed on a Poroshell 120 C8-end-capped (100 × 4.6 mm, 2.7 µm) column protected by 2.7 µm guard cartridges 12.5 × 4.6 mm (Agilent Technologies, USA). The SPE process was carried out on the manual SPE system, VACMaster 96 sample processing Manifold (IST-Biotage, Sweden) using Oasis-MCX 30 mg cartridges or 96 well plates (Waters, USA). The Manifold system uses vacuum to get the liquid through the SPE column or plate. The SpeedVac system (Thermo Fisher Scientific) consisting of a condensation vacuum combined with a centrifugator was used to evaporate the eluates.

Chromatography

The mobile phase consisted of a gradient mode comprising components A (TCA 1M:TEA:HPLC water, 1:0.25:998.75; adjusted to pH 3.4 using H3PO4 85%) and B (ACN). The buffer was filtered through 0.20 µm, regenerated cellulose membrane, (Sartorius, Goettingen, Germany) and degassed for 30 minutes in a sonic bath (AL 04-12, Advantage Lab, Switzerland) before loading to pump A. The gradient elution, flow rate, oven temperature and wavelength for detecting the analytes were shown in Table 1. The injection volume was 50 µL.

Table 1: Chromatographic conditions for piperaquine and mefloquine.

Time (min)	Pump A (%)	Pump B (%)	Flow rate (mL/min)	Wave-length (nm)	Oven tem-perature (°C)
Gradient program	ACN	TCA 1M: TEA: water (1:0.25: 998.75) pH 3.4		283 (MFQ) 347 (PPQ, QN)	35
0	14	86	1		
2	14	86	1		
9	60	40	1		
11	60	40	1		
12	14	86	1		
16	14	86	1		

Preparation of standards

Stock solutions of PPQ and QN (1 mg/mL) were prepared in FA 2%. MFQ (1 mg/mL) was prepared in FA-MeOH (1:1, v/v). Working solutions, ranging from 0.2 to 40 µg/mL and 1 to 200 µg/mL for PPQ and MFQ,

respectively, were prepared from the stock solution using FA as dilution solution. CCs and QC were prepared by diluting (1/20) the respective working solutions with blank plasma to obtain 8 CC points at 0, 10, 50, 100, 200, 500, 1,000 and 2,000 ng/mL (PPQ) and 0, 50, 250, 500, 1,000, 2,500, 5,000 and 10,000 ng/mL (MFQ). The same dilution process was applied to get the quality control at LOQ as well as QCL, QCM, and QCH concentrations of 10, 30, 300 and 1,500 ng/mL for PPQ and 50, 150, 1,500 and 7,500 ng/mL for MFQ.

Sample preparation

Aliquots of thawed plasma samples were mixed for 20 seconds. Then, 100 µL of QN/IS 1 µg/mL in FA, and 600 µL of FA were added to 200 µL of plasma sample. After being mixed for 15 seconds, the mixture rested on the bench for 2 minutes prior to being centrifuged at 10,000 rpm for 4 minutes. The sample mixture was loaded on the Oasis, MCX 96 fixed well plate, which was pre-treated with 1 mL of MeOH and 1 mL of FA. The SPE plate was washed in turn with 0.5 mL of FA, 0.5 mL of MeOH and then dried for 2 minutes. PPQ, MFQ and QN/IS were eluted by using 1.6 mL of MeOH-NH4OH 25% (95:5, v/v) and collected in a collection plate. The eluates were evaporated using Speed-VAC system with pulsed ventilation at 40°C. Finally, the residues were reconstituted in 100 µL of reconstitution solvent, which was prepared by mixing TCA 1M (100 µL), ACN (1.4 mL) and a buffer of pH 3.4 to achieve the total volume of 10 mL. The mixtures were sonicated and centrifuged prior to the injection.

Bioanalytical method validation

A method validation of PPQ and MFQ in plasma was performed in accordance with USA FDA bioanalytical method validation guidance [31].

Selectivity, specificity, carryover and sensitivity: The selectivity and specificity were performed at the LOQ and tested the ability of the method to differentiate the analytes (PPQ, MFQ and QN/IS) towards endogenous plasma interferences from at least 6 different donors as well as some anti-malarial drugs and antipyretic which are currently administered to malaria patients in Viet Nam. No interfering components were considered to be present when the signal was less than 20% of the LOQ for analytes and no more than 5% for IS. Carryover was evaluated by injecting two blank samples after the five highest calibration standard (ULOQ) were run. The signal in the blank sample run after injection of the highest calibration standard should not be greater than 20% of the LOQ. Sensitivity was defined by the LOD and the LOQ. The LOD was expressed as the concentration produced a signal three times higher than the noise of a blank sample. The LOQ was defined as the lowest concentration of analyte, which could be determined with acceptable accuracy and precision. The analyte signal of the sample with the LOQ should be from 5-10 times higher than the signal of a blank sample resulting from the determination of six replicate spiked samples.

Linearity: The linearity assay was assessed by analyzing the calibration curve consisted of eight calibration standards (including the blank sample). For each analyte, calibration curves were obtained by calculating the peak-area ratios of PPQ, MFQ to IS against the corresponding concentrations. The different regression models (linear regressions, quadratic regressions) with or without data transformations, and different weighting were evaluated using data obtained during the validation of the assays for PPQ and MFQ quantification in plasma. The best regression model was selected using model options in HPLC System Manager Software-EZChrom Elite version 3.18 (Hitachi-Japan). Back calculations were made to determine the concentrations of PPQ, MFQ in the QC validation sets and clinical samples.

Extraction recoveries: Extraction recoveries of analytes and IS were measured at four different levels (corresponding to the LOQ, QC samples with low, medium, and high concentrations) and determined by comparing the average response of extracted QC samples with that of an unprocessed standard solution at the same concentrations as QC samples.

Precision and accuracy: The intra- and inter-day precision and accuracy were performed by testing four replicates of samples at four levels of the quality control samples (LOQ, QCL, QCM and QCH). Five independent runs were performed and evaluated. Intra-, inter-assay precisions were calculated using a single factor ANOVA and expressed as the mean RSD (%), which was equal to (SD/mean) × 100. Accuracy as mean relative error (%) was calculated by comparing the measured average concentration with the nominal concentrations at each QC level (%=[estimated concentration/nominal value] × 100). Acceptance criteria were as follows: accuracy error had to be within 85-115% of the nominal value, and intra, inter assay precisions had to be less than 15%.

Dilution effect: The ability to dilute samples above the ULOQ (i.e., dilution integrity of over the curve samples) was investigated by analyzing four replicates at 2 × ULOQ for PPQ and MFQ by 1:5 dilutions for plasma.

Stability: The stability study was carried out to test the stability of PPQ, MFQ under different conditions.

Stability of stock and working solutions: Stability was determined by comparing in quadruplicate the peak area of PPQ and MFQ at QCL and QCH concentrations between solutions stored in plastic tubes in the fridge (2°C-4°C) for one week and the fresh solution at identical levels. The difference between the mean peak areas had to be within ± 15% [31]. Stability of working solutions in different containers was evaluated. The peak areas of each analyte in quadruplicate at QCL and QCH in the glass vials and plastic tubes stored in the fridge for one month were compared to evaluate variable adsorption of the analytes to both devices if any.

Stability of plasma samples: The stability study was undertaken to test the stability of PPQ and MFQ under different storage conditions, including heat evaporation at 56°C for 60 minutes, 24 hour at room temperature (25°C), the stability in injection-ready samples in the LC autosampler at room temperature for up to 24 hour, three freeze-thaw cycles (the samples were frozen at -80°C for 24 hour for the following freezing cycles and thawed at room temperature for one hour per cycle) and long-term stability at -80°C for 1 month. All of the stability experiments were evaluated by comparing the PPQ and MFQ concentrations area response (analytes/IS) of five different replicates of QCL and QCH against freshly prepared identical samples. Spiked samples were considered stable if the deviation from the nominal values was within ± 15% 31].

RESULTS AND DISCUSSION

Sample preparation and chromatography

For sample preparation processing, because PPQ, MFQ and QN are basic compounds with pKa ≥ 10, ISOLUTE cation exchange sorbents-CBA and Oasis MCX were tested for sample treatment. CBA sorbent was not an appropriate sorbent to retain PPQ due to PPQ has many pKa and it is not totally ionized at pH 7.0. Noticeably at pH 7.0, the CBA sorbent is totally ionized, which probably ensures the ion-exchange retention of analytes on CBA sorbent. Consequently, PPQ was lost during SPE process using CBA sorbent. MCX is a strong mixed-mode cation exchange, water-wettable, providing dual modes of retention: ion exchange and reverse on an organic copolymer that is suitable sorbent to treat plasma for PPQ and MFQ. As resulted, MCX provided the clean eluates with reasonable recovery ranging from over 64.36% to 87.77% for PPQ and 70.34% to 88.61% for MFQ in all QC levels for plasma samples.

Due to the different polarity of analytes, the method was developed using gradient delivery of mobile phase. The various concentrations of acetonitrile changed over the time and pH of buffer were

focused on the best specificity and resolution between analytes (PPQ, MFQ), IS and interferences. The separation was performed on reverse-phase column Poroshell 120 C8-end-capped (100 × 4.6 mm, 2.7 µm) column protected by 2.7 µm guard cartridges 12.5 × 4.6mm (Agilent Technologies, USA) and various combinations of acetonitrile and phosphate buffer as well as TCA buffer compared at different pH (2.0 to 4.0). The retention time of PPQ was decreased when decreasing pH buffer, while QN, MFQ were just slightly influenced by pH. At pH 2.0 to 2.5, PPQ was early eluted and overlapped with interferences in the matrices. pH 3.4 with the optimal gradient program was selected to separate the interested compounds. In addition, because MFQ was a non-polar compound and needed high level of ACN in the mobile phase to elute through the reverse phase column, the combination of phosphate buffer at different concentrations with high level of ACN probably leads to the precipitation of the salt, which may clog the columns and tubing of the system. Therefore, the best combination was Poroshell 120 C8-end-capped (100 × 4.6 mm, 2.7 µm) and TCA buffer (TCA 1M:TEA:HPLC water, 1:0.25:998.75; adjusted to pH 3.4 using H3PO4 85%) and ACN with gradient elution, which was described in Table 1. The developed LC method allowed to completely separate PPQ and MFQ with a total run time of 16 minutes, including a 5 minute-column re-equilibration step. Figure 1 shows an overlay of chromatograms from blank plasma, blank plasma with IS, a spiked plasma sample at QCM (PPQ, 300 ng/mL; MFQ, 1500 ng/mL) and a patient plasma sample at 52 hours (PPQ, 253.87 ng/mL) and 8 hours (MFQ, 2193.5 ng/mL).

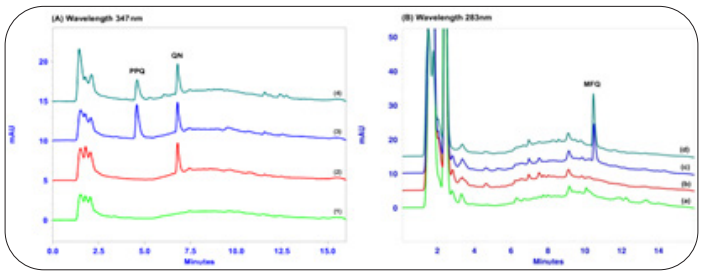

Figure 1: The chromatograms of analytes (PPQ, MFQ) and QN/IS at wavelength 343nm (A) and 283nm (B). A: Blank plasma (1), Blank plasma with IS (2), Spiked plasma with PPQ and IS (3), Patient sample at 52hrs (4). B: Blank plasma (a), Blank plasma with IS (b), Spiked plasma with MFQ and IS (c), Patient sample at 8hrs (d).

Specificity and sensitivity

The chromatograms PPQ, MFQ and QN/IS in human plasma were shown in Figure 1. The analytes were well defined and separated from matrix contaminants, with a good symmetrical shape at the respective retention time of 4.8 minutes (PPQ), 7.2 minutes (QN/IS) and 10.50 minutes (MFQ). PPQ and QN/IS were monitored at wavelength 343 nm and MFQ at wavelength 283nm. For any of the validation batches, no interfering compounds from plasma were found to co-eluate with PPQ, MFQ or the QN/IS. This was also confirmed along the entire clinical samples analysis. A number of current antimalarial drugs (dihydroartemisinin, artesunate, chloroquine, desethylchloroquine, primaquine) and acetaminophen, which are potentially administered to local patients, were also tested. However, no chromatographic interferences from the related drugs investigated can be detected in the LC system.

The LOD and LOQ using 200 µL samples were 5 ng/mL, 10 ng/mL for PPQ and 30 ng/mL, 50 ng/mL for MFQ. At the LOQ, in plasma samples, the intra-assay precision of PPQ and MFQ were 7.96%, and 2.16%, respectively.

In general, these results show comparable sensitivity to previously published methods for measurement of PPQ in plasma. However, it was successfully achieved with a limited sample volume of 200 µL versus 250-500 µL [15-17] on previous studies. For MFQ, in the present study, LOQ was more or equal sensitive than those of previously published bio-analytical methods [23,25,26,32]. In the recent years, LC-MS/MS has become the routine bio-analytical method because it can provide the extremely LOQ as well as its selectivity and specificity. When LC-MS/MS was applied to quantify PPQ and MFQ, the LOQ were 1-1.5 ng/mL using 50µl plasma [18,19] for PPQ and 2.5 ng/mL using 200 µl plasma [28] for MFQ. The results should be adequate since thresholds of PPQ at day 7 and MFQ at day 28 typically were around 30 ng/mL [2] and 500 ng/mL [14,33], respectively. The previously published bio-analytical methods for determination of PPQ and MFQ in different biological matrices were shown in Table 2.

Recovery, accuracy and precision

The recoveries of PPQ and MFQ in plasma were calculated at four QC levels (LOQ, QCL, QCM and QCH), on 4 replicates, over 5 occasions. The mean recoveries of PPQ and MFQ in all QC levels were 64.36%-87.77%; 70.34-88.61% for plasma. The recovery of the QN/IS, calculated over all the validation batches, was 87.1 ± 3.8% (± SD) and independent of PPQ, MFQ concentration. The intra and inter assay relative standard deviations were less than 8% over all QC ranges for PPQ and MFQ in plasma. The back calculated accuracies varied between 99.97% and 104.6% for PPQ, and 99.85% and 103.2% for MFQ at all QC levels. The detailed results of the assay were presented in Table 3.

Dilution effect

To investigate the matrix dilution effects, PPQ and MFQ spiked samples (n=4) were prepared at a concentration exceeding by two-fold the highest calibration level, then diluted 5 times with blank plasma. Those samples were then processed as described in sample preparation and chromatography sessions. The accuracies (% ± SD) were 98.48 ± 0.83 and 101.8 ± 1.55 in plasma for PPQ and MFQ, respectively. The results indicated that plasma samples containing PPQ and MFQ above the high level of calibration can be adequately diluted with blank plasma prior to the HPLC system without any influence on the analysis of PPQ and MFQ.

Linearity

The linearity of calibration consisted of 8 points (including blank sample) and was determined in the range of 10-2000 ng/

Table 2: Methods for the analysis of PPQ and MFQ using HPLC in biological fluids.

Reference	Drugs	Matrix	Extraction method	LC Column	Mobile phase	Detection	Run time (min)	Sample volume (µL)	Analytical range (ng/mL)	LOQ (ng/mL)
Hung T.Y. (2003) [16]	Piperaquine	Plasma	LLE	Waters XTerra RP18 (100 × 4.6 mm)	Water-ACN-trifluoro-acetic acid-triethylamine (93:7:0.025:0.008, v/v)-0.1% sodium chloride, w/v.	UV	14	1000	5-500	5
Lindegardh, N. (2003) [15]	Piperaquine	Plasma	SPE	Zorbax SB-CN (250 × 4.6 mm)	Phosphate buffer 01M, pH 2.5-ACN (88:12, v/v)	UV	8	500	13-2678	13
Lindegardh, N.(2005) [17]	Piperaquine	Plasma	SPE	Chromolith Performance (100 × 4.6 mm)	Phosphate buffer 01M, pH 2.5-ACN (92:8, v/v)	UV	2	250/1000	10-5000	10/2.5
Choemang A. (2019) [22]	Piperaquine	Plasma	LLE	Reverse-phase C18	Trichloroacetic acid 0.1%-ACN (85:15, v/v)	UV	16	150	10-1000	10
Singhal P. (2007) [18]	Piperaquine	Plasma	Precipitation	ChromolithSpeedROD RP-18e (50 × 4.6 mm)	Aminium acetate (10mM)-methanol-formic acid-ammonia (25:75:2:0.15, v/v)	MS/MS	2.5	50	1-250	1
Lindegardh, N.(2008) [19]	Piperaquine	Plasma	SPE	GEMINI C18 (50 × 2 mm)	Amonium bicarbonate (2.5mM, pH 10)-ACN (15:85, v/v)	MS/MS	2.5	50	1.5-500	1.5
Aziz M.Y. (2017) [21]	Piperaquine and metabolite	Plasma	Precipitation	Ascentis Express C18 column (3 cm × 2.1 mm)	0.1% formic acid in water and 0.1% formic acid in ACN (gradient elution)	MS/MS	7	100	3.9-2508nM	3.9nM
Guenzi A. (1989) [23]	Mefloquine and PMT	Plasma	LLE	Bondapak (300 × 3.9 mm, 10 µm)	ACN– 0.1 M KH2PO4, pH 3.0 (35:65, v/v)	UV	14	1000	10-800	10
Green M.D. (1999) [25]	Mefloquine and metabolite	Whole blood, plasma and serum	Precipitation and SPE	SymmetryShield RP18 (250 × 4.6 mm)	ACN– 0.1 M KH2PO4, (40:60, v/v), adjusted to pH 6.0 with ammonium hydroxide	UV	15	200	100-1000	100

Lai C.S. (2007) [26]	Mefloquine and AST, DHA	Plasma	SPE	Inertsil C8-3 150 × 4.6 mm, 5 µm	MeOH-CAN-0.05 M KH2PO4 adjusted to pH 3.9 with 0.5% orthophosphoric acid (50:8:42,v/v/v)	UV	16*	500	75–1500	25
Mannemala, S.S (2015) [32]	Mefloquine and 4 other drugs[a]	Plasma	Precipitation	Gemini C18 (150mm × 4.6 mm, 5µm)	dipotassium orthophosphate buffer/ACN/MeOH (20:38:42, v/v) adjusted to a pH of 3.0.	PDA	10	250	150-3000	150
Blessborn D. (2010) [30]	Mefloquine and 6 other drugs[b]	Dried blood spot	LLE and SPE	Gemini C18 (150 mm × 2 mm)	(A) ACN:ammonium formiate (20 mM with 1 vol-% formic acid) (5:95 v/v) and (B) ACN:ammoniumformiate (10 mM with 1 vol-% formic acid) (80:20,v/v). Gradient elution.	Ion-trap MS	15	100	50-3000	50
Gallay J. (2017) [27]	Mefloquine and 8 other drugs[c]	Dried blood spot	LLE	XSelect® UPLC HSS T3 75 × 2.1 mm, 2.5 µm	2 mM ammonium acetate + 0.1% FA (mobile phase A) and ACN+ 0.1% FA (mobile phase B).	MS/MS	12*	10	20-5000	20
Hodel E.M. (2009) [28]	Piperaquine and mefloquine + 12 other drugs[d]	Plasma	Precipitation	Atlantis® dC18 (2.1 mm × 50 mm)	20 mM ammonium formate (buffer A) and ACN (solvent B), both containing 0.5% formic acid (FA). Stepwise gradient elution.	MS/MS	21	200	2-4000 (PPQ) and 2.5-500 (MFQ)	2 (PPQ) 2.5 (MFQ)

AST: artesunate, DHA: dihydroartemisinin; PMT: pyrimethamine LLE: liquid-liquid extraction; LOQ: lower limit of quantification; MS/MS: tandem mass spectrometry; PDA: Photo-diode Array; SPE: solid-phase extraction.
a: artemether, lumefantrine, pyrimethamine, sulfadoxine, and mefloquine
b: quinine, mefloquine, sulfadoxine, pyrimethamine, lumefantrine, chloroquine and its metabolite desethylchloroquine
c: mefloquine, amodiaquine, chloroquine, quinine, sulfadoxine, pyrimethamine, lumefantrine and 2 active metabolites (N-desethyl-amodiaquine, desbutyl-lumefantrine)
d: artemether, artesunate, dihydroartemisinin, amodiaquine,N-desethyl-amodiaquine, lumefantrine, desbutyl-lumefantrine, piperaquine, pyronaridine, mefloquine, chloroquine, quinine, pyrimethamine and sulfadoxine
*: based on chromatograms

Table 3: Precision, accuracy and recovery of the HPLC method for the determination of piperaquine and mefloquine in human plasma samples (ANOVA, n=20)..

		Concentration (ng/mL)	Precision (RSD)		Accuracy (%)		Recovery %
			Inter-assay	Inter-assay	Inter-assay	Inter-assay	
PPQ	LOQ	10	5.76	7.96	99.97	103.7	64.36
0	QCL	30	4.71	3.92	102.2	103.6	82.20
0	QCM	300	2.80	2.51	103.2	102.3	83.61
0	QCH	1500	1.67	1.96	103.6	104.6	87.77
MFQ	LOQ	50	7.00	2.16	103.2	99.85	75.75
0	QCL	150	1.91	3.18	102.1	101.6	70.34
0	QCM	1500	4.54	2.96	101.7	100.3	78.13
0	QCH	7500	3.35	1.32	101.1	101.2	88.61

RSD: Relative Standard Deviation (%). LOQ, QCL, QCM, QCH: limit of quantification, quality control at low, medium, high concentrations.

mL and 50-10000 ng/mL for PPQ and MFQ, respectively. Linear calibration curves were generated by 1/amount (PPQ) and 1/amount2 (MFQ) weighted and log-log transformed data quadratic regression analysis. The final regression model resulted in high coefficient of regression (r2>0.99) for all the PPQ, MFQ calibration curves for plasma.

Stability

Stock solutions and working solutions of PPQ, MFQ and IS stored in PE containers remained stable at 2°C-4°C for one week (the variation in the analytical responses of PPQ, MFQ were no more than 2% and IS was less than 5% compared to the initial values). In addition, a significant and variable adsorption of PPQ to glass tubes was observed, the PPQ responses were 7.81% (QCH)-52.83% (QCL) lower when working solutions were stored in glass tubes compared to storage in PE tubes at -20°C for one month, whilst no significant change was observed for MFQ and IS. The results have reconfirmed that PPQ is highly unstable in glassware compared to polyethylene devices as reported in previous publications [18,20]. For the stability of drugs in auto sampler, the SPE eluates evaporated and reconstituted in solvent-ready for injection kept at ambient temperature in the auto sampler were stable up to 24 hours. The stability of drugs after 3 freeze and thaw cycle in plasma showed that plasma samples containing PPQ and MFQ at concentration 30 ng/mL, 1500 ng/mL and 1500 ng/mL, 7500 ng/mL were found to be stable after 3 consecutive freeze/thaw cycles and when being stored in a freezer at -80°C for at least 1 month. The variation in PPQ and MFQ content was found to be less than 10% from the initial values. The results of stability of PPQ and MFQ in plasma were presented in Table 4.

Method performance during a clinical study

The total precision for all quality controls (n=21 at each level) during the analysis of PPQ and MFQ were 5.22, 3.94 and 4.52% at 30, 300 and 1,500 ng/mL, and 3.90, 5.11 and 2.83% at 150, 1,500, and 7,500 ng/mL respectively.

Clinical applicability

The validated method was successfully applied to determine PPQ and MFQ concentrations in human plasma collected from uncomplicated P. falciparum patients treated with ACT (DHA: 2-10 mg/kg, PPQ: 16-27 mg/kg for three days) and TACT (ACT plus MFQ: 7-11mg/kg for three days) in Binh Phuoc province (Ethical approval: 3951/UBND-VX). Up to 14 blood samples were collected from an individual patient at different time points: 0, 1, 2, 4, 6, 8, 12, 24, 52 hours, and at day 4, 7, 14, 28 and 42. Totally, 168 clinical samples collected from 12 patients were analyzed and validated against freshly prepared CC and QC in blank plasma. The acceptance criteria on the validation QC sets was<15% for precision and between 85 and 115% for accuracy.

In general, the large inter-individual differences of PPQ and MFQ levels were found in plasma. The median (range) day 7 plasma concentration of PPQ in 12 patients was 37.21(18.94-96.21) ng/mL, which was in agreement with previous studies [1,34]. The median (range) day 28 and day 42 plasma concentration of MFQ were 872.3(531.7-1162.9) ng/mL and 647.1(304.5-695.9) ng/mL, respectively. The initial results indicated the adequate MFQ exposure and are above the 500 ng/mL plasma threshold considered as the required concentration to kill MFQ-sensitive P. falciparum [14,33]. In Vietnam, MFQ was used both as a mono therapy and in an ACT (artesunate-mefloquine) since the 1990s. However, in 2005, the National Malaria Control Program of Viet Nam switched to local DHA-PPQ as the first-line treatment for falciparum malaria due to the ceased foreign financial support that leads to the shortage of MFQ. The preliminary results of the present study demonstrated that MFQ, with its high levels at day 28 and day 42, would be an effective and potential long-acting drug in the combination with ACT regimen to treat the patients with uncomplicated malaria in Vietnam. The representative mean plasma concentration profiles of PPQ and MFQ, following ACT and TACT oral administration to uncomplicated P. falciparum patients were presented in Figure 2.

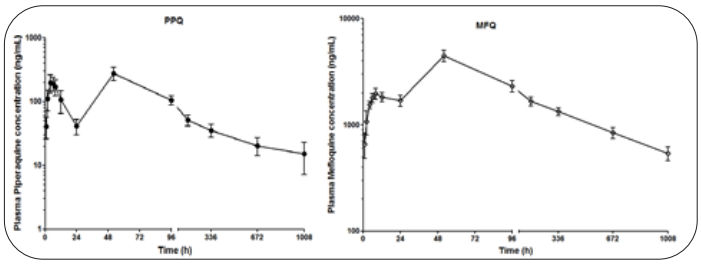

Figure 2: Mean (± SEM) plasma concentration–time profiles of piperaquine (n=12) and mefloquine (n=7) following oral administration of ACT and TACT regimens in uncomplicated P. falciparum patients. SEM: Standard Error of Mean.

Table 4: Variation of PPQ, MFQ concentrations in plasma under different storage conditions.

Conditions	PPQ (n=5)		MFQ (n=5)	
	QCL % (RSD) (0.03 µg/mL)	QCH % (RSD) (1.5 µg/mL)	QCL %(RSD) (0.15 µg/mL)	QCH %(RSD) (7.5 µg/mL)
RT-24H	97.70 (5.46)	90.96 (4.46)	108.4 (3.32)	100.6 (5.13)
EXT-24H	99.76 (6.23)	101.7 (1.34)	92.54 (4.89)	94.60 (1.20)
Heat 56°C for 60 minutes	99.91 (4.76)	98.68 (3.66)	99.28 (5.33)	100.7 (1.54)
C3	100.7 (7.02)	96.43 (1.14)	100.6 (2.39)	102.5 (5.77)
M1 (-80°C)	92.25 (5.37)	90.47 (2.34)	100.1 (3.09)	96.87 (5.12)

Results in % recovery = [(mean value in stability sample/mean value in reference] × 100 and RSD: Relative Standard Deviation. Storage conditions are: RT-24H: Unprocessed samples left at room temperature for 24hrs. EXT-24H: SPE eluates evaporated, reconstituted in reconstitution solvent and kept at room temperature in autosampler for 24 hours. C3: third freeze/thaw cycle. M1: Storage for 1 month at -80°C.

CONCLUSION

A bioanalytical method for the simultaneous determination of PPQ and MFQ in plasma has been developed and validated. The assay has been proven sensitive and reproducible and used only 0.2 mL of plasma. The method has been implemented for the analysis of clinical samples with satisfying performance data for the daily control samples. We hope that the determination of PPQ and MFQ in plasma from uncomplicated malaria patients in Viet Nam will help to elucidate and improve knowledge on the P.falciparum resistance to PPQ as well as the efficacy of MFQ against P. falciparum when using in triple artemisinin combination therapy. The results may contribute to find an appropriate and effective regimen for the treatment of uncomplicated malaria in Vietnam.

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CONFLICTS OF INTEREST

The authors declare no competing interests.

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