Journal of Mass Spectrometry and Advances in the Clinical Lab 31 (2024) 19–26

Contents lists available at ScienceDirect

Journal of Mass Spectrometry and Advances in the Clinical Lab

journal homepage: www.sciencedirect.com/journal/journal-of-mass-spectrometry-and-advances-in-the-clinical-lab

Research Article

A high-throughput LC-MS/MS assay for piperaquine from dried blood spots: Improving malaria treatment in resource-limited settings

Daniel Blessborn [a,b,*], Natpapat Kaewkhao [a], Joel Tarning [a,b]

[a] Mahidol Oxford Tropical Medicine Research Unit, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand
[b] Centre for Tropical Medicine and Global Health, Nuffield Department of Clinical Medicine, University of Oxford, Oxford, UK

ARTICLE INFO

Keywords:
Piperaquine
Dried blood spots
Eurartesim
Filter paper
Hematocrit
LC-MS/MS

ABSTRACT

Background: Malaria is a parasitic disease that affects many of the poorest economies, resulting in approximately 241 million clinical episodes and 627,000 deaths annually. Piperaquine, when administered with dihydroartemisinin, is an effective drug against the disease. Drug concentration measurements taken on day 7 after treatment initiation have been shown to be a good predictor of therapeutic success with piperaquine. A simple capillary blood collection technique, where blood is dried onto filter paper, is especially suitable for drug studies in remote areas or resource-limited settings or when taking samples from children, toddlers, and infants.
Methods: Three 3.2 mm discs were punched out from a dried blood spot (DBS) and then extracted in a 96-well plate using solid phase extraction on a fully automated liquid handling system. The analysis was performed using LC-MS/MS with a calibration range of 3 – 1000 ng/mL.
Results: The recovery rate was approximately 54–72 %, and the relative standard deviation was below 9 % for low, middle and high quality control levels. The LC-MS/MS quantification limit of 3 ng/mL is sensitive enough to detect piperaquine for up to 4–8 weeks after drug administration, which is crucial when evaluating recrudescence and drug resistance development. While different hematocrit levels can affect DBS drug measurements, the effect was minimal for piperaquine.
Conclusion: A sensitive LC-MS/MS method, in combination with fully automated extraction in a 96-well plate format, was developed and validated for the quantification of piperaquine in DBS. The assay was implemented in a bioanalytical laboratory for processing large-scale clinical trial samples.

1. Introduction

Piperaquine, an antimalarial drug developed in China in the 1960 s [1], was initially widely used as monotherapy. However, due to increasing parasite resistance against the drug, its popularity declined. Nevertheless, in the early 2000s, the drug was reintroduced as a combination therapy with dihydroartemisinin (Artekin®) and received regulatory approval from the European Medicines Agency (EMA) in 2011 under the name Eurartesim™ [2–6]. This drug combination has demonstrated excellent efficacy, with dihydroartemisinin rapidly clearing most of the parasites during the initial days of therapy, while piperaquine, with its longer half-life, eliminates the remaining parasites to prevent recrudescence. Additionally, this combination therapy is relatively inexpensive compared to other available antimalarial drugs [7]. It is recommended as one of the first-line treatments against uncomplicated malaria caused by *Plasmodium falciparum* by the World Health Organization (WHO) [8].

Malaria is an infection transmitted by a mosquito of the *Anopheles* family. It is caused by infection of red blood cells by a protozoan parasite known as *Plasmodium* [9]. Symptoms such as fever, chills, headache, and vomiting occur when the red blood cells rupture and release toxins. Hemoglobin serves as a crucial nutritional source for the parasite, and it

Abbreviations: ANOVA, Analysis of variance; DBS, Dried blood spot; EDTA, Ehylenediaminetetraacetic acid; EMA, European Medicines Agency; IS, Internal standard; LC-MS/MS, Liquid chromatography tandem mass spectrometry; LC-UV, liquid chromatography-ultraviolet detection; LLOQ, Lower limit of quantification; LOD, Limit of detection; MF, Matrix factor; OC, Over curve; QC, Quality control; RE, Relative Error; RH, Relative humidity; RSD, Relative standard deviation; SD, Standard deviation; SPE, Solid phase extraction; ULOQ, Upper limit of quantification; WHO, World Health Organization.

* Corresponding author at: Mahidol-Oxford Tropical Medicine Research Unit, 8th Floor, 50th Anniversary Chalermprakiat Building, 420/6 Rajvithi Rd., Bangkok 10400 Thailand.
E-mail address: danielb@tropmedres.ac (D. Blessborn).

https://doi.org/10.1016/j.jmsacl.2023.12.004
Received 21 June 2023; Received in revised form 4 December 2023; Accepted 21 December 2023
Available online 30 December 2023

is suggested that piperaquine inhibits the heme-digestion pathway in the parasite's food vacuole [10].

Eurartesim™ is typically administered once a day for three days, with the number of tablets per dose (usually 1–4) dependent on the patient's weight. Each tablet contains 40 mg of dihydroartemisinin and 320 mg of piperaquine tetraphosphate. Pharmacokinetic studies have been conducted in both healthy volunteers (single and multiple doses) and malaria patients. Piperaquine is rapidly absorbed, reaching peak concentrations between 2 and 6 h after administration [11–13]. Median peak plasma concentrations of approximately 250 ng/mL are observed in patients receiving the standard three-day treatment, with 99 % of drug measurements reported to be below 1,000 ng/mL [11,12]. The terminal elimination half-life is in the range of 13–28 days [14–18]. Some studies have compared the uptake of the drug when piperaquine is administered with or without food. While two studies did not find any statistically significant difference in exposure [14,19], one study demonstrated that a high-fat, high-calorie intake significantly impacted drug exposure [20]. However, this required significant amounts of fat, and considering the symptoms of malaria and the low-fat content of standard meals in endemic regions, the food effect is unlikely to have a significant impact on the clinical use of piperaquine. In the treatment of drug-sensitive malaria parasites, piperaquine concentrations on day 7 have been shown to be a good predictor of therapeutic success, with successful treatment generally associated with a venous plasma concentration of 27–30 ng/mL [21,22]. In a malaria prevention study conducted in healthy adults using dihydroartemisinin-piperaquine on a monthly or bi-monthly basis, it was found that all participants who developed malaria had a piperaquine venous plasma concentration below 31 ng/mL [23]. When analyzed in healthy volunteers, piperaquine showed approximately two-fold higher concentrations in venous blood and three-fold higher concentrations in capillary blood compared to venous plasma [24].

Most quantification methods for piperaquine have been developed for plasma samples [25–28]. However, there is one report that utilized capillary blood collection, where blood was dried on filter paper (also known as a dried blood spot or DBS methodology) [29]. The DBS methodology offers several advantages, including less invasive and easy-to-perform sampling using finger or heel prick (which is ideal for remote sampling sites), as well as a small sample volume that is suitable for vulnerable populations like young children, toddlers, and infants. Additionally, dried blood spots generally provide increased sample stability, allowing for easy transportation and storage without the need for cold-chain logistics. In a previously published DBS method, 100 μL of blood was used, and liquid chromatography-ultraviolet detection (LC-UV) was employed as the detection method with a quantification limit of approximately 25 ng/mL [29]. Our objective was to use 50 μL of capillary blood, which is more suitable for studying small children, and to achieve a sensitivity that enables the quantification of samples for at least the standard 28 days of follow-up in efficacy studies.

2. Materials and methods

2.1. Chemicals and reagents

All reagents and chemicals were of analytical grade except where otherwise stated. Piperaquine was obtained from Guangzhou University of Traditional Chinese Medicine (Guangzhou, China). The internal standard (IS) was a stable isotope labeled IS, piperaquine-d6, obtained from Sigma-Tau (Pomezia, Italy). Water, acetonitrile, and methanol were obtained from JT Baker (Phillipsburg, USA). Formic acid (98–100 %), sodium hydroxide (pellets) and triethylamine were obtained from BDH (Poole, UK). Ammonia (25 %), *ortho*-phosphoric acid (85 %) and perchloric acid (70–72 %) were obtained from Merck (Merck KGaA, Darmstadt, Germany).

2.2. Equipment

Filter paper used in this method was Whatman 31 ET Chr chromatography paper (cat. no: 3031–915) from Whatman international (Maidstone, UK). Paper spots were punched with a BSD600 duet puncher (BSD Robotics, Queensland, Australia) and extraction with a mixed phase cation exchange solid phase extraction MPC-SD Empore 96-wellplate standard well 1 mL (3 M Empore, 3 M Centre, St. Paul, MN, USA).

2.3. Preparation of standards and working solutions

Piperaquine and IS stock solutions (1 mg/mL in its base form) and working solutions were prepared in Methanol:Water:Formic acid (50:49.5:0.5, v/v/v) and stored in methanol washed cryo tubes at −80 °C. All solutions were allowed to equilibrate to room temperature before use.

2.4. Preparation of calibration standards and Quality control samples

Blank blood (EDTA or heparin as an anticoagulant) was obtained from healthy human volunteers at the Healthy Volunteer Ward of the Hospital for Tropical Medicine in Bangkok, Thailand. Potential blood donors who showed interest in participating in the study signed written consent forms and underwent screening assessments, including vital sign measurements and routine laboratory blood tests. The donated blood tubes were blinded at the Healthy Volunteer Ward and then transferred to the clinical laboratory.

Fresh whole blood (EDTA) was used to prepare calibration standards at concentrations of 3, 9, 30, 100, 400, and 1000 ng/mL. Quality control (QC) samples were also prepared, including a lower limit of quantification (LLOQ) sample at 3 ng/mL, a low QC sample at 9 ng/mL (QC_{low}), a middle QC sample at 40 ng/mL (QC_{middle}), a high QC sample at 800 ng/mL (QC_{high}), an upper limit of quantification (ULOQ) sample at 1000 ng/mL, and a limit of detection (LOD) sample at 1 ng/mL. Over curve (OC) samples at a concentration of 2000 ng/mL were also prepared. The calibrators and QCs were prepared separately using separate stock solutions. The middle QC samples were positioned in the lower range of the calibration curve, as this is where most of the clinical samples are expected to fall. The volume of the working solution in whole blood was kept below 2 % in all samples. The spiked blood was mixed and allowed to rest for 30 min at room temperature (approximately 22 °C) to allow for potential equilibration processes to occur. It was then mixed again before use.

Dried blood spots were prepared by spotting 50 μL of the spiked blood onto filter paper. The spots were allowed to dry at room temperature (approximately 40–50 % relative humidity (RH)) for at least 2 h before being transferred to a dry cabinet set at approximately 22 °C and 20 % RH) for storage until analysis.

2.5. Extraction procedure

Sample preparation and solid phase extraction (SPE) were performed using a Freedom Evo 200 platform (TECAN, Mannedorf, Switzerland). Prior to use, pipette tips, 96-well plates, and seal mats were all washed with methanol. From each DBS sample, three 3.2 mm punches were made using a BSD600 duet puncher, with all three discs collected into one well. A total of 375 μL IS (0.44 ng/mL) in phosphate buffer pH 2.0 (50 mM) were added to each well, except for the double blank, which only used 375 μL of phosphate buffer. This was followed by the addition of 150 μL of perchloric acid (0.3 M) and 75 μL of acetonitrile to each well. The 96-well plate was then covered with a seal mat and mixed for 60 min at 1500 rpm on a Mixmate® (Eppendorf, Hamburg, Germany). After mixing, the plate was centrifuged for 5 min at 1100 × g. Approximately 500 μL of the processed sample was transferred to a 96-well SPE plate and extracted according to Table 1. The eluate was

Table 1
Solid phase extraction of piperaquine dried blood spot samples.

Solid phase extraction step	Extraction solvent	Volume (mL)
Activation	Methanol	2.0
Conditioning	phosphate buffer pH 2.0 (50 mM)	0.5
Loading	Sample	0.5
Washing	Methanol:Phosphate buffer pH 2.0 (50 mM) (80:20, v/v)	1.0
Elution	Methanol:Triethylamine (98:2, v/v)	0.95

evaporated under a gentle stream of nitrogen at 70 °C using a TurboVap® 96 (Biotage, Uppsala, Sweden) until complete dryness, and then reconstituted in 250 μL of the mobile phase (acetonitrile: ammonium bicarbonate (2.5 mM) pH 10 (85:15, v/v)). The 96-well plate was mixed for 10 min at 1000 rpm on a Mixmate® and placed in the autosampler (20 °C) to equilibrate for at least 40 min before analysis.

2.6. Instrumentation and chromatographic conditions

The liquid chromatography (LC) system used was an Agilent 1200, consisting of a binary LC pump, a vacuum degasser, a temperature-controlled micro-well plate autosampler set at 20 °C, and a temperature-controlled column compartment set at 20 °C (Agilent Technologies, CA, USA). Data acquisition and quantification were performed using Analyst 1.4.2 software (Sciex, MA, USA). The compounds were analyzed under isocratic conditions using a Phenomenex Gemini C18 column (50 mm × 2 mm, 5 μm) protected by a pre-column Security guard Gemini C18 (4 mm × 2 mm) (Phenomenex, Torrance, CA, USA). The flow rate was set at 500 μL/min. The mobile phase consisted of acetonitrile:ammonium bicarbonate 2.5 mM pH 10 (85:15, v/v) with an injection volume of 5 μL and a total runtime of 2.5 min.

For liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis, an API 5000 triple quadrupole mass spectrometer (Sciex, MA, USA) with a TurboV ionization source interface was used. The ion spray voltage was set to 5500 V, and the dry temperature was set at 600 °C. The curtain gas was set to 35 psi, and the nebulizer and ionization source gases were set to 50 and 45 psi, respectively. Quantification was performed using selected reaction monitoring (SRM) with collision energy of 45 V and the transition *m/z* 535.1- >288.1 for piperaquine and *m/z* 541- >294.1 for the internal standard piperaquine-d6. Other MS parameters included a declustering potential (DP) of 205 V, an entrance potential (EP) of 10 V, and a collision cell exit potential (CXP) of 22 V.

2.7. Validation procedure

2.7.1. Accuracy and precision

The accuracy and precision of the method were evaluated by analyzing 5 replicate sets of 5 concentrations (3, 9, 40, 800, and 1000 ng/mL, LLOQ, QC_{low}, QC_{middle}, QC_{high}, and ULOQ, respectively) in 4 separate runs. Dilution integrity was also assessed by diluting OC samples at 2000 ng/mL. This was done by punching once from the OC sample, followed by punching twice from a blank blood spot into the same well. Another well was processed containing only blank spots. The eluates from the two wells were then combined to achieve a 1:6 dilution.

Accuracy was determined by comparing the measured average concentration of the QC samples from each run with the nominal (true) concentration, and it was calculated as the mean % relative error. Precision of the method (within-run, between-run, and total run variation) was calculated using analysis of variance (ANOVA). The within-run variation was calculated as the square root of the mean square due to error divided by the grand mean, while the between-run variation was calculated as the square root of the mean square due to factor divided by the grand mean. Precision was expressed as a percentage, represented by the relative standard deviation (RSD).

2.7.2. Linearity

Linearity was evaluated by analyzing calibration standards in duplicate across four different runs. A regression model was selected based on the back-calculated concentrations of the calibration curve that provided the best prediction of the QC samples across the entire calibration range.

2.7.3. Sensitivity and selectivity

Sensitivity was assessed by determining the LOD, which was set to approximately 3 times the signal response of the blank sample. The LLOQ was set to at least 10 times the signal response of the blank sample.

Selectivity was evaluated by analyzing blank samples obtained from seven different blood donors, considering both heparin and EDTA as anticoagulants. This assessment helped to determine the selectivity of the method and ensure that there were no interfering components from the blood samples or the anticoagulants themselves.

2.7.4. Recovery

Recovery was calculated by comparing the signal response of pre-spiked QC samples (5 replicates at each concentration level) with the signal response of extracted blank DBS after the addition of analytical standards (post-spiked) to achieve the same nominal concentration of piperaquine as the QC sample. This process simulated a 100 % extraction recovery to determine the percentage of the analyte that was successfully recovered during the extraction procedure.

2.7.5. Matrix effects

Matrix effects were examined using two approaches. Firstly, post-column infusion and injection of blank samples from seven different blood donors (evaluating both heparin and EDTA as anticoagulants) were performed to qualitatively determine the presence of any significant matrix effects at, or near, the retention time of piperaquine [30,31]. Secondly, a quantitative assessment was carried out using the simplified approach described by Matuszewski et al. [32].

The matrix factor was calculated by comparing the peak response of extracted blank DBS samples after the addition of analytical standards (post-spiked) to the average peak response of the analyte in a matrix-free reference solution (n = 6) at the same nominal concentrations. For each donor, one sample was evaluated at the QC_{low} concentration of 9 ng/mL and one sample at the QC_{high} concentration of 800 ng/mL (n = 1).

If the ratio of the matrix effect (response of the post-extract addition sample divided by the response in neat solution) is less than 0.85 or greater than 1.15, it would indicate the presence of a matrix effect. Additionally, if a matrix effect is detected, the RSD of the IS normalized matrix factor, calculated by dividing the matrix factor for the analyte by the matrix factor for the IS, should not exceed ± 15 % when considering different lots of blank matrices.

2.7.6. Carry-over and crosstalk

The carry-over effect was evaluated by analyzing blank extracted DBS directly following the last injected ULOQ calibration standard. The carry-over effect should not exceed 20 % of the signal response at the LLOQ.

Crosstalk between the IS and piperaquine was investigated by extracting a blank DBS with the IS, and then monitoring the signal response of piperaquine in the LC-MS/MS system. Crosstalk from piperaquine to the IS was investigated by injecting a neat, high-concentration solution of piperaquine and monitoring the signal response of the IS. The signal response should not be more than 20 % of the LLOQ for piperaquine and should not be more than 5 % of the signal response of the IS in the LLOQ sample.

2.7.7. Stability

The stability of piperaquine in DBS was assessed during three freeze/thaw cycles. The spots were frozen at −80 °C overnight, thawed, kept at room temperature for 4 h, and then frozen again. Three spots were

analyzed from each cycle using high and low QC concentrations.

Short-term stability testing was conducted for 5 days, comparing room temperature storage with 4 °C and frozen −80 °C storage. Long-term storage stability testing was evaluated after 7 months and again after 3 years to estimate long-term stability. For stability samples, the mean concentration at each QC level should be within ± 15 % of the nominal concentration to meet the stability criteria.

2.7.8. Dried blood spot specific tests

Additional tests specific to DBS included evaluating the effects of hematocrit (erythrocyte volume fraction) and punch position to assess the homogeneity of the blood spot. To create different hematocrit levels, three tubes of fresh EDTA whole blood were centrifuged, and plasma was either added to or removed from the blood to achieve hematocrit levels of 20 %, 40 % and 50 %. The blood was then spiked with the same concentration as QC_{low} and QC_{high}. The spiked blood was mixed on a sample tube rocker and applied as 100 μL spots onto paper.

Measurements of spot diameter were taken, and three punches were made in the center or close to the edge of the DBS for each hematocrit and QC level combination. Four replicates of each combination were evaluated. The average concentration from each experiment was compared to the reference concentration at the 50 % hematocrit level. This comparison was calculated as (sample - reference) divided by reference to determine any impact of hematocrit level or punch position. The deviation should not exceed ± 15 % of the nominal concentration.

2.8. Clinical dried blood spot samples

The developed assay was utilized to quantify piperaquine in DBS samples collected from a clinical study conducted in a malaria-endemic area in Indonesia, Southeast Asia [33]. The clinical trial was registered at ClinicalTrials.gov under the identifier NCT01669941 and also at ISRCTN under the number ISRCTN34010937. Ethical approval for the study was obtained from Liverpool School of Tropical Medicine (approval number 12.28), Eijkman Institute for Molecular Biology (project N:57), and Litbangkes, Ministry of Health, Jakarta (approval number LB02.01/5.2/KE059/2013).

In the clinical trial, the researchers investigated the intermittent preventive treatment of pregnant women during the second and third trimesters using a fixed-dose combination of 40 mg dihydroartemisinin/320 mg piperaquine tetraphosphate per tablet (Eurartesim, Sigma-Tau, Rome, Italy). The treatment regimen consisted of a standard 3-day course, with the dosage adjusted based on the participant's weight to approximately 4 mg/kg dihydroartemisinin and 18 mg/kg of piperaquine (base) per day. The first dose was administered under supervision, while the remaining two doses were taken at home.

Blood samples were collected in the form of DBS approximately one month after each treatment cycle during monthly visits. Additionally, a few patient samples were collected pre-dose and at other time points during the study.

3. Result and discussion

As described in previous publications, special attention should be given when working with piperaquine due to its tendency to adsorb to glass surfaces and stick to metallic surfaces within the LC system [28,34]. To mitigate these issues, it is recommended to use plastic materials for the preparation of stock and working solutions. In the LC system, using PEEK tubing or surface-treated injection needles and tubing to prevent direct contact between piperaquine and bare metal is also advised.

However, in our experience, the use of plastic materials can sometimes lead to the appearance of "ghost peaks", which are small peaks detected at the retention time of piperaquine. To avoid this, all plasticware, such as pipette tips, tubes, 96-well plates, and plastic caps on bottles, should be thoroughly washed with methanol before use.

To ensure accurate concentration measurements, it is important to use fresh unfrozen blood for the preparation of calibrators and QC samples before applying them as blood spots on filter paper to mimic study samples. Additionally, before punching out a sub-sample from a DBS, it is crucial to ascertain whether the blood applied has completely saturated the filter paper to create a homogenous blood spot. This is achieved by inspecting both sides of the DBS to ensure its uniformity at the punch site. In a saturated DBS, the same amount of blood will be obtained with each punch.

3.1. Validation

This method builds upon two previous publications. The first is a DBS method by Malm et al. [29], where the DBS extraction process was updated to accommodate smaller spot sizes, make the solvents compatible with LC-MS, and adapt volumes for use in a 96-well plate. The second publication by Lindegardh et al. [27] describes a plasma method, which provided the basis for adopting the LC and MS methods for use in the DBS method.

As stated in the previous publication [27], it is crucial to evaporate the elution solvent used in SPE to complete dryness. Residues of triethylamine, if present, may cause suppression effects around the tail of the piperaquine peak. Since the retention time of the stable isotope IS is slightly faster than that of piperaquine, any suppression would impact piperaquine to a greater extent than the IS, potentially affecting the accuracy of quantification. Therefore, it is important to ensure complete evaporation of the elution solvent to prevent such suppression effects.

3.1.1. Precision and accuracy

After implementing all the precautions mentioned above, the validation of the piperaquine assay in DBS demonstrated satisfactory accuracy and precision. Both the accuracy and precision of the method remained well within the acceptable criteria, with deviations of less than 15 % (for the LLOQ, a deviation of ± 20 % is considered acceptable). The results obtained from the validation met the required standards, indicating the reliability of the method for quantifying piperaquine in DBS samples. Detailed information on the accuracy and precision can be found in Table 2.

3.1.2. Linearity

Previous studies have reported peak venous plasma concentrations of piperaquine in the range of 170–400 ng/mL [11,12,19,22,35]. Additionally, a study by Ashley et al. [24] demonstrated that concentrations of piperaquine in capillary blood samples can be up to three times higher than in venous plasma samples. Given these findings, it was necessary to set the calibration range to cover these higher concentrations, with the ULOQ defined as 1000 ng/mL.

The linearity of the assay was assessed using calibration standards ranging from 3 to 1000 ng/mL. The regression model that provided the

Table 2
Assay accuracy and precision of piperaquine dried blood spot samples.

Concentration	Within-run precision (RSD)	Between-run precision (RSD)	Total-run precision (RSD)	Accuracy (% RE)
LLOQ, 3 ng/mL	11.3	10.2	11.2	−0.9
QC_{low}, 9 ng/mL	7.7	8.7	7.9	6.1
QC_{middle}, 40 ng/mL	4.3	7.7	5.0	3.9
QC_{high}, 800 ng/mL	4.3	3.1	4.1	−0.4
ULOQ, 1000 ng/mL	3.3	5.7	3.8	−2.7
OC (2000 ng/mL); diluted 1:6	9.0	7.6	8.8	−6.3

RSD = Relative Standard Deviation; RE = Relative Error.

best prediction of the back-calculated values of the calibration points and the best prediction of QC samples across the entire calibration range was a linear regression with $1/x^2$ weighting. The resulting equation was y = 0.893x + 0.059, with an excellent correlation coefficient (R = 0.997).

During the validation process, the overall accuracy and RSD for each calibration level were within 7 %, as evaluated using duplicate calibration points in four analytical tests. This demonstrates the satisfactory accuracy and precision of the assay across the calibration range.

3.1.3. Sensitivity and selectivity

The sensitivity of the assay was estimated to have a LOD of approximately 1 ng/mL, determined by achieving a signal-to-noise ratio of approximately 3 times the signal response of blank samples. The LLOQ was determined to be 3 ng/mL, with a signal response that was approximately 10 times the noise level.

Selectivity was assessed by analyzing seven blank samples collected from different blood donors, using both heparin and EDTA as anticoagulants. Fig. 1 demonstrates that there were no significant interferences observed, indicating the high selectivity of the assay.

3.1.4. Recovery

Recovery was calculated as: Recovery = (Response of pre-spiked DBS sample) / (Response of blank DBS post extract addition (post-spiked)). Average recoveries were in the range of 54–72 % for spiked blood extracted DBS over the QC concentration range for piperaquine (Table 3).

3.1.5. Matrix effects

Matrix effects were examined qualitatively using post-column infusion techniques [30,31]. Extracts from blank DBS samples obtained from the seven healthy volunteers, with heparin and EDTA as anticoagulants, were injected. No visible matrix effects were observed that would impact the signal around the retention time of piperaquine and IS.

To quantitatively assess the presence of any matrix effects, a method described by Matuszewski et al. [32] was employed. The MS response of the extracted blank DBS samples, following the addition of analyte (post-spiked), was compared to the MS response of a spiked neat matrix-free reference solution at the same concentration.

Table 3
Recovery of piperaquine in DBS (pre-spiked/blank extracted post spiked).

Concentration/ Sample:	No: 1	No: 2	No: 3	No: 4	No: 5	Average	RSD
QC_{low}, 9 ng/mL	77 %	71 %	70 %	76 %	67 %	72 %	5.7
QC_{high}, 800 ng/mL	62 %	56 %	50 %	53 %	49 %	54 %	9.7
IS for QC_{low}, 0.44 ng/mL	106 %	108 %	104 %	103 %	98 %	104 %	3.9
IS for QC_{high}, 0.44 ng/mL	97 %	94 %	87 %	90 %	83 %	90 %	6.2

QC, Quality Control; IS, internal standard; No: 1–5, Individual sample.

$$\text{Matrix factor} = (\text{Response of blank DBS post extract addition (post - spiked)}) / (\text{Average peak response in neat matrix free reference solution})$$

The normalized matrix effect, represented as the ratio of the matrix factor for the analyte to the matrix factor for the IS (MF-analyte/MF-IS), remained within the predetermined limit of ± 15 % variation, indicating the absence of matrix effects. Table 4 provides more comprehensive information regarding the normalized matrix effect values.

3.1.6. Carry-over and crosstalk

To assess carry-over effects, blank DBS were analyzed directly following two high-level calibration samples. The analysis revealed no detectable signal for piperaquine or the IS that would indicate any carry-over effect.

Similarly, crosstalk between piperaquine and the IS, was examined using the criterion described in section 2.7.6 of the Materials and Methods. Based on the assessment, no significant crosstalk was detected between piperaquine and the IS. More detailed information and verification can be found in Supplemental Figs. 1–3.

3.1.7. Stability

The stability of piperaquine in DBS was assessed to simulate pre-analytical sample handling situations such as transportation and storage. A study by Hung et al. had previously shown that freeze and thaw cycles could potentially affect the stability of piperaquine in DBS, with a

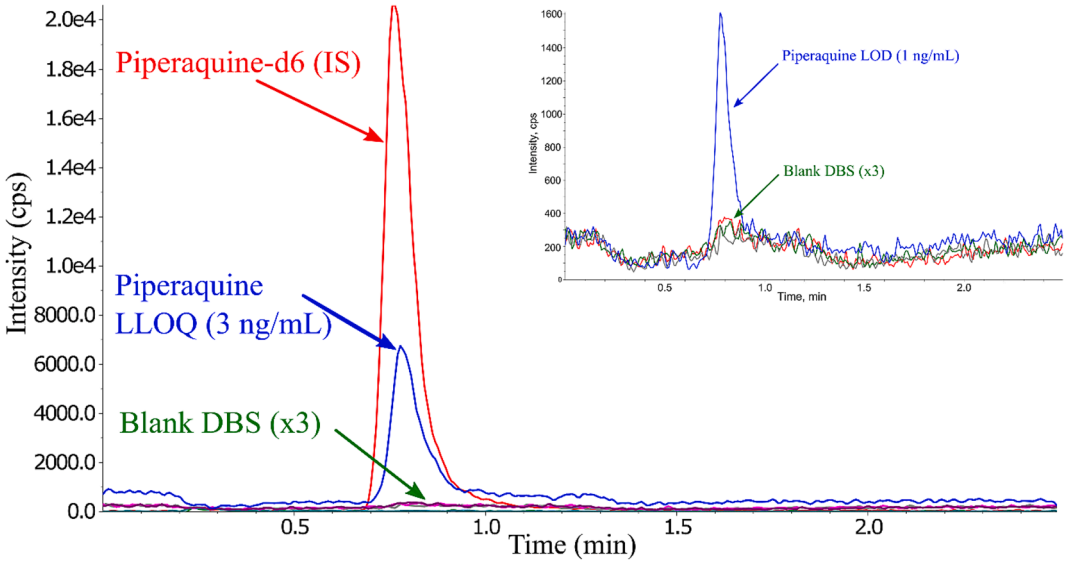

Fig. 1. Overlay of three sources of blank dried blood spot (EDTA) and piperaquine 3 ng/mL with IS 17.4 ng/mL. The inserted LOD figure originate from a separate run, where one DBS disc was punched from a LLOQ sample followed by two blank DBS discs into the same well. (Heparin blanks are equally free from endogenous peaks).

Table 4
Matrix effects from donors A-G in DBS.

Concentration/Donor:	A	B	C	D	E	F	G	Average	RSD
QC_{low}, 9 ng/mL	0.91	0.92	0.95	0.87	0.79	0.88	0.89	0.89	5.60
QC_{high}, 800 ng/mL	1.03	0.97	1.10	1.01	1.00	0.93	0.97	1.00	5.36
IS for QC_{low}	0.98	0.94	0.96	0.88	0.83	0.89	0.92	0.91	5.40
IS for QC_{high}	1.07	1.00	1.10	1.00	0.98	0.93	0.98	1.01	5.83
Normalized QC_{low}/IS	0.94	0.98	0.99	0.99	0.95	0.99	0.97	0.97	2.16
Normalized QC_{high}/IS	0.96	0.97	1.00	1.01	1.02	1.00	0.99	0.99	2.11

Donor A-D were collected using heparin as anticoagulant and donor E-G used EDTA as anticoagulant.

decrease of −10 % for the QC_{low} and −20 % for the QC_{high} in the third cycle. However, in our study, no degradation of piperaquine was detected during the three freeze–thaw cycles (to −80 °C), and the concentrations remained within ± 15 % of the nominal concentration.

Short-term stability testing of piperaquine in DBS was conducted for 5 days at ambient temperature and at 4 °C. Samples stored in the freezer at −80 °C were also compared. No degradation of piperaquine was observed under any of these conditions.

For long-term stability, samples were stored at ambient temperature (20–25 °C, 40–60 % RH) and in the refrigerator (4–6 °C) for a duration of 7 months, in comparison to a reference set stored as frozen DBS at −80 °C. No signs of degradation were detected at any of the storage temperatures. Although a desiccant bag was initially placed in each plastic bag to maintain dryness, it was consumed within one month due to the high water vapor transmission rate of the plastic bags used. However, even without replacing the desiccant, the samples stored in an air-conditioned room showed no adverse effects.

Extended long-term stability testing of samples stored in a dry cabinet at approximately 20 °C and 20 % RH for 3 years against a freshly prepared control was also conducted. No signs of degradation were observed, with the QC_{low} at 102 % and the QC_{high} at 92 %. Therefore, the long-term stability of piperaquine in DBS is considered very good when stored in a dark environment at around 20 °C and low humidity.

It is important to note that temperature and humidity conditions during storage play a crucial role in maintaining stability, and higher temperatures or humidity levels could potentially compromise stability. However, these factors were not specifically investigated in this study.

3.1.8. *Dried blood spot specific tests*

Pre-analytical factors that could potentially influence the quantification of DBS were evaluated, including the effects of hematocrit and spot homogeneity.

To facilitate the punching of both the center and the edge of the same spot, a spot volume of 100 µL was used. The average diameter of the 100 µL spots at different hematocrit levels (50 %, 40 %, and 20 %) was measured as follows: 17.5 ± 0.08 mm for 50 % hematocrit, 17.8 ± 0.13 mm for 40 % hematocrit, and 18.9 ± 0.18 mm for 20 % hematocrit. These measurements indicate that lower hematocrit values result in larger spots and, consequently, a smaller volume of blood would be obtained with each punch.

However, despite the differences in spot volume due to varying hematocrit levels, hematocrit did not have a significant impact on the quantification of piperaquine. The greatest difference in quantification, calculated as (sample - reference) / reference, was only 6 % between the reference (50 % hematocrit) and the 20 % hematocrit sample. This information is crucial, as it indicates that the impact of hematocrit variation, which is often lower in malaria patients, on the quantification of piperaquine is minimal [36].

Moreover, the location of the punches (center or close to the edge) in different hematocrit samples did not demonstrate any major impact on the quantification. The difference between the center reference sample and the edge samples was only 5 %, and this difference was well within the normal variation observed with the method.

3.2. *Clinical dried blood spot samples*

A total of 795 DBS samples were analyzed, including 32 samples collected on day 3 of the 3-day treatment course, representing the time around the predicted peak concentrations of piperaquine. These samples had a mean ± SD concentration of 512 ± 329 ng/mL, ranging from 49.3 to 1470 ng/mL. Among these samples, two were found to have concentrations above the ULOQ of the calibration curve.

During the first monthly treatment cycle, 228 samples were collected approximately one month after the last dose. These samples had a mean ± SD concentration of 34 ± 22.9 ng/mL, ranging from 2.55 to 156 ng/mL. Out of the 228 samples, only 2 were found to have concentrations below the LLOQ.

An in-depth analysis of the pharmacokinetic properties of piperaquine during intermittent preventive treatment for malaria in pregnancy has been published elsewhere [37]. An example chromatogram comparing a pre-dose sample and a one-month post-dose sample from the same participant can be seen in Fig. 2. In this study, a total of 33 QC samples at each concentration level were analyzed, and all were found to be within ± 15 % accuracy. The method demonstrated robustness and reliability, as evidenced by the low relative standard deviations of 4.4 %, 3.3 %, and 3.9 % for the low, medium, and high QC samples, respectively. Overall, the method performed well and met the required criteria for accuracy and precision.

4. Conclusion

This method was specifically developed for capillary blood collections conducted in remote areas and resource-limited settings where access to electricity or advanced equipment may be limited. It is particularly suitable for sampling vulnerable patients such as young children, toddlers, and infants who may not be able to provide large blood volumes. The minimum blood volume required for a blood spot is 30 µL, although a recommendation is to use 50 µL of blood to ensure a completely homogenous punching area.

The LLOQ of 3 ng/mL allows accurate measurement of piperaquine concentrations for up to 4–8 weeks after administration of a standard oral treatment regimen. This enables the evaluation of piperaquine

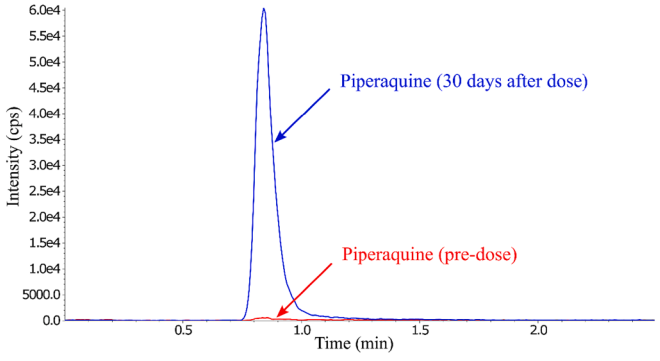

Fig. 2. Overlay of two samples from the same participant, pre-dose sample and 30 days after the 3-day treatment course measuring 44 ng/mL. (IS not shown).

levels at the time of recurrent infection or at the end of the standard 28-day follow-up period in efficacy trials.

The size of the blood spot or the position of the punch did not have a significant impact on the quantification of piperaquine. Similarly, comparing 50 % hematocrit to 20 % hematocrit showed minimal impact on quantification, with differences of less than 6 % between samples.

Piperaquine in DBS demonstrated stability for at least 3 years when stored in a dark environment with low humidity. However, it is important to consider the specific characteristics of piperaquine, including its potential adsorption to metallic surfaces and glassware, as well as potential interferences from plastic materials and triethylamine residues in the LC-MS analysis. Adequate precautions should be taken to mitigate these effects and ensure accurate quantification of piperaquine.

Funding information

This research was supported in whole, or in part, by the Wellcome Trust [091901/Z/10/Z], [220211]. For the purpose of Open Access, the author has applied for a CC BY public copyright license to any Author Accepted Manuscript version arising from this submission.

IRB and ethics statement

All volunteers provided written informed consent for their participation in the study, according to the principles of the Declaration of Helsinki. Ethical approval from the Liverpool School of Tropical Medicine (LSTM REC 12.28), Eijkman Institute for Molecular Biology (REC: 57), and Litbangkes, Ministry of Health, Jakarta (LB02.01/5.2/KE059/2013).

CRediT authorship contribution statement

Daniel Blessborn: Methodology, Investigation, Validation, Writing – original draft, Writing – review & editing. **Natpapat Kaewkhao:** Methodology, Investigation, Validation, Writing – review & editing. **Joel Tarning:** Resources, Project administration, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmsacl.2023.12.004.

References

[1] L. Chen, F.Y. Qu, Y.C. Zhou, Field observations on the antimalarial piperaquine, Chin Med J (Engl). 95 (4) (1982) 281–286. PubMed PMID: 6813038.
[2] Ashley EA, Krudsood S, Phaiphun L, Srivilairit S, McGready R, Leowattana W, et al. Randomized, controlled dose-optimization studies of dihydroartemisinin-piperaquine for the treatment of uncomplicated multidrug-resistant falciparum malaria in Thailand. J Infect Dis. 2004;190(10):1773-8Epub 2004/10/23. doi: JID32824 [pii] 10.1086/425015. PubMed PMID: 15499533.
[3] Denis MB, Davis TM, Hewitt S, Incardona S, Nimol K, Fandeur T, et al. Efficacy and safety of dihydroartemisinin-piperaquine (Artekin) in Cambodian children and adults with uncomplicated falciparum malaria. Clin Infect Dis. 2002;35(12):1469-76. Epub 2002/12/10. doi: 10.1086/344647. PubMed PMID: 12471565.
[4] T.T. Hien, C. Dolecek, P.P. Mai, N.T. Dung, N.T. Truong, L.H. Thai, et al., Dihydroartemisinin-piperaquine against multidrug-resistant Plasmodium falciparum malaria in Vietnam: randomised clinical trial, Lancet 363 (9402) (2004) 18–22, https://doi.org/10.1016/s0140-6736(03)15163-x.
[5] H. Karunajeewa, C. Lim, T.Y. Hung, K.F. Ilett, M.B. Denis, D. Socheat, et al., Safety evaluation of fixed combination piperaquine plus dihydroartemisinin (Artekin) in Cambodian children and adults with malaria, Br J Clin Pharmacol. 57 (1) (2004) 93–99. PubMed PMID: 14678346.
[6] Ubben D, Poll EM. MMV in partnership: the Eurartesim(R) experience. Malaria journal. 2013;12:211. Epub 2013/06/21. doi: 10.1186/1475-2875-12-211. PubMed PMID: 23782869; PubMed Central PMCID: PMCPMC3691732.
[7] Myint HY, Ashley EA, Day NP, Nosten F, White NJ. Efficacy and safety of dihydroartemisinin-piperaquine. Trans R Soc Trop Med Hyg. 2007;101(9):858-66. Epub 2007/07/31. doi: S0035-9203(07)00163-0 [pii] 10.1016/j.trstmh.2007.05.018. PubMed PMID: 17659311.
[8] WHO. Guidelines for malaria Geneva: World Health Organization; 2022 [updated 3 June 2022]. 396]. Available from: https://www.who.int/publications/i/item/guidelines-for-malaria. Accessed 26 Oct 2022.
[9] Warrell DA, Gilles HM. Essential malariology. 4th ed. London: Arnold; 2002. x, 348 p. [14] p. of plates p.
[10] T.M. Davis, T.Y. Hung, I.K. Sim, H.A. Karunajeewa, K.F. Ilett, Piperaquine: a resurgent antimalarial drug, Drugs 65 (1) (2005) 75–87. PubMed PMID: 15610051.
[11] Chotsiri P, Wattanakul T, Hoglund RM, Hanboonkunupakarn B, Pukrittayakamee S, Blessborn D, et al. Population pharmacokinetics and electrocardiographic effects of dihydroartemisinin-piperaquine in healthy volunteers. Br J Clin Pharmacol. 2017;83(12):2752-66. Epub 2017/07/12. doi: 10.1111/bcp.13372. PubMed PMID: 28695570; PubMed Central PMCID: PMCPMC5698590.
[12] R.M. Hoglund, L. Workman, M.D. Edstein, N.X. Thanh, N.N. Quang, I. Zongo, et al., Population pharmacokinetic properties of piperaquine in falciparum malaria: an individual participant data meta-analysis, PLoS medicine. 14 (1) (2017).
[13] K.C. Kobylinski, P. Jittamala, B. Hanboonkunupakarn, S. Pukrittayakamee, K. Pantuwatana, S. Phasomkusolsil, et al., Safety, pharmacokinetics, and mosquito-lethal effects of ivermectin in combination with dihydroartemisinin-piperaquine and primaquine in healthy adult Thai subjects, Clinical pharmacology and therapeutics. 107 (5) (2020) 1221–1230.
[14] T.N. Hai, S.F. Hietala, N. Van Huong, M. Ashton, The influence of food on the pharmacokinetics of piperaquine in healthy Vietnamese volunteers, Acta Trop. 107 (2) (2008) 145–149, https://doi.org/10.1016/j.actatropica.2008.05.013.
[15] T.Y. Hung, T.M. Davis, K.F. Ilett, Measurement of piperaquine in plasma by liquid chromatography with ultraviolet absorbance detection, J. Chromatogr. 791 (1–2) (2003) 93–101. PubMed PMID: 12798169.
[16] H.A. Karunajeewa, K.F. Ilett, I. Mueller, P. Siba, I. Law, M. Page-Sharp, et al., Pharmacokinetics and efficacy of piperaquine and chloroquine in Melanesian children with uncomplicated malaria, Antimicrobial Agents and Chemotherapy. 52 (1) (2008) 237–243, https://doi.org/10.1128/AAC.00555-07.
[17] C. Liu, R. Zhang, X. Hong, T. Huang, S. Mi, N. Wang, Pharmacokinetics of piperaquine after single and multiple oral administrations in healthy volunteers, Yakugaku Zasshi 127 (10) (2007) 1709–1714. PubMed PMID: 17917428.
[18] J. Tarning, E.A. Ashley, N. Lindegardh, K. Stepniewska, L. Phaiphun, N.P. Day, et al., Population pharmacokinetics of piperaquine after two different treatment regimens with dihydroartemisinin-piperaquine in patients with Plasmodium falciparum malaria in Thailand, Antimicrob. Agents Chemother. 52 (3) (2008) 1052–1061. PubMed PMID: 18180343.
[19] B.R. Moore, J.M. Benjamin, S. Salman, S. Griffin, E. Ginny, M. Page-Sharp, et al., Effect of coadministered fat on the tolerability, safety, and pharmacokinetic properties of dihydroartemisinin-piperaquine in Papua New Guinean children with uncomplicated malaria, Antimicrobial agents and chemotherapy. 58 (10) (2014) 5784–5794, https://doi.org/10.1128/AAC.03314-14.
[20] S.E. Reuter, A.M. Evans, S. Shakib, Y. Lungershausen, B. Francis, G. Valentini, et al., Effect of food on the pharmacokinetics of piperaquine and dihydroartemisinin, Clinical Drug Investigation 35 (9) (2015) 559–567, https://doi.org/10.1007/s40261-015-0312-8.
[21] R.N. Price, A.R. Hasugian, A. Ratcliff, H. Siswantoro, H.L. Purba, E. Kenangalem, et al., Clinical and pharmacological determinants of the therapeutic response to dihydroartemisinin-piperaquine for drug-resistant malaria, Antimicrob. Agents Chemother. 51 (11) (2007) 4090–4097. PubMed PMID: 17846129.
[22] D.J. Creek, V. Bigira, S. McCormack, E. Arinaitwe, H. Wanzira, A. Kakuru, et al., Pharmacokinetic predictors for recurrent malaria after dihydroartemisinin-piperaquine treatment of uncomplicated malaria in Ugandan infants, J Infect Dis. 207 (11) (2013) 1646–1654, https://doi.org/10.1093/infdis/jit078.
[23] K.M. Lwin, A.P. Phyo, J. Tarning, W. Hanpithakpong, E.A. Ashley, S.J. Lee, et al., Randomized, double-blind, placebo-controlled trial of monthly versus bimonthly dihydroartemisinin-piperaquine chemoprevention in adults at high risk of malaria, Antimicrobial Agents and Chemotherapy. 56 (3) (2012) 1571–1577, https://doi.org/10.1128/AAC.05877-11.
[24] E.A. Ashley, K. Stepniewska, N. Lindegardh, A. Annerberg, J. Tarning, R. McGready, et al., Comparison of plasma, venous and capillary blood levels of piperaquine in patients with uncomplicated falciparum malaria, Eur J Clin Pharmacol. 66 (7) (2010) 705–712, https://doi.org/10.1007/s00228-010-0804-7.
[25] M.Y. Aziz, K.J. Hoffmann, M. Ashton, LC-MS/MS quantitation of antimalarial drug piperaquine and metabolites in human plasma, Journal of Chromatography. 1063 (2017) 253–258, https://doi.org/10.1016/j.jchromb.2017.06.035.
[26] L.L. Kjellin, G. Dorsey, P.J. Rosenthal, F. Aweeka, L. Huang, Determination of the antimalarial drug piperaquine in small volume pediatric plasma samples by LC-MS/MS, Bioanalysis 6 (23) (2014) 3081–3089, https://doi.org/10.4155/bio.14.254.
[27] N. Lindegardh, A. Annerberg, N.J. White, N.P. Day, Development and validation of a liquid chromatographic-tandem mass spectrometric method for determination of piperaquine in plasma stable isotope labeled internal standard does not always compensate for matrix effects, Journal of Chromatography. 862 (1–2) (2008) 227–236, https://doi.org/10.1016/j.jchromb.2007.12.011.
[28] N. Mwebaza, V. Cheah, C. Forsman, R. Kajubi, F. Marzan, E. Wallender, et al., Determination of piperaquine concentration in human plasma and the correlation of capillary versus venous plasma concentrations, PLoS One. 15 (5) (2020), https://doi.org/10.1371/journal.pone.0233893.
[29] M. Malm, N. Lindegardh, Y. Bergqvist, Automated solid-phase extraction method for the determination of piperaquine in capillary blood applied onto sampling

paper by liquid chromatography, Journal of Chromatography. 809 (1) (2004) 43–49, https://doi.org/10.1016/j.jchromb.2004.05.032.
[30] R. Bonfiglio, R.C. King, T.V. Olah, K. Merkle, The effects of sample preparation methods on the variability of the electrospray ionization response for model drug compounds, Rapid Commun Mass Spectrom. 13 (12) (1999) 1175–1185, doi: 10.1002/(SICI)1097-0231(19990630)13:12<1175::AID-RCM639>3.0.CO;2-0.
[31] J.H. Ye, L.H. Pao, Using visualized matrix effects to develop and improve LC-MS/MS bioanalytical methods, taking TRAM-34 as an example, PLoS One. 10 (4) (2015), https://doi.org/10.1371/journal.pone.0118818.
[32] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS, Anal Chem. 75 (13) (2003) 3019–3030, https://doi.org/10.1021/ac020361s.
[33] R. Ahmed, J.R. Poespoprodjo, D. Syafruddin, C. Khairallah, C. Pace, T. Lukito, et al., Efficacy and safety of intermittent preventive treatment and intermittent screening and treatment versus single screening and treatment with dihydroartemisinin–piperaquine for the control of malaria in pregnancy in Indonesia: a cluster-randomised, open-label, superiority trial, Lancet Infect. Dis. 19 (9) (2019) 973–987, https://doi.org/10.1016/s1473-3099(19)30156-2.
[34] P. Singhal, A. Gaur, A. Gautam, B. Varshney, J. Paliwal, V. Batra, Sensitive and rapid liquid chromatography/tandem mass spectrometric assay for the quantification of piperaquine in human plasma, Journal of Chromatography. 859 (1) (2007) 24–29, https://doi.org/10.1016/j.jchromb.2007.09.021.
[35] T.Y. Hung, T.M. Davis, K.F. Ilett, H. Karunajeewa, S. Hewitt, M.B. Denis, et al., Population pharmacokinetics of piperaquine in adults and children with uncomplicated falciparum or vivax malaria, Br J Clin Pharmacol. 57 (3) (2004) 253–262. PubMed PMID: 14998421.
[36] N.J. White, Anaemia and malaria, Malaria Journal 17 (1) (2018) 371, https://doi.org/10.1186/s12936-018-2509-9.
[37] P. Chotsiri, J.R. Gutman, R. Ahmed, J.R. Poespoprodjo, D. Syafruddin, C. Khairallah, et al., Piperaquine pharmacokinetics during intermittent preventive treatment for malaria in pregnancy, Antimicrobial Agents and Chemotherapy. 65 (3) (2021), https://doi.org/10.1128/AAC.01150-20.