Journal of Pharmaceutical and Biomedical Analysis 245 (2024) 116154

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.journals.elsevier.com/journal-of-pharmaceutical-and-biomedical-analysis

# Development and validation of an ultra-performance liquid chromatography-tandem mass spectrometry method for the quantification of the antimalarial drug pyronaridine in human whole blood

Wietse M. Schouten [a], Ignace C. Roseboom [a], Luc Lucas [a], Japhet Kabalu Tshiongo [b,c,d], Hypolite Muhindo Mavoko [b], Kassoum Kayentao [e], Hilde Rosing [a], Alwin D.R. Huitema [a,f,g], Jos H. Beijnen [a,h], Thomas P.C. Dorlo [a,i,*]

[a] Department of Pharmacy & Pharmacology, Antoni van Leeuwenhoek/The Netherlands Cancer Institute, Amsterdam, the Netherlands
[b] Department of Tropical Medicine University of Kinshasa (UNIKIN), Kinshasa, Congo
[c] Amsterdam University Medical Centres, Department of Medical Microbiology and Infection Prevention, Laboratory for Experimental Parasitology, Academic Medical Centres at the University of Amsterdam, Amsterdam, the Netherlands
[d] Amsterdam Institute for Infection and Immunity, Infectious Diseases Programme, Amsterdam, the Netherlands
[e] Malaria Research and Training Center (MRTC), University of Sciences, Techniques and Technologies of Bamako (USTTB), Bamako, Mali
[f] Department of Pharmacology, Princess Maxima Center, Utrecht, the Netherlands
[g] Department of Clinical Pharmacy, University Medical Center Utrecht, Utrecht University, Utrecht, the Netherlands
[h] Utrecht Institute of Pharmaceutical Sciences, Utrecht University, Utrecht, the Netherlands
[i] Department of Pharmacy Uppsala University, Uppsala, Sweden

ARTICLE INFO

*Keywords:*
Pyronaridine
UPLC-MS/MS
Whole blood
Pharmacokinetics
Liquid-liquid extraction

ABSTRACT

Malaria remains a major health concern, aggravated by emerging resistance of the parasite to existing treatments. The World Health Organization recently endorsed the use of artesunate-pyronaridine to treat uncomplicated malaria. However, there is a lack of clinical pharmacokinetic (PK) data of pyronaridine, particularly in special populations such as children and pregnant women. Existing methods for the quantification of pyronaridine in biological matrices to support PK studies exhibit several drawbacks. These include limited sensitivity, a large sample volume required, and extensive analysis time. To overcome these limitations, an ultra-performance reversed-phase liquid chromatography tandem-mass spectrometry method to determine pyronaridine was developed and validated according to international guidelines. The method enabled fast and accurate quantification of pyronaridine in whole blood across a clinically relevant concentration range of 0.500–500 ng/mL ($r^2 \geq 0.9963$), with a required sample volume of 50 μL. Pyronaridine was extracted from whole blood using liquid-liquid extraction, effectively eliminating the matrix effect and preventing ion enhancement or suppression. The method achieved a satisfactory reproducible sample preparation recovery of 77%, accuracy (as bias) and precision were within ±8.2% and ≤5.3%, respectively. Stability experiments demonstrated that pyronaridine was stable for up to 315 days when stored at −70°C. Adjustments to the chromatographic system substantially reduced carry-over and improved sensitivity compared to prior methods. The method was successfully applied to quantify pyronaridine in whole blood samples from a selection of pregnant malaria patients participating in the PYRAPREG clinical trial (PACTR202011812241529) in the Democratic Republic of the Congo, demonstrating its suitability to support future PK studies. Furthermore, the enhanced sensitivity allows for the determination of pyronaridine up to 42 days post-treatment initiation, enabling assessment of the terminal elimination half-life.

## 1. Introduction

Malaria, a parasitic, vector-borne disease primarily occurring in the (sub)tropics, remains one of the most urgent global health priorities [1, 2]. The mainstay of current antimalarial treatment is artemisinin-based combination therapy, which involves a fast-acting

* Corresponding author at: Department of Pharmacy & Pharmacology, Antoni van Leeuwenhoek/The Netherlands Cancer Institute, Amsterdam, the Netherlands.
*E-mail address:* thomas.dorlo@farmaci.uu.se (T.P.C. Dorlo).

https://doi.org/10.1016/j.jpba.2024.116154
Received 19 January 2024; Received in revised form 12 April 2024; Accepted 15 April 2024
Available online 16 April 2024

artemisinin-derivative to rapidly kill the parasite combined with a longer-acting drug to prevent parasite recrudescence [3,4]. The World Health Organization recently reinforced a strong recommendation for the use of the oral fixed-dose combination artesunate-pyronaridine (Pyramax, PA) for the treatment of uncomplicated malaria caused by *Plasmodium falciparum* and *P. vivax* in adults and infants [5,6]. To further optimize therapy and enable the safe use of this combination therapy in all malaria patients, there is an urgent need for more pharmacokinetic (PK) data, particularly for the longer-acting component pyronaridine.

Pyronaridine exhibits a high affinity for red blood cells, with a reported whole blood to plasma partitioning factor median of 10.5 (range 4.9–17.8) in rabbits and a mean value of 9.0±0.83 in humans [7,8]. Consequently, whole blood has been recommended to be used as biomatrix for PK studies on pyronaridine [7]. Only a few analytical methods have been developed to quantify pyronaridine in various biomatrices from various species [7–15]. The most commonly used method is based on liquid chromatography-tandem mass spectrometry (LC-MS/MS) with human whole blood as the biomatrix [8,10,14,15]. The majority of these studies reported poor sensitivity (>5.0 ng/mL), leading to limitations in characterizing the terminal elimination phase and in the interpretation of drug exposure during malaria recrudescence at the end of the clinical follow-up at 42 days [8,10]. Moreover, all of these studies required a sample volume of above 100 µL. Malaria patients are anemic, and thus, it is recommended to minimize the amount of blood collected. Furthermore, a prolonged analysis time (>10 min) is commonly observed for the majority of published methods, leading to limited sample throughput [10,14]. Most PK studies have used the assay developed by Naik *et al.* despite the mentioned drawbacks [10]. Blessborn *et al.* developed a complex automated solid-phase extraction (SPE) sample preparation [15]. This particular sample preparation demands a sample volume of 100 µL and requires specific equipment, which is relatively costly and mostly unavailable. Hence, there is a need for a simple, rapid, and sensitive ultra-performance LC-MS/MS (UPLC-MS/MS) method, preferably capable of measuring concentrations of pyronaridine up to the end of clinical and pharmacodynamic follow-up at 42 days, and only requiring a small whole blood sample volume to facilitate PK studies in severely anemic malaria patients.

The aim of this study was therefore to develop a sensitive and accurate bioanalytical method to quantify pyronaridine in a small volume of human whole blood in a clinically relevant range of 0.500–500 ng/mL with a short analysis time. To illustrate the clinical suitability of this bioanalytical method, pyronaridine PK curves of pregnant malaria patients treated with pyronaridine-artesunate are presented.

## 2. Materials and methods

### 2.1. Chemicals

Acetonitrile (ACN), methanol (MeOH), formic acid (FA), and water were all ULC/MS grade and purchased from Biosolve (Valkenswaard, The Netherlands). Pyronaridine tetraphosphate and the internal standard, stable-isotopically labelled [$^{13}C_2D_4$]-pyronaridine tetraphosphate (SIL), were both purchased from Toronto Research Chemicals (Toronto, Canada). Artesunate was purchased from Alsachim (Illkirch-Graffenstaden, France). Ammonia (25%) and tert-butyl methyl ether (TBME) were supplied by Merck Chemicals (Amsterdam, The Netherlands). Blank whole blood was collected in $K_2$EDTA blood containers from healthy volunteers at the Netherlands Cancer Institute (Amsterdam, The Netherlands).

### 2.2. Stock solutions and working solutions

Pyronaridine (Fig. 1) and the SIL were dissolved in 1% FA in water-MeOH (1:1, v/v) to obtain a potency-corrected stock solution with a 1 mg/mL concentration, corrected for tetraphosphate. Two separate stocks were prepared for pyronaridine to make the calibration standards and the quality control (QC) samples. The working solution of the SIL was prepared by diluting the stock solution to a final concentration of 100 ng/mL (SIL100) in 1% FA in water-MeOH (1:1, v/v). All stock solutions and working solutions were stored at −20°C.

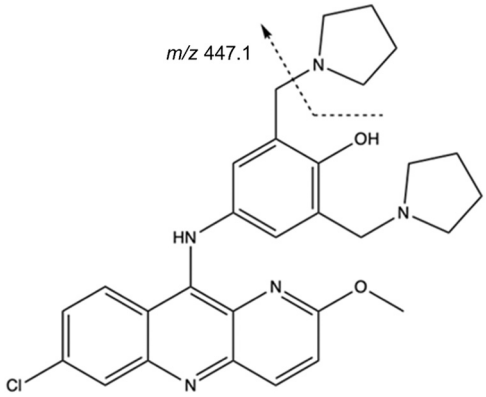


**Fig. 1.** Chemical structure of pyronaridine. The dashed arrow indicates the proposed fragmentation of the product ion *m/z* 447.1.

### 2.3. Calibration standards and quality control samples

Calibration standards and QC samples were both prepared in control human whole blood ($K_2$EDTA). Eight calibration standard concentrations were made by diluting the working solutions 20-fold in whole blood to obtain the following concentrations: 0.500, 1.00, 2.50, 10.0, 25.0, 100, 400, and 500 ng/mL. QC samples were prepared the same way as the calibration standards to obtain the QC-LLOQ (lower limit of quantification), QC-LOW, QC-MID, QC-HIGH and with the following concentrations: 0.500, 1.50, 20.0, and 375 ng/mL, respectively. All calibration standards and QC samples were prepared in a total volume of 1.0 mL and stored at −70°C.

### 2.4. Sample preparation

The analyte was extracted from the biomatrix using liquid-liquid extraction (LLE). For this, 50 µL of each whole blood sample was transferred to a reaction tube of 2.0 mL, and 20 µL of SIL100 was added except for the double blank. Next, 250 µL of 100 mM ammonium hydroxide was added to increase the pH of the samples. Samples were thoroughly vortex mixed for 10 sec and subsequently, 1 mL of TBME was added to each sample. After shaking (10 min at 1250 rpm), the samples were centrifuged for 5 min at 15,000 rpm. The water layer of the samples was snap-frozen with a dry-ice bath before transferring the organic layer to a clean tube. The organic TBME layer was evaporated under a gentle stream of nitrogen in a water bath set at 40°C. Next, the dried samples were reconstituted in 200 µL reconstitution solvent (RS), composed of 1% (v/v) FA in water-ACN-MeOH (95:2.5:2.5, v/v/v), by vortex mixing until the dried extract was fully dissolved. After centrifuging (5 min at 15,000 rpm), clear supernatant was transferred to a polypropylene vial with an insert of which 1 µL was injected into the UPLC-MS/MS system.

### 2.5. UPLC equipment

The chromatographic system consisted of an Agilent 1290 Infinity II system, which included a binary UPLC pump, an on-line degasser, an autosampler, and a column oven (Agilent, Santa Clara, CA, USA). The column oven was set to operate at 40°C while the autosampler was set to 5°C. Chromatographic separation was achieved using an ACQUITY UPLC BEH C18 column (50 ×2.1 mm, 1.7 µm particle size, Waters, Milford, MA, USA) preceded by an in-line filter (0.2 µm, Waters) at a flow rate of 0.4 mL/min. Mobile phase A consisted of 1% (v/v) FA in

water, while mobile phase B consisted of ACN-MeOH (1:1, v/v). The gradient program used to elute pyronaridine was as follows: 1.0 min isocratic elution at 10% B, linear increase to 40% B in 2.0 min, isocratic at 40% B for 0.5 min, down to 10% in 0.05 min, and conditioning at 10% for 0.95 min, resulting in a total run time of 4.5 min. A mixture consisting of 0.1% (v/v) FA in ACN-isopropanol-MeOH–water (25:25:25:25, v/v/v/v) was used as autosampler wash solvent.

### 2.6. MS equipment

Detection of pyronaridine was performed using a QTRAP6500 (Sciex, Framingham, MA, USA). This mass spectrometer (MS) was equipped with a Turbo ionspray (TIS) source operating in positive ion mode. The MS operating settings are summarized in Table 1. Multiple reaction monitoring (MRM) mode was used to detect and quantify pyronaridine and the SIL. For pyronaridine, the transition *m/z* 518.3 → 447.1 was used, and for the SIL *m/z* 524.3 → 453.1 was used for quantification. Data acquisition was performed using Analyst™ software (Sciex, version 1.7.2).

### 2.7. Validation procedure

Validation was based on the relevant ICH M10 bioanalytical guideline [16]. The following validation experiments were deemed relevant and were performed during the bioanalytical validation: calibration model, lower limit of quantification, accuracy and precision, carry-over, dilution integrity, selectivity (including cross analyte/SIL internal standard interference, endogenous interference, co-medication interference of artesunate), hematocrit (Hct) effect, matrix effect, extraction recovery, and stability under various conditions. Each run consisted of two sets of calibration standards and two sets of QC samples (QC-LOW, QC-MID and QC-HIGH), except for the accuracy and precision experiments, in which 5 sets of QC samples were used.

### 2.8. Clinical application

The goal of developing and validating this bioanalytical assay was to support a PK study in a Phase III clinical trial (PYRAPREG clinical trial, Pan-African Clinical Trials Registry ID: PACTR202011812241529) focused on evaluating efficacy, safety, and PK of PA in the treatment of pregnant malaria patients in various sub-Saharan African countries, including the Democratic Republic of the Congo (DRC) [17]. The study protocol has been reviewed and approved by all relevant local and/or national medical ethics committees, including the DRC National Ethics Committee (approval reference number 169/CNES/BN/PMMF/2019). The PA regimen consisted of three daily doses of 540 or 720 mg pyronaridine and 180 or 240 mg artesunate. Whole blood PK samples were taken before the first dose, 1 h, 2 h, 6 h, and 10 h after the first dose, on day 2 and 3 before the dose, and on day 7, 14, 21, 28, 42. $K_2$EDTA whole blood samples were immediately stored at −70°C and transported frozen on dry ice. To determine the clinical applicability of the bioanalytical method, two PK curves (consisting of 12 different sampling time points) of patients enrolled in the clinical trial were randomly selected and pyronaridine whole blood concentrations were quantified using the validated method.

**Table 1**
Above: general mass spectrometric parameters. Below: Analyte specific mass spectrometric parameters for pyronaridine and the SIL, [$^{13}C_2D_4$]-pyronaridine.

| **Mass-spectrometer** | | |
|---|---|---|
| **Run duration** | 4.5 min | |
| **Polarity** | Positive | |
| **Ionspray voltage** | 5500 V | |
| **Nebulizer gas** | 60 psi | |
| **Turbo gas/heater gas** | 30 psi | |
| **Curtain gas** | 30 psi | |
| **Collision gas** | 10 psi | |
| **Temperature** | 500 °C | |
| | Pyronaridine | SIL |
| **Transition (*m/z*)** | 518.3 → 447.1 | 524.3 → 453.1 |
| **Collision energy (V)** | 10 | 10 |
| **Collision exit potential (V)** | 23 | 23 |
| **Declustering potential (V)** | 101 | 101 |
| **Entrance potential (V)** | 16 | 16 |
| **Dwell time (msec)** | 100 | 100 |

Abbreviations: SIL = stable-isotopically labelled [$^{13}C_2D_4$]-pyronaridine tetraphosphate.

## 3. Results and discussion

### 3.1. Development of the bioanalytical assay

During direct infusion of pyronaridine into the MS system, the molecular ion ($M+H^+$) was observed at *m/z* 518.3. This *m/z* was used as the precursor ion to create fragment ions, of which *m/z* 447.1 was the most abundant. The precursor ion of the SIL was observed accordingly at *m/z* 524.3, which fragmented into *m/z* 453.1. The loss of 71.1 Da corresponds to the loss of the pyrrolidine moiety. The proposed fragmentation pattern of pyronaridine is shown in Fig. 1.

It was challenging to find an appropriate column to achieve sufficient retention with an acceptable peak shape. Due to the basicity and lipophilicity of pyronaridine, secondary interactions between the column and the analyte were observed for many different columns, which contributed to peak tailing. Several columns with different separation mechanisms (C18, Phenylhexyl, HILIC, Shielded C18) were evaluated to investigate which column showed the least tailing. Eventually, the ACQUITY UPLC BEH C18 column was found superior in terms of peak symmetry, with a 2.5-fold reduction in peak asymmetry factor compared to other columns. The addition of 1% FA instead of 0.1% FA in mobile phase A further reduced the tailing and improved the peak shape significantly. This resulted in a peak with an asymmetry factor of 3.6 that was found acceptable and was easy to integrate over the complete calibration range.

Another hurdle was to control carry-over to ensure data integrity. Blessborn *et al.* had already reported issues relating to carry-over, which appeared to originate from the needle of the autosampler [15]. A substantial amount of carry-over, which was 160% of the area of the LLOQ in the first double blank after injection of the upper limit of quantification (ULOQ), was noticed. Several tests were performed to determine the cause of the carry-over. This revealed the memory effect of the analytical column as the most plausible cause of the carry-over. Carry-over could be prevented by using a linearly increased gradient elution instead of a block gradient elution. This, in turn, resulted in a slight increase in peak tailing but was deemed acceptable. In addition, there was an increase in baseline offset observed in the double blank after injection of the ULOQ sample. Therefore, it was investigated whether the baseline offset could affect the quantification of an LLOQ sample after injection of the ULOQ sample. The LLOQ was accurately determined, meeting the acceptance criteria of ±20% for bias and ≤20% for precision. Consequently, the baseline offset caused by carry-over had no effect on the quantification.

The use of whole blood as a biomatrix complicates sample preparation. Simple protein precipitation is inadequate to remove all matrix components of whole blood and may result in column clogging. SPE could be used to obtain clean final extracts. However, this is a time-consuming sample preparation without the use of automated SPE equipment. Therefore, the aim was to use a LLE method using TBME. Since pyronaridine is an alkalic and lipophilic compound, these characteristics were exploited by using an alkaline buffer to ensure that pyronaridine is uncharged. Consequently, pyronaridine accumulates in the organic TBME layer over the water layer. This organic layer can be transferred to a clean reaction tube by snap-freezing the water layer.

Different concentrations (10/100/1000 mM) of ammonium hydroxide in water were added to the whole blood samples in a 5:1 ratio to increase the pH. The highest recovery (76%) was obtained by using the 100 mM ammonium hydroxide solution (compared to 61% for the 1000 mM ammonium hydroxide solution and 2% for the 10 mM ammonium hydroxide solution).

This LLE method also increased the sensitivity of the assay. The organic layer was evaporated, and the dried sample was then

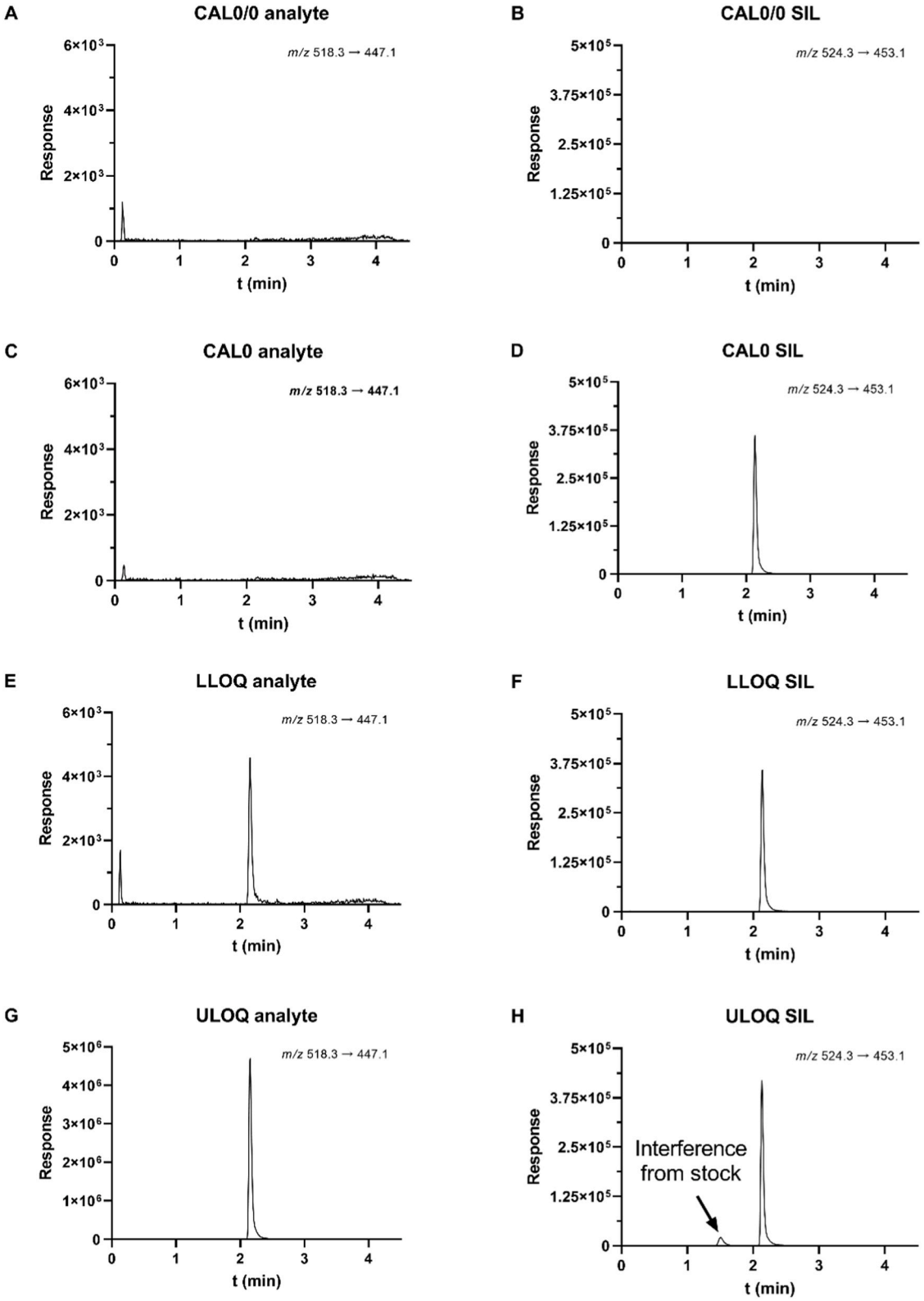


**Fig. 2.** Representative MRM chromatograms of pyronaridine and the SIL for the double blank (A and B), the blank (C and D), the lower limit of quantification (LLOQ) of 0.500 ng/mL (E and F), and the upper limit of quantification (ULOQ) of 500 ng/mL (G and H), at their transitions. The interference from pyronaridine is highlighted with an arrow at t=1.5 min (H).

reconstituted in 1% (v/v) FA in water-ACN-MeOH (95:2.5:2.5, v/v/v). If necessary, the sensitivity of the assay could theoretically even be further enhanced by either increasing the injection volume or decreasing the amount of RS.

### 3.2. Validation procedures

#### 3.2.1. Calibration model, lower limit of quantification

The method was linear over a concentration range of 0.500 ng/mL to 500 ng/mL. Linear regression was chosen as a model with a $1/x^2$ weighting, where x is the analyte concentration. A linear fit with a correlation coefficient ($r^2$) of at least 0.9963 was obtained in three separate analytical runs. The signal-to-noise ratio at the LLOQ level (0.500 ng/mL) was, at minimum, above 36. Representative MRM chromatograms of the double blank, single blank, LLOQ, and ULOQ are depicted in Fig. 2.

#### 3.2.2. Accuracy and precision

To determine assay performance, QC samples (QC-LLOQ, QC-LOW, QC-MID, and QC-HIGH) were quantified in quintuplicate in three separate runs. The intra/inter-run accuracies were determined by calculating the bias (%) from the nominal concentrations. The intra/inter-run precisions were calculated using a one-way ANOVA test expressed as the coefficient of variation (CV%). The accuracy and precision data are summarized in Table 2. The intra- and inter-assay bias was ±8.2% and ±3.4% over all concentration levels. Intra- and inter-assay precision was ≤5.3% and ≤5.6% over all concentration levels. The acceptance criteria for the intra/inter-run accuracies and the intra/inter-run precision values should be within ±15% and ≤15% (±20% and ≤20% at the LLOQ), respectively. Therefore, all accuracy and precision acceptance criteria were met.

#### 3.2.3. Carry-over

The carry-over of pyronaridine and the SIL was determined by injecting two double blanks after the ULOQ (500 ng/mL) of the calibration standard in three consecutive runs. This was assessed by comparing the mean of the peak areas of pyronaridine and the SIL of five QC LLOQ samples with the peak area of the two double blanks. The peak areas in the double blanks compared to the QC LLOQ samples should be ≤ 20% for pyronaridine and ≤ 5% for the SIL. In Section 3.1, extensive experiments to investigate the carry-over were already described and concluded that it was not affecting the performance of the assay.

#### 3.2.4. Dilution integrity

The dilution of samples was performed (in quintuplicate) by diluting spiked human whole blood at a concentration of 5000 ng/mL (10 times the ULOQ) with blank human whole blood in a 20-fold dilution. The intra-run accuracy and precision for the dilution should be within the acceptance criteria of ±15% and ≤15%, respectively. The measured diluted samples were within these acceptance criteria, with an intra-run bias of −10.5% and an intra-run precision (CV) of 1.5%.

#### 3.2.5. Selectivity

The cross-analyte/SIL internal standard experiment determined whether there was interference from pyronaridine in the SIL transition and vice versa. This was obtained by spiking blank whole blood exclusively with pyronaridine at the ULOQ level (500 ng/mL) and, in a separate sample, solely with SIL at its normal concentration level (100 ng/mL). There was a concentration-dependent peak in the SIL transition (around t=1.5 min, indicated by the arrow in Fig. 2H). This peak originated from the stock or working solutions of pyronaridine. Since the retention time differed between the interference peak and SIL (1.5 min for interference, 2.1 min for the SIL), the peak area of the SIL was not influenced. In addition, the SIL did not show any interference in the pyronaridine transition.

Any influence of endogenous material in the pyronaridine and SIL transitions was determined using blank human whole blood obtained from six different individuals. Double blank and LLOQ (0.500 ng/mL) samples were processed for each batch. No interference peaks were present in the double blank at the pyronaridine and SIL transition. The deviation for these LLOQ samples was ±16.0%, except for one batch, which showed a bias of −22.0%. This was deemed acceptable according the guidelines.

In addition, the potential influence of artesunate, the co-formulated drug in PA, on the bioanalytical performance was investigated. This was executed by spiking artesunate at its highest concentration (200 ng/mL) after a 4 mg/kg dose, as reported in the clinical study report of a phase I dose escalation study, to an LLOQ sample (0.500 ng/mL) [12]. The intra-assay bias and precision for these LLOQ samples were ±6.9% and ≤3.7%, respectively. Thus, the addition of the co-medication did not influence the quantification of the LLOQ in any way.

At last, the influence of different Hct levels was assessed. The patient population of the clinical trial for which this assay is being developed is a population of pregnant malaria patients. Both pregnancy and malaria potentially lead to a reduction in Hct levels, with large inter-patient variability [18,19]. Therefore, it was investigated if different Hct levels affected the performance of the bioanalytical method. For this experiment, different clinically relevant Hct levels (0.20, 0.30, and 0.40) were spiked with a working solution to obtain QC-LOW and QC-HIGH [12,20]. There was no evidence that different Hct levels in this range influenced the quantification of pyronaridine. The bias of QC-LOW and

**Table 3**
Hematocrit effect on assay performance, determined at QC-LOW (1.50 ng/mL) and QC-HIGH (375 ng/mL) levels (n=3).

| Hematocrit | Nominal concentration (ng/mL) | Mean measured concentration (ng/mL) | Accuracy (Bias %) | Precision (CV %) |
|---|---|---|---|---|
| **0.20** | 1.50 | 1.51 | 0.4 | 1.4 |
| | 375 | 399 | 6.3 | 0.3 |
| **0.30** | 1.50 | 1.51 | 0.4 | 4.0 |
| | 375 | 378 | 0.8 | 0.5 |
| **0.40** | 1.50 | 1.48 | -1.3 | 2.7 |
| | 375 | 397 | 5.9 | 3.5 |

Abbreviations: CV% = coefficient of variation.

**Table 2**
Assay performance data for pyronaridine in human whole blood. The accuracy (bias in %) and the precision (CV %) were analyzed at four concentrations in quintuplicate in three consecutive analytical runs (n=15).

| | | Intra-assay (n=5, determined in 3 single runs) | | Inter-assay (n=15) | |
|---|---|---|---|---|---|
| Nominal concentration (ng/mL) | Mean measured concentration (ng/mL) | Accuracy (Bias %) | Precision (CV %) | Accuracy (Bias %) | Precision (CV %) |
| **0.500** | 0.517 | ±8.2 | ≤4.3 | 3.4 | 5.6 |
| **1.50** | 1.46 | ±5.2 | ≤5.3 | -2.4 | 1.7 |
| **20.0** | 19.9 | ±1.4 | ≤2.1 | 0.3 | 0.6 |
| **375** | 369 | ±1.9 | ≤1.7 | -1.5 | -* |

Abbreviations: CV% = coefficient of variation.
* No additional variation was found by performing the assay between days (the mean square between groups is less than the mean square within groups).

QC-HIGH of the different Hct levels was all ±6.3%, while the accuracy (CV%) was ≤3.5%, as summarized in Table 3.

### 3.2.6. Matrix effect and recovery

The matrix effect was determined in whole blood to evaluate potential ion enhancement or suppression. For this experiment, control human whole blood from six different individuals was used. These batches were used as matrix present samples and were processed as double blank samples. Matrix absent samples were prepared in triplicate by processing water as double blank samples. Both matrix present and absent samples were post-extraction spiked with QC working solutions to their appropriate concentrations. To assess the matrix effect, the peak areas of pyronaridine and the SIL in matrix present and matrix absent samples were compared at equal concentrations. The matrix factors (MF) of pyronaridine for QC-LOW and QC-HIGH were 1.03 and 1.01, respectively (Table 4). The normalized MFs, achieved by dividing the MF of pyronaridine by the MF of the SIL, were 1.01 (CV% 2.0) and 1.02 (CV% 3.4) for QC-LOW and QC-HIGH, respectively. These MF values were within the acceptance criteria, indicating that there was no ion enhancement or suppression caused by interfering endogenous compounds present in the processed matrix.

The sample preparation recovery in human whole blood samples was determined by comparing the peak areas of pyronaridine in the matrix present samples to processed whole blood samples (as described in Section 2.4). The total overall recovery, accounting for the matrix effect and sample preparation, was calculated by comparing the peak areas of pyronaridine in the matrix absent samples to the processed whole blood samples, both at QC-LOW and QC-HIGH concentration levels. The sample preparation recovery of pyronaridine was 77.2 and 77.0% (CV% 2.6–3.7), whereas the total overall recovery was 80.0 and 78.7% (CV% of 1.3%), for QC LOW and HIGH, respectively (Table 4).

### 3.2.7. Stability

For stability experiments, stability samples (QC-LOW and QC-HIGH) were processed and analysed against a freshly spiked calibration curve. The mean concentration should fall within ±15% of the nominal concentration. The stability of pyronaridine in different matrices stored under several analytically relevant conditions is summarized in Table 5. Pyronaridine was found to be stable in whole blood for up to 131 days when stored at −20°C and at least 315 days when stored at −70°C.

Storage of aliquots (50 µL) at −20°C for 209 days was proven to be unsuccessful, as the whole blood samples exhibited discoloration and transformed into a solid form. This transformation is hypothesized to negatively impact the extraction recovery of the analyte from the whole blood. Furthermore, during the analysis of these transformed samples, shifted and distorted peaks were observed, resulting in an unquantifiable concentration of pyronaridine. Consequently, study samples, calibration standards, and QC samples are advised to be stored in larger volumes (>500 µL) at −70°C and aliquoted before sample preparation.

Pyronaridine in whole blood was stable after storage for 24 hours at room temperature (nominally 20°C). Additionally, subjecting aliquoted samples to three freeze-thaw cycles, each involving freezing for a minimum of 12 hours at −70°C followed by thawing at room temperature, did not affect the stability of pyronaridine in whole blood. The final extract solution remained stable for at least 7 days when stored at a nominal temperature of 2–8°C. The stock solutions and working solutions were stable for at least 352 days and 59 days, respectively, when stored at −20°C, and the stock solution was stable for at least 24 hours at room temperature.

**Table 5**
Stability parameters for pyronaridine in biomatrix, final extract, stock solution and working solution with the accuracy (bias %) and precision (CV %) (n=3 samples per concentration level).

| Matrix | Condition | Nominal Concentration (ng/mL) | Accuracy (Bias %) | Precision (CV %) |
|---|---|---|---|---|
| Human whole blood | -20°C, 131d | 1.50 | -2.7 | 1.8 |
| | | 375 | -1.1 | 2.7 |
| | -70°C, 315d | 1.50 | -11.8 | 3.5 |
| | | 375 | 1.4 | 3.0 |
| | -20°C, 3 F/T cycles | 1.50 | 2.2 | 1.4 |
| | | 375 | -0.4 | 0.4 |
| | RT, 24 h | 1.50 | -6.2 | 2.9 |
| | | 375 | -3.1 | 0.3 |
| Final extract | 2–8°C, 7d | 1.50 | -2.2 | 2.1 |
| | | 375 | -2.5 | 1.3 |
| Stock solution | -20°C, 352d | $1.00 \times 10^6$ | -1.5 | 0.8 |
| | RT, 24 h | $1.00 \times 10^6$ | 1.8 | 3.5 |
| Working solution | -20°C, 59d | 30.0 | 0.4 | 2.0 |

Abbreviations: CV% = coefficient of variation; d = days; F/T = freeze/thaw; h = hours; RT = room temperature at nominally 20°C.

### 3.3. Clinical applicability

The clinical application of the bioanalytical method was evaluated using PK whole blood samples (12 samples per individual) obtained from two randomly selected non-HIV-infected pregnant women with malaria receiving a conventional PA regimen as part of the PYRAPREG trial. The measured pyronaridine whole blood concentration versus time profile is shown in Fig. 3. Patient 1 reached a maximum concentration ($C_{max}$) of 382 ng/mL at 2 hours post-administration, while Patient 2 had

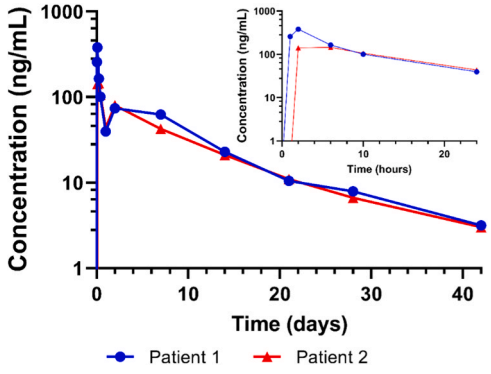


**Fig. 3.** The pyronaridine concentration in whole blood versus the time (ranging from t=0 hours to t=42 days) in two patients treated with PA (720 mg pyronaridine, 240 mg artesunate, PO, QD) for three consecutive days. The concentration-time profile of pyronaridine during the first 24 hours is shown in the upper right corner.

**Table 4**
Matrix effect (as factor) and recovery (as %) for pyronaridine in human whole blood, determined at QC-LOW (1.50 ng/mL) and QC-HIGH (375 ng/mL) levels.

| | Matrix effect (as factor) | | | Recovery (as %) | |
|---|---|---|---|---|---|
| Nominal concentration (ng/mL) | MF Pyronaridine (area-based) | MF SIL (area-based) | SIL-normalized MF (area ratio based) | Sample Preparation Recovery | Total Overall Recovery |
| **1.50** | 1.03 (CV% 1.7) | 1.04 (CV% 1.7) | 1.01 (CV% 2.0) | 77.2% (CV% 3.7) | 80.0% (CV% 1.3) |
| **375** | 1.01 (CV% 1.1) | 1.00 (CV% 2.4) | 1.02 (CV% 3.4) | 77.0% (CV% 2.6) | 78.7% (CV% 1.3) |

Abbreviations: MF= Matrix factor; CV% = coefficient of variation; SIL = stable-isotopically labelled [$^{13}C_2D_4$]-pyronaridine tetraphosphate.

a $C_{max}$ of 145 ng/mL at 6 hours post-administration. The two patients displayed similar elimination profiles. All pharmacokinetic results from this clinical trial will be reported in more detail elsewhere. Almost all observed whole blood concentrations from the patients, even the day 42 sample, were within the validated range (between 0.5 and 500 ng/mL). Only 3 out of 24 samples were below the LLOQ, all of which were sampled within 1 hour post-dose, confirming the suitability of the newly developed method. Due to the simple extraction method and short analysis time, 250 patient samples can be processed and measured in a single day, showcasing its high throughput without requiring expensive machinery as in previously reported methods [8–10,15]. Furthermore, the enhanced sensitivity of the method could facilitate extended sampling beyond 42 days, and thereby contribute to a deeper comprehensive understanding of late parasite recrudescence in PA-treated malaria patients.

## 4. Conclusion

An easy, accurate, and robust bioanalytical assay for determining the concentration of pyronaridine in human whole blood was developed and validated. LLE extraction with TBME was utilized to extract pyronaridine out of whole blood using UPLC-MS/MS for separation and detection with an accuracy of ±8.2% and a precision of ≤5.3%. Clinical samples must be stored at −70°C and aliquoted before analysis, according to the stability data. This increased the stability of pyronaridine to at least 315 days. This assay was shown to be sufficiently sensitive and reliable to quantify pyronaridine in 50 μL whole blood samples obtained from pregnant patients enrolled in a PK Phase III clinical trial. The small sample volume required (50 μL) means that this method is also suitable for the quantification of pyronaridine in the whole blood of typically anemic malaria patients, infants, and pregnant women.

## Funding

This project is part of the EDCTP2 program supported by the European Union (grant number RIA2017MC-2025 - PYRAPREG). TD is supported by the Swedish Research Council (VR grant number 2022–01251) and the Dutch Research Council (NWO/ZonMw Veni project no. 91617140).

## CRediT authorship contribution statement

**Wietse M. Schouten:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Conceptualization. **Ignace C. Roseboom:** Writing – review & editing, Methodology, Conceptualization. **Luc Lucas:** Writing – review & editing, Validation, Methodology, Conceptualization. **Japhet Kabalu Tshiongo:** Writing – review & editing, Resources. **Hypolite Muhindo Mavoko:** Writing – review & editing, Resources. **Kassoum Kayentao:** Writing – review & editing, Resources. **Hilde Rosing:** Writing – review & editing, Validation, Supervision, Methodology, Conceptualization. **Alwin D.R. Huitema:** Writing – review & editing, Supervision. **Jos H. Beijnen:** Writing – review & editing, Supervision. **Thomas P.C. Dorlo:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

## 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.

## Acknowledgments

We gratefully acknowledge the pregnant malaria patients from the Democratic Republic of the Congo who participated in the PK study without any self-interest and whose whole blood samples were used for the clinical application of this methodology. We would also like to thank the clinical site team of the University of Kinshasa (UNIKIN), Democratic Republic of the Congo, for organizing the collection and transport of patient whole blood samples, in particular Lise Kuseke, Doudou Yobi and Freddy Kabasele.

## References

[1] M.A. Phillips, J.N. Burrows, C. Manyando, R.H. van Huijsduijnen, W.C. Van Voorhis, T.N.C. Wells, Malaria, Nat. Rev. Dis. Prim. 3 (1) (2017) 17050.
[2] World Health Organization (WHO), World Malaria Report 2021. ⟨https://www.who.int/teams/global-malaria-programme/reports/world-malaria-report-2021⟩, 2021 (accessed 01 November 2023).
[3] F. Nosten, N.J. White, Artemisinin-Based Combination Treatment of Falciparum Malaria, Am. Soc. Trop. Med. Hyg. (2007).
[4] C. Chang, Chapter 9 - Artemisinins and Pyronaridine Phosphate Combination, in: L. Guoqiao, L. Ying, L. Zelin, Z. Meiyi (Eds.), Artemisinin-Based and Other Antimalarials, Academic Press, 2018, pp. 571–607.
[5] World Health Organization (WHO), The Use of Artesunate-Pyronaridine for the Treatment of Uncomplicated Malaria. ⟨https://fctc.who.int/publications/i/item/WHO-HTM-GMP-2019.13⟩, 2019 (accessed 01 November 2023).
[6] World Health Organization (WHO), Guidelines for Malaria. ⟨https://www.who.int/publications-detail-redirect/guidelines-for-malaria⟩, 2023 (accessed 01 November 2023).
[7] Y.-C. Chen, L. Fleckenstein, Improved assay method for the determination of pyronaridine in plasma and whole blood by high-performance liquid chromatography for application to clinical pharmacokinetic studies, J. Chromatogr. B 752 (2001) 39–46.
[8] S.A. Charman, A. Andreu, H. Barker, S. Blundell, A. Campbell, M. Campbell, G. Chen, F.C.K. Chiu, E. Crighton, K. Katneni, J. Morizzi, R. Patil, T. Pham, E. Ryan, J. Saunders, D.M. Shackleford, K.L. White, L. Almond, M. Dickins, D.A. Smith, J. J. Moehrle, J.N. Burrows, N. Abla, An in vitro toolbox to accelerate anti-malarial drug discovery and development, Malar. J. 19 (1) (2020) 1–27.
[9] D. Blessborn, N. Lindegårdh, Ö. Ericsson, U. Hellgren, Y. Bergqvist, Determination of pyronaridine in whole blood by automated solid phase extraction and high-performance liquid chromatography, Ther. Drug Monit. 25 (3) (2003) 264–270.
[10] H. Naik, P. Imming, M.S. Schmidt, D.J. Murry, L. Fleckenstein, Development and validation of a liquid chromatography-mass spectrometry assay for the determination of pyronaridine in human blood for application to clinical pharmacokinetic studies, J. Pharm. Biomed. Anal. 45 (1) (2007) 112–119.
[11] E.M. Hodel, B. Zanolari, T. Mercier, J. Biollaz, J. Keiser, P. Olliaro, B. Genton, L. A. Decosterd, A single LC-tandem mass spectrometry method for the simultaneous determination of 14 antimalarial drugs and their metabolites in human plasma, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 877 (10) (2009) 867–886.
[12] B. Tan, H. Naik, I.J. Jang, K.S. Yu, L.E. Kirsch, C.S. Shin, J.C. Craft, L. Fleckenstein, Population pharmacokinetics of artesunate and dihydroartemisinin following single-and multiple-dosing of oral artesunate in healthy subjects, Malar. J. 8 (1) (2009) 304.
[13] J. Lee, J. Son, S.J. Chung, E.S. Lee, D.H. Kim, In vitro and in vivo metabolism of pyronaridine characterized by low-energy collision-induced dissociation mass spectrometry with electrospray ionization, J. Mass Spectrom. 39 (9) (2004) 1036–1043.
[14] C.A. Morris, S.R. Dueker, P.N. Lohstroh, L.Q. Wang, X.P. Fang, D. Jung, L. Lopez-Lazaro, M. Baker, S. Duparc, I. Borghini-Fuhrer, R. Pokorny, J.S. Shin, L. Fleckenstein, Mass balance and metabolism of the antimalarial pyronaridine in healthy volunteers, Eur. J. Drug Metab. Pharmacokinet. 40 (1) (2015) 75–86.
[15] D. Blessborn, K. Kaewkhao, L. Song, N.J. White, N.P.J. Day, J. Tarning, Quantification of the antimalarial drug pyronaridine in whole blood using LC–MS/MS — Increased sensitivity resulting from reduced non-specific binding, J. Pharm. Biomed. Anal. 146 (2017) 214–219.
[16] European Medicines Agency (EMA), ICH guideline M10 on bioanalytical method validation and study sample analysis. ⟨https://www.ema.europa.eu/en/ich-m10-bioanalytical-method-validation-scientific-guideline⟩, 2022 (accessed 01 November 2023).
[17] D. Moussa, T. Japhet Kabalu, M. Hypolite Mavoko, T. Halidou, S. Esperanca, T. Maminata, V. Anifa, M. Salesio, K. Berenger, D. Edgard Diniba, E. Annette, D. Hamadoun, K. Mohamed, P. Mireia, G. Raquel, M. Clara, P.C.D. Thomas, S. Issaka, M. Petra, S. Henk, D. Umberto, K. Alessandro, Kassoum, Efficacy and safety of pyronaridine-artesunate (PYRAMAX) for the treatment of P. falciparum uncomplicated malaria in African pregnant women (PYRAPREG): study protocol for a phase 3, non-inferiority, randomised open-label clinical trial, BMJ Open 13 (10) (2023) e065295.
[18] W.E. Harrington, K.A. Moore, A.M. Min, M.E. Gilder, N.W. Tun, M.K. Paw, J. Wiladphaingern, S. Proux, K. Chotivanich, M.J. Rijken, N.J. White, F. Nosten, R. McGready, Falciparum but not vivax malaria increases the risk of hypertensive disorders of pregnancy in women followed prospectively from the first trimester, BMC Med. 19 (1) (2021) 98.
[19] A.K. Matthew, O.O.A. James, D. Bolanle, O.O. Stephen, Hamatological parameters and malaria parasite infection among pregnant women in Northwest Nigeria, Asian Pac. J. Trop. Dis. 3 (1) (2013) 47–50.
[20] V.N. Chandrashekar, K. Punnath, K.K. Dayanand, R.N. Achur, S.B. Kakkilaya, P. Jayadev, S.N. Kumari, D.C. Gowda, Malarial anemia among pregnant women in

the south-western coastal city of Mangaluru in India, Inform. Med. Unlocked 15 (2019) 100159.