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Quantification of the antimalarial drug pyronaridine in whole blood using LC–MS/MS — Increased sensitivity resulting from reduced non-specific binding

Daniel Blessborn[a,b,*], Karnrawee Kaewkhao[a], Lijiang Song[a,b,1], Nicholas J. White[a,b], Nicholas P.J. Day[a,b], Joel Tarning[a,b]

[a] Mahidol-Oxford Tropical Medicine Research Unit, Faculty of Tropical Medicine, Mahidol University, Bangkok, 10400, Thailand
[b] Centre for Tropical Medicine and Global Health, Nuffield Department of Clinical Medicine, University of Oxford, Oxford, UK

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ABSTRACT

Malaria is one of the most important parasitic diseases of man. The development of drug resistance in malaria parasites is an inevitable consequence of their widespread and often unregulated use. There is an urgent need for new and effective drugs. Pyronaridine is a known antimalarial drug that has received renewed interest as a partner drug in artemisinin-based combination therapy. To study its pharmacokinetic properties, particularly in field settings, it is necessary to develop and validate a robust, highly sensitive and accurate bioanalytical method for drug measurements in biological samples. We have developed a sensitive quantification method that covers a wide range of clinically relevant concentrations (1.5 ng/mL to 882 ng/mL) using a relatively low volume sample of 100 μL of whole blood. Total run time is 5 min and precision is within ±15% at all concentration levels. Pyronaridine was extracted on a weak cation exchange solid-phase column (SPE) and separated on a HALO RP amide fused-core column using a gradient mobile phase of acetonitrile–ammonium formate and acetonitrile-methanol. Detection was performed using electrospray ionization and tandem mass spectrometry (positive ion mode with selected reaction monitoring). The developed method is suitable for implementation in high-throughput routine drug analysis, and was used to quantify pyronaridine accurately for up to 42 days after a single oral dose in a drug-drug interaction study in healthy volunteers.

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1. Introduction

Malaria is one of the most important infectious diseases in the world, resulting in an estimated 214 million cases and 438,000 deaths in 2015 [1]. Children under the age of five bear the main burden of malaria-attributed deaths. Artemisinin-based combination therapy (ACT) is the World Health Organization recommended first-line treatment against *P. falciparum* malaria [2]. The fast-acting artemisinin component kills the majority of parasites during the first days of treatment. As, this class of drug has a short biological half-life [3,4], it is necessary to combine it with a long-acting partner drug to eliminate residual parasites and ensure cure.

Pyronaridine is a synthetic antimalarial drug, developed in the 1970s, which has been used as a mono-therapy in China. It has a long biological half-life (13–17 days) and is therefore suitable to use in ACTs [5–7]. The fixed-dose combination of artesunate and pyronaridine is a newly developed and deployed ACT, which has shown excellent efficacy against both *P. falciparum* and *P. vivax* malaria [8].

Sensitive and accurate bioanalytical methods are crucial for high-quality pharmacokinetic studies. There are a few methods published for the quantification of pyronaridine in plasma [9–12], and most of these require a sample volume of 200–250 μL of plasma and present a quantitation limit of 10 ng/mL or higher [11,12]. Hodel et al. reported a LC–MS/MS method able to quantify pyronaridine at 1 ng/mL [10], but the run time was reported to be over 20 min making it unsuitable for high throughput routine analysis of clinical studies. An in vivo study in rabbits showed a high blood-to-plasma distribution ratio (4.9-17.8) for pyronaridine [13], suggesting that whole blood rather than plasma would be the preferred sample matrix for drug measurements [6,14]. Several

* Corresponding author at: Mahidol-Oxford Tropical Medicine Research Unit, Faculty of Tropical Medicine, Mahidol University, Bangkok, 10400, Thailand.
E-mail address: danielb@tropmedres.ac (D. Blessborn).
[1] Current address: Department of Chemistry, University of Warwick, Coventry, UK.

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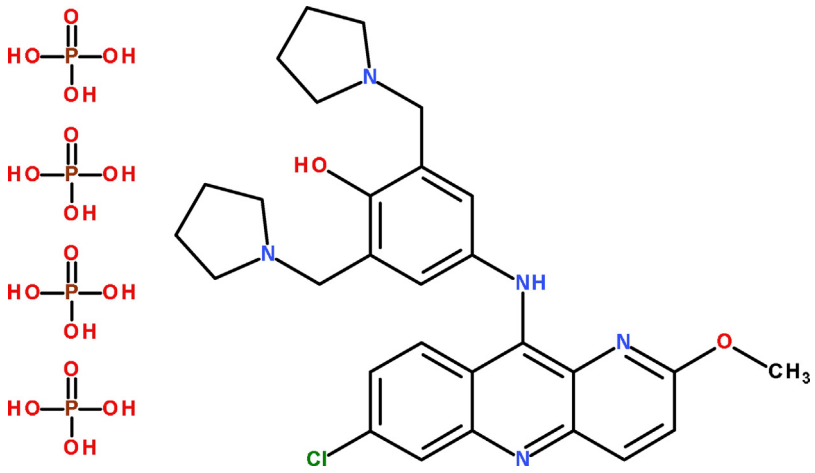

Fig. 1. Chemical structure of pyronaridine tetraphosphate.

quantification methods have been reported for whole blood, but with a relatively poor sensitivity (i.e. 20–30 ng/mL) [13,15,16]. A recent LC–MS/MS method [17] used a sample volume of 300 μL, resulting in a lower limit of quantification (LLOQ) of 5.7 ng/mL. However, liquid-liquid extraction was employed which is laborious and difficult to automate. Another crucial drawback in the previously reported LC–MS/MS methods is that none of them used stable isotope-labeled internal standards, and could therefore be compromised by matrix effects.

The aim of this work was to develop a sensitive and accurate quantification method of pyronaridine in low volume whole blood samples that is suitable for implementation into high-throughput routine drug quantification in clinical trials.

2. Materials and methods

2.1. Materials

Pyronaridine tetraphosphate (Fig. 1) and its stable isotope labeled internal standard, pyronaridine-$^{13}C_2D_4$ (SIL-PYN), was provided by the Worldwide Antimalarial Resistance Network Reference program [18]. LC–MS grade of water, acetonitrile and methanol were obtained from JT Baker (Phillipsburg, USA). All other chemicals and reagents were of analytical grade. Formic acid (98%), ammonium formate and ammonium carbonate were from Sigma–Aldrich (St. Louis, USA). Acetic acid was obtained from Merck (Darmstadt, Germany). Blank blood in fluoride-oxalate, EDTA, Na-heparin, Li-heparin, and fluoride-heparin was collected from healthy volunteers at the Faculty of Tropical Medicine, Mahidol University, Thailand.

2.2. Sample preparation

2.2.1. Preparation of standards and working solution

Stock solutions (1 mg/mL) of pyronaridine and SIL-PYN were prepared in methanol-formic acid 1% (50:50 v/v) and stored in methanol-washed cryo vials at −80 °C. Working solutions were prepared in human plasma-water (50:50 v/v) and used for spiking of whole blood. All solutions were allowed to equilibrate to room temperature before use.

2.2.2. Preparation of calibrators and quality control samples

Fresh fluoride-oxalate whole blood was used to prepare calibration standards at a concentration range of 1.47–882 ng/mL and LLOQ at 1.47 ng/mL, upper limit of quantification (ULOQ) at 882 ng/mL and over-curve samples (OC) at 1807 ng/mL. Quality control (QC) samples at 4.67, 57.2 and 452 ng/mL were prepared from a second stock solution. The final volume of working solution in whole blood was less than 5% in all samples.

2.2.3. Extraction procedure

Sample preparation and SPE was performed on a Freedom Evo 200 platform (TECAN, Mannedorf, Switzerland). Pipette tips, 96-well plates and seal mats were all methanol-washed before use. Whole blood samples (100 μL) were manually aliquoted to the 96-well plate. Then, by liquid handler, 100 μL plasma-water 50:50 v/v was added in the first well (i.e. double blank) and 100 μL of internal standard (30 ng/mL) in plasma-water 50:50 v/v was added to all other wells. Four hundred microliters of ammonium carbonate (20 mM, pH 8.8) was added to all wells and then covered with a seal mat. The 96-well plate was mixed for 2 min at 1000 rpm and centrifuged for 2 min at 1100 × g. Then 500 μL of the centrifuged sample was transferred to a 96 well plate solid phase CBA extraction plate (Biotage, Uppsala, Sweden) and extracted according to the following procedure; (1) conditioning of the SPE cartridge with 1 mL methanol followed by 1 mL ammonium carbonate (20 mM, pH 8.8), (2) loading of the whole blood sample (500 μL), (3) washing with 1 mL ammonium carbonate (20 mM, pH 8.8) followed by 1.5 mL methanol-ammonium carbonate 20 mM (80:20 v/v) and finally 1 mL methanol-water (50:50 v/v), (4) elution with 0.9 mL methanol-formic acid (98:2 v/v). The eluates were evaporated under a gentle stream of nitrogen at 70 °C (Caliper TurboVap® 96) until dryness and reconstituted in 400 μL mobile phase A (see below section 2.3.1). The extracted and reconstituted samples were mixed for 2 min at 1000 rpm, centrifuged for 2 min at 1100 × g and placed in the autosampler to equilibrate for at least 30 min before analysis.

2.3. Instrumentation and chromatographic conditions

2.3.1. Chromatography

The LC system was an Agilent 1260 infinity system consisting of a binary LC pump, a vacuum degasser, a temperature-controlled micro-well plate autosampler set at 4 °C and a temperature-controlled column compartment set at 40 °C (Agilent technologies, CA, USA). Data acquisition and processing were performed using Analyst 1.5.2 (Applied Biosystems/MDS Sciex, CA, USA). The compounds were analyzed on a Halo amide fused-core column (50 mm × 2.1 mm; I.D. 2.7 μm) with a Halo amide pre-column (5 mm × 2.1 mm; I.D. 2.7 μm) at a flow rate of 400 μL/min (Advanced Materials Technology, Wilmington, DE, USA). The mobile phase consisted of solvent A: acetonitrile–ammonium for-

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