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Semi-quantitative measurement of the antimalarial lumefantrine from untreated dried blood spots using LC–MS/MS

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ABSTRACT

Study of the clinical effects of combination therapy for malaria is aided by the ability to measure concentrations of individual partner drugs. Existing methods for measurement of the antimalarial drug lumefantrine (LF) in dried blood spots (DBS) on filter paper rely on chemical pretreatment of the paper to facilitate drug elution. However, in the absence of pretreatment, DBS may still offer some utility for semi-quantitative measurements and pharmacokinetic–pharmacodynamic (PK–PD) analyses. We present a method for semi-quantitation of LF in DBS on untreated filter paper using liquid chromatography tandem mass spectrometry. Optimal recovery was achieved by extraction with acetone-water-formic acid (90:5:5). The range of quantitation was 100–20,000 ng/ml. Mean intra- and inter-day accuracy values were 86.6% (coefficient of variation [CV]: 10.1%) and 91.8% (CV: 16.1%), therefore we propose the assay as semi-quantitative. Clinical application was demonstrated in exploratory PK–PD analyses of a drug efficacy trial of artemether-lumefantrine in children with uncomplicated falciparum malaria using post-treatment day 7 samples, parasite clearance times estimated from serial blood smears, and recurrence of malaria out to 35 days. The median day 7 concentration among children (n = 71) was 111 ng/ml (interquartile range: 100–194 ng/ml). We used a truncated calibration curve of 100–5000 ng/ml for calculations due to low observed concentrations. Calculations using the full calibration curve yielded similar values (+1% avg. deviation). Controlling for participant age, sex, and parasite burden, each log increase in LF day 7 concentration corresponded to a decrease of 7.1 h in mean parasite clearance time (95% confidence interval: 0.1–14.3 h, P = 0.05). A nested case-control study of participants (n = 18) with and without recurrent malaria showed mean post-treatment day 7 concentrations of 181 ng/ml and 235 ng/ml, respectively, but the difference was not significant ($P = 0.64$). A method for semi-quantitation of LF from post-treatment day 7 collections of DBS on untreated filter paper demonstrated clinical application in exploratory PK–PD analyses of parasite clearance and reinfection. Use of DBS will endure in certain study settings by virtue of their ease of collection and resilience. Their utility should continue to be explored as our instruments gain in sensitivity and as clinical pharmacology inquiries are pursued to the field.

1. Introduction

Malaria remains endemic in 91 countries and territories, infecting over 200 million people and causing nearly a half-million deaths in 2016, mainly due to infection with *Plasmodium falciparum* in young children in sub-Saharan Africa [1]. Control and elimination of malaria are achieved primarily through vector control with indoor residual spraying and distribution of insecticide-treated bed nets, and case management with artemisinin-based combination therapies (ACTs) [2]. In the absence of a sufficiently effective vaccine, drugs remain a keystone in control and elimination programs. Evaluations of the pharmacokinetic (PK) and pharmacodynamic (PD) characteristics of ACTs are aided by the ability to determine concen-

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trations of the individual constituent drugs in treated individuals. In the resource-limited settings where malaria is prevalent, solutions that eschew reliance on electricity, multistep processing, and cold-chain transport are desirable. Whole blood collected as dried blood spots (DBS) on filter paper offers a means of producing resilient field samples.

Among current ACTs, artemether-lumefantrine (AL) is the most widely deployed. Typical peak concentrations of lumefantrine (LF) seen clinically range from 4000–9000 ng/ml and median day 7 concentrations are reported between 216 and 528 ng/ml [3–7], with lower concentrations observed in younger children and in assays of DBS matrices on paper [5,6]. The terminal elimination half-life of LF is 3–4 days, and prior PK–PD studies estimated a threshold day 7 concentration of LF for protection against re-infection ranging from 175 to 280 ng/ml [4,8,9].

Highly hydrophobic, LF adsorbs to filter paper used in DBS collections; existing methods of extraction from DBS therefore rely on pretreatment of the collection card with tartaric or phosphoric acid to promote drug elution [10,11]. However, determinations of LF using DBS on filter paper may provide useful PK data even if pretreatment was not done. Therefore, as annex to a therapeutic efficacy trial of AL conducted in rural Zambia from 2014 to 2015, we developed a method for LF determinations from DBS on untreated filter paper and applied it for a pilot PK–PD analysis of malaria parasite clearance and reinfection following standard AL therapy.

2. Materials and methods

2.1. Chemicals and materials

LF was acquired from AK Scientific, Inc. (California, USA) with a production date of September 2, 2013. The deuterated LF (LF-D_9) internal standard was provided as a gift from Novartis Pharma. Co. (New Jersey, USA), prepared in 50% acetonitrile and 0.5% formic acid on May 25, 2010. Acetone, acetonitrile, high performance liquid chromatography (HPLC) grade water, and ammonium formate were purchased from Fisher Scientific (New Jersey, USA) and ethyl acetate, formic acid, and Whatman 903 protein saver cards from Sigma-Aldrich (Missouri, USA). All solvents were HPLC grade, and chemicals were American Chemical Society (ACS) reagents. Human whole blood with ethylene-diamine-tetra-acetic acid (EDTA) was purchased from Biological Specialty, Co. (Colmar, Pennsylvania, USA).

2.2. Calibration curve standards and quality control samples

Calibration curve standards and quality control samples were prepared in 3 batches at approximately 3 months, 2 months, and 1–3 days prior to assay. Standards of 100, 200, 500, 1000, 5000, 10,000 and 20,000 ng/ml were prepared from spiked whole blood applied in 25 or 50 μl aliquots to Whatman 903 protein saver filter paper with drying times of 3–18 h. Quality controls of 300, 3000 and 17,000 ng/ml were similarly prepared. All samples were kept in clear plastic storage bags with desiccant. The 3- and 2-month-old samples were stored at −80 °C and then ambient temperature for 1–3 weeks, and the 1–3-day-old samples were kept at ambient temperature.

2.3. Clinical specimens

We obtained DBS from patients enrolled in a therapeutic efficacy trial of AL in Zambia, collected 7 days after the first treatment dose. Consent was obtained from parents or legal guardians of participating children according to an Institutional Review Board-approved protocol. Trial results will be published in a separate manuscript. Briefly, 100 children aged 6–59 months with uncomplicated *Plasmodium falciparum* malaria were treated with the standard 6-dose regimen of AL dosed according to weight. Children were enrolled over the 8-month period December 2014 to July 2015. Indoor residual spraying of participants' households was prevalent and bed net use was common (98% and 87%). Finger prick capillary blood was spotted onto Whatman 903 protein saver filter paper and allowed to dry at ambient temperature and humidity for 3–4 h. Blood was spotted in volumes of approximately 25–50 μl per spot at intervals of 6 h for 48 h following start of treatment then weekly for 5 w. They were stored with desiccant at −20 °C until July 2016, at which time they were transported with desiccant at ambient temperature to the laboratory and placed in −80 °C storage after approximately four weeks outside of frozen storage. Assays were done using the 3-month-old calibrators and quality controls except where noted.

2.4. Extraction procedure

A 3 mm paper punch was made from the DBS using a handheld 3 mm round hole puncher (Fiskars Brands, Inc., Middleton, WI, USA). The punch and 10 μl of LF-D_9 (100 ng/ml) were added to a 1.5 ml Eppendorf tube. The mixture was extracted with different combinations of water and organic solvents: 50% acetonitrile with 0.5% formic acid, acetonitrile with 5% formic acid, 50% acetone with 5% $ZnSO_4$ and 5% formic acid, ethyl acetate with 5% formic acid, methanol with 5% formic acid, acetone (50%, 90% and 100%) with 5% formic acid, and methoxyethoxy ethanol with 5% formic acid. Rotation (5–30 min) was compared to vortex mixing (5–30 min), with and without sonication (3 min). All samples were centrifuged for 3 min at 25,000 × *g*. The liquid phase was transferred in volumes of 20–40 μl to autosampler vials for liquid chromatography tandem mass spectrometry (LC–MS/MS).

2.5. Analytical procedure

Instrument and chromatographic conditions were adopted from a published method for plasma samples [12]. Briefly, the LC–MS/MS instrumentation comprised twin PE 200 micro-LC pumps and PE autosampler (Perkin-Elmer, Connecticut, USA) and the API 2000 triple quadrupole MS system (AB Sciex, Ontario, Canada). A Zorbax C_8 column (50 × 2.1 mm, 5 μm; Agilent Technologies Inc., California, USA) was used for chromatographic separation. Solvent A was aqueous ammonium formate 10 mM, pH 4.0, and solvent B was acetonitrile with 0.1% formic acid. Elution by gradient LC was done according to the schedule 0–1 min: 50% solvent B, 1–4 min: 50–100% solvent B, 4–6 min: 100% solvent B, 6–6.1 min: 100–50% solvent B, 6.1–8 min: 50% solvent B. Injection volumes were 10 μl and the flow rate was 0.4 ml/min with LF retention times of 3.5 min. The precursor-product ion pairs were mass-to-charge ratios (m/z) of 528 → 510 for LF and m/z 537 → 519 for the internal standard. Chromatographic data were analyzed using Analyst 1.6.2 (Danaher Co., Washington, DC, USA).

2.6. Method validation and assessments

Validation was performed by evaluating back-calculated concentrations of the calibrator (standard curve) samples, intra- and inter-day precision and accuracy of control samples, matrix effect, and extraction recovery. The calibration curve was validated in three runs with seven concentrations over several days at ambient temperature. To assess storage recovery, we compared control samples stored for 1 day, 6 days, and 3 months to freshly prepared samples. We assessed for variation due to differences in weight among punches by examining weight as a covariate in quantitation calculations and by comparing different punches of the same or similar weight to those of different weights. We assessed for differ-