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Journal of Pharmaceutical and Biomedical Analysis 41 (2006) 213–218
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
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Development and validation of an automated solid phase extraction and liquid chromatographic method for the determination of piperaquine in urine

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Received 6 August 2005; received in revised form 20 October 2005; accepted 20 October 2005
Available online 28 November 2005

Abstract

A sensitive and specific bioanalytical method for determination of piperaquine in urine by automated solid-phase extraction (SPE) and liquid chromatography (LC) has been developed and validated. Buffered urine samples (containing internal standard) were loaded onto mixed phase (cation-exchange and octylsilica) SPE columns using an ASPEC XL SPE robot. Chromatographic separation was achieved on a Chromolith Performance RP-18e (100 mm × 4.6 mm I.D.) LC column with phosphate buffer (pH 2.5; 0.1 mol/L)–acetonitrile (92:8, v/v). Piperaquine was analysed at a flow rate of 3 mL/min with UV detection at 347 nm. A linear regression model on log–log transformed data was used for quantification. Within-day precision for piperaquine was 1.3% at 5000 ng/mL and 6.6% at 50 ng/mL. Between-day precision for piperaquine was 3.7% at 5000 ng/mL and 7.2% at 50 ng/mL. Total-assay precision for piperaquine over 4 days using five replicates each day ($n = 20$) was 4.0%, 5.2% and 9.8% at 5000, 500 and 50 ng/mL, respectively. The lower limit of quantification (LLOQ) was set to 3 ng/mL using 1 mL of urine, which could be lowered to 0.33 ng/mL when using 9 mL of urine and an increased injection volume.

Keywords: Piperaquine; Liquid chromatography; Solid-phase extraction; Validation; Method development; Urine

1. Introduction

Piperaquine (PQ), 1,3-bis-[4-(7-chloroquinolyl-4)-piperazinyl-1]-propane, is an antimalarial compound belonging to the 4-aminoquinolines (Fig. 1). Produced by the Shanghai Research Institute of Pharmaceutical Industry in 1966, it has a chemical structure identical to compound 13228 RP earlier synthesized by Rhone Poulence, France. PQ replaced chloroquine as first-line monotherapy in southern China during the 1980s until resistance emerged [1–5]. PQ has recently received renewed interest as a suitable partner to artemisinin derivatives in artemisinin-based combination theraphy (ACT) treatment of malaria. Artekin®, a combination of PQ and dihydroartemisinin (DHA) registered in China and Cambodia, has over the last couple of years shown good efficacy in the treatment of uncomplicated *P. falciparum* malaria [6–9]. Although PQ has been used for decades, published preclinical and pharmacokinetic data are scarce. To the best of our knowledge, only two pharmacokinetic studies have been published [10,11]. The studies suggest a long terminal half-life ($t_{1/2} = 20$–23 days) both in healthy volunteers and patients receiving Artekin®. Only three of the adult patients studied ($n = 38$) had quantifiable plasma concentrations at the last sampling point 35 days after the last dose [10]. The relatively high variation at the lower limit of quantification (between-day variation at LLOQ > 20%) combined with a small number of subjects might incorporate an uncertainty when estimating the terminal elimination phase [10]. The healthy volunteers display a similar terminal elimination half-life of 20–21 days when followed for 42 days [11]. The long terminal elimination

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doi:10.1016/j.jpba.2005.10.027

half-life combined with sensitivity challenges for published methods limit the characterisation of PQ pharmacokinetics [10–15]. To date only five assays have been published for the determination of PQ in biological samples but none of them permits quantification in urine. Three of these assays quantify PQ in plasma, one quantifies PQ in whole blood and one quantifies PQ in capillary blood applied onto sampling paper [12–16]. A urine assay with non-invasive sampling and thus unlimited sample volumes would theoretically lead to improved assay sensitivity. Therefore it may allow quantification of PQ for a longer duration of time after administration enabling a more accurate evaluation of the terminal elimination phase compared to published studies based on quantification in plasma [10,11]. The assay for quantification of PQ in urine was validated according to international guidelines [17]. The assay utilises mixed phase solid-phase extraction (SPE) columns, which include strong cation binding properties (sulphonic acid) and lipophilic binding properties (octylsilica). This offers an excellent retention mechanism when extracting a drug in a matrix containing an excess of salts. Salt competes with the drug for the ionic binding sites and could cause a partial loss of drug in the absence of a lipophilic binding site. The ability of binding through both liphophilic and ionic interactions increases the recovery of PQ from the urine matrix compared to a traditional octylsilica or cation column. The aim of this work was to develop a sensitive method suitable for quantification of PQ in urine.

2. Experimental

2.1. Chemicals

PQ was obtained from Guangzhou University of Traditional Chinese Medicine (Guangzhou, China) (Fig. 1). The internal standard (IS), 3-methyl-4-(3-hydroxy-4-diethylaminopropyl)-7-chloroquinoline, was obtained from Glaxo Wellcome (Hertfordshire, UK) (Fig. 1). Acetonitrile (HPLC-grade), methanol (pro analysis) and HPLC-water were obtained from JT Baker (Phillipsburg, USA). Triethylamine (HiPerSolv for HPLC) was obtained from BDH (Poole, UK). The phosphate buffer solutions were prepared by mixing appropriate amounts of sodium hydroxide and *ortho*-phosphoric acid (Merck, Darmstadt, Germany) with HPLC water (JT Baker, Phillipsburg, USA).

2.2. Solid-phase extraction

Extraction was carried out on an automated ASPEC XL SPE robot (Gilson Inc., Middletown, USA) using MPC (mixed phase strong cation-exchange and octylsilica) columns (4 mm, 1 mL) (3 M Empore, 3 M Centre, St. Paul, USA). The ASPEC system uses a positive air pressure instead of vacuum to force the liquid through the column.

2.3. LC instrumentation and chromatographic conditions

The LC system used was a LaChrom Elite system consisting of a L2130 LC pump, a L2200 injector, a L2300 column oven set at 25 °C and a L2450 DAD detector (Hitachi, Tokyo, Japan). The detector was set at 347 nm and data acquisition was performed using LaChrom Elite software (VWR International, Darmstadt, Germany). The chromatographic separation was achieved on a Chromolith Performance RP-18e (100 mm × 4.6 mm I.D.) column (VWR International, Darmstadt, Germany) protected by a Chromolith Guard Cartridge RP-18e (10 mm × 4.6 mm I.D.) (VWR International, Darmstadt, Germany) using a mobile phase containing phosphate buffer (pH 2.5; 0.1 mol/L)–acetonitrile (92:8, v/v) at a flow rate of 3 mL/min.

2.4. Preparation of standards and quality control (QC) samples

A concentrated stock solution of PQ (1 mg/mL) was prepared in phosphoric acid (0.05 mol/L). Working solutions of PQ ranging from 0.45 to 500 μg/mL were prepared by serial dilution of the stock solution in phosphoric acid (0.05 mol/L). Working solution of PQ (0.2 mL) was added to blank urine (9.8 mL) to yield spiked calibration standards at six different concentrations ranging from 9 to 10,000 ng/mL (not including blank urine). The calibration standards were prepared in batches of 10 mL, divided into aliquots and stored at −80 °C until analysis. Quality control (QC) samples for determination of accuracy and precision in urine were prepared at three concentrations (50, 500 and 5000 ng/mL) in batches of 80 mL and divided into 1 mL aliquots. The aliquots were stored at −80 °C until analysis. These samples (i.e. 50 and 5000 ng/mL) were also used to evaluate long-term, short-term and freeze/thaw stability.

2.5. Sample preparation

Urine samples (1 mL) were thawed for 60 min and diluted with an equal volume of phosphate buffer (pH 2.15; 0.05 mol/L) containing IS (1 μmol/L). The micro tubes were placed on a vortex mixer for approximately 10 s, left undisturbed for 5 min and centrifuged at 19,870 × *g* for a few seconds. After centrifugation, the supernatants were decanted into new tubes and diluted with 2 mL of phosphate buffer (pH 2.15; 0.05 mol/L).

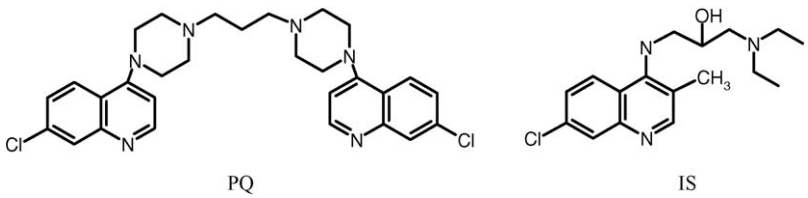

Fig. 1. Structure of piperaquine (PQ) and 3-methyl-4-(3-hydroxy-4-diethylaminopropyl)-7-chloroquinoline (IS).

Table 1
ASPEC SPE procedure for extraction of PQ in urine

SPE step	Liquid dispensed	Dispensing volume (mL)	Dispensing flow (mL/min)	Pressuring air volume (mL)
Condition	Methanol	0.5	3.0	–
	Phosphate buffer (pH 2.15; 0.05 mol/L)	0.3	3.0	–
Sample load	Buffered urine	4.0	1.5	1.1
Wash	HPLC water	0.3	2.0	1.6
	Phosphate buffer (pH 2.15; 0.05 mol/L)–methanol (20:80, v/v)	0.5	1.0	1.6
Elute	Methanol–triethylamine (98:2, v/v)	0.7	1.0	0.7

An ASPEC XL SPE system was used to load the samples onto SPE columns and extraction was achieved according to Table 1. The SPE eluates were evaporated to dryness at 70 °C under a gentle stream of air and reconstituted in 100 μL phosphate buffer (pH 2.5; 0.1 mol/L)–acetonitrile (95:5, v/v). Reconstituted SPE eluates were placed on a vortex mixer for approximately 20 s and transferred to silanised glass inserts (La-Pha-Pak, National Scientific, UK). Depending on the sensitivity requirements, a volume of 15 or 50 μL was injected into the LC system.

2.6. Method validation

2.6.1. Accuracy, precision and recovery

The accuracy and precision of the method was evaluated by analysis of five replicates of spiked urine (50, 500 and 5000 ng/mL) over 4 days. Concentrations were predicted using a calibration curve prepared on the same day as the QC samples and stored at −80 °C until analysis. Calibration curves were constructed using peak–height ratio (PQ/IS) against the corresponding concentration for six spiked urine standards, ranging from 9 to 10,000 ng/mL. Log–log transformed linear regression of peak–height ratio (PQ/IS) against PQ concentration was used for quantification. Accuracy and precision when using 1, 3 and 9 mL of urine was validated by analysing five replicates of spiked urine at 50, 17 and 6 ng/mL, respectively. The extraction recovery was determined over 4 days using the same QC samples as used for precision and accuracy. Duplicates of PQ in reconstitution solvent (corresponding to nominal QC concentrations; 50, 500, and 5000 ng/mL) were directly injected into the LC system. The PQ peak height responses of the direct injections were used to construct a calibration curve and to predict PQ concentrations. The predicted concentrations of extracted QC samples were compared to back-calculated concentrations of direct injections to determine recovery.

2.6.2. Lower limit of quantification and limit of detection

The lower limit of quantification (LLOQ) was selected at the lowest concentration at which the assay precision and accuracy was within 20% [17]. LLOQ was validated using five replicates of 1 mL urine (15 and 50 μL injection volume) and five replicates of 9 mL urine (50 μL injection volume). The limit of detection (LOD) was determined at the concentration at which the signal-to-noise ratio exceeded 3:1.

2.6.3. Stability

Short-term stability of PQ (50 and 5000 ng/mL) in urine, urine–phosphate buffer (SPE loading matrix) and in reconstitution solvent (auto-sampler condition) was evaluated during 24 h at room temperature. Freeze/thaw stability was evaluated during three freeze/thaw cycles at 50 and 5000 ng/mL. Long-term stability of PQ in urine (50 and 5000 ng/mL) was evaluated at 8 °C over 14 days, −17 and −80 °C for up to 120 days. A freshly prepared calibration curve on the day of analysis was used to predict concentrations. The analyte was considered stable if more than 85% of the initial value at day zero was recovered. [17].

2.6.4. Selectivity

Blank urine samples from six different healthy volunteers were analysed, and the chromatograms examined for endogenous compounds that could interfere at the retention time of PQ.

2.7. Clinical applicability

This method was used to analyse PQ and possible metabolites in clinical urine samples. One healthy Caucasian male volunteer received a single oral dose of Artekin® (three tablets each containing 320 mg PQ phosphate and 40 mg DHA) together with a fatty meal, in order to facilitate the absorption of PQ [11,18]. Urine was collected during pre-specified time intervals up to 123 days after administration and stored at −80 °C until analysis. Triplicates of QC samples at three levels were analysed in the analytical run to ensure satisfactory method performance in accordance with guidelines for routine drug analysis [17].

3. Results and discussion

Urine sampling has the advantage of almost unlimited sample volumes compared to using plasma or blood in clinical studies. The collection of urine can be performed by the patient in home environment without supervision and will therefore facilitate sampling in rural areas. Collecting large volumes of urine (i.e. 24 h sampling) will not entail increased suffering for the patient. Thus, the use of urine and large sample volumes when quantifying PQ is both ethically and practically justified. The urine dilution factor and SPE breakthrough of urine load was investigated prior to validation to evaluate the use of larger volumes and more concentrated urine samples. The internal standard compensates fully for any loss of PQ at higher urine load and the

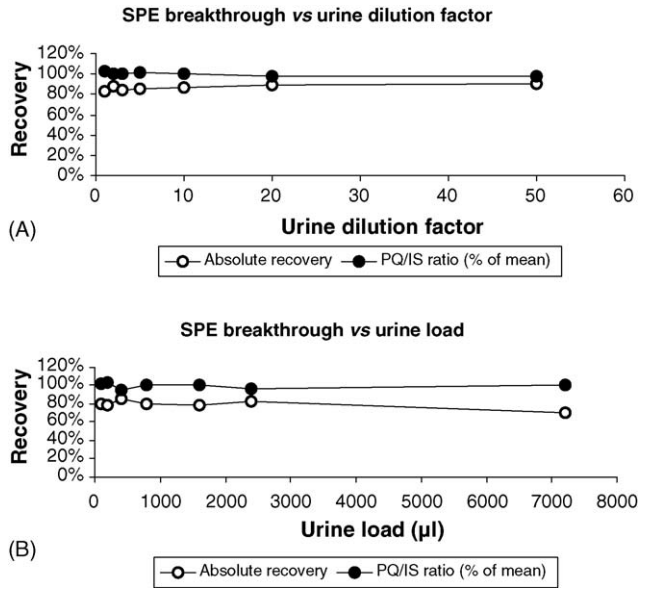

Fig. 2. Effect of urine dilution factor (B) and urine load SPE breakthrough (A) in spiked urine ($n = 2$).

assay is more or less independent upon dilution factor, which can be seen in Fig. 2. Thus, to improve sensitivity, up to 9 mL urine can be used in the assay. The mixed phase SPE columns with strong cation binding properties mixed with an octylsilica part of lipophilic character facilitate a high recovery even when using a matrix with large amounts of salts.

3.1. Method validation

3.1.1. Accuracy, precision and recovery

Based on overall performance and simplicity, log–log transformed linear regression without weighting was considered the most appropriate model [19]. The accuracy, precision and recovery of the assay are summarized in Table 2. The within-day, between-day and total-assay precision was well within stipulated limits according to FDA guidelines [17]. Total-assay precision over all four days ($n = 20$) was 3.9%, 5.6% and 9.2% at 5000, 500 and 50 ng/mL, respectively. Five replicates of 1, 3 and 9 mL of spiked urine at 50, 17 and 6 ng/mL, respectively, was also analysed to validate the precision and accuracy within and between different urine volumes at low PQ concentrations. The use of 1, 3 or 9 mL of urine did not influence the precision or the accuracy of the method. A precision (i.e. relative standard deviation (RSD)) of 2.4%, 4.3% and 6.1% and accuracy (i.e. deviation from nominal value) of 4.2%, 2.6% and 3.1% were achieved using 1, 3 and 9 mL, respectively. The recovery was reproducible over 4 days and independent upon concentration (86% mean recovery).

Table 3
Lower limit of quantification (LLOQ) of the assay ($n = 5$)

	9 ng/mL	3 ng/mL	0.33 ng/mL
Urine sample volume (mL)	1.0	1.0	9.0
Injection volume (µL)	15.0	50.0	50.0
Precision (%)	4.8	5.6	10.0
Accuracy (%)	1.0	1.5	0.9
LOD (ng/mL)	4.5	1.5	0.15

3.1.2. Lower limit of quantification and lower limit of detection

The LLOQ of the assay is summarized in Table 3. The validation protocol generated a LLOQ of 9 ng/mL (1 mL of urine; 15 µL injection), which could be lowered to 0.33 ng/mL when using 9 mL of urine and 50 µL injection. The precision and accuracy was well within stipulated requirements (i.e. <20%) (17). At LOD the signal-to-noise ratio was greater than 3:1 in accordance with international guidelines (17).

3.1.3. Stability

PQ has a good bench top stability in elution solvent (methanol–triethylamine, 98:2, v/v) for up to 24 h [12]. The stock solution is stable for at least 50 days when stored at 4 °C protected from light [13]. PQ was found stable in urine for at least three freeze/thaw cycles, which is in accordance with previous reports using other matrices [12,14–15]. The mean differences between initial concentration and that after three cycles were 1% and 9% at 50 ng/mL ($n = 3$) and 5000 ng/mL ($n = 3$), respectively. QC samples were found to be stable for at least 14 days when stored at 8 °C and for at least 120 days when stored at −17 and −80 °C. PQ was found to be stable in urine, in reconstitution solvent (auto-sampler) and in buffered urine (SPE conditions) at ambient temperature for at least 24 h.

3.1.4. Selectivity

Analysed blank urine shows no interfering peaks at the retention time of PQ. Other commonly used antimalarials and their metabolites (i.e. amodiaquine, monodesethyl-amodiaqine, atovaquone, chloroquine, desethyl-chloroquine, mefloquine, carboxy-mefloquine, proguanil, 4-chlorophenylbiguanid (proguanil metabolite), cycloguanil (proguanil metabolite), primaquine, pyrimethamine, pyronaridine, sulfadoxine, and trimethoprim) do not interfere with the quantification of PQ with the current LC-settings [12].

Table 2
Validation performances

	QC 1 (50 ng/ml)	QC 2 (500 ng/ml)	QC 3 (5000 ng/ml)
Predicted concentration (mean)	49.9	535	5510
Within-day precision (%), $n = 20$	5.9	5.3	1.4
Between-day precision (%), $n = 4$	8.9	5.1	4.3
Total-assay precision (%)	9.2	5.6	3.9
Accuracy (%)	0.3	7.0	10.14
Recovery (mean %)	88	80	89

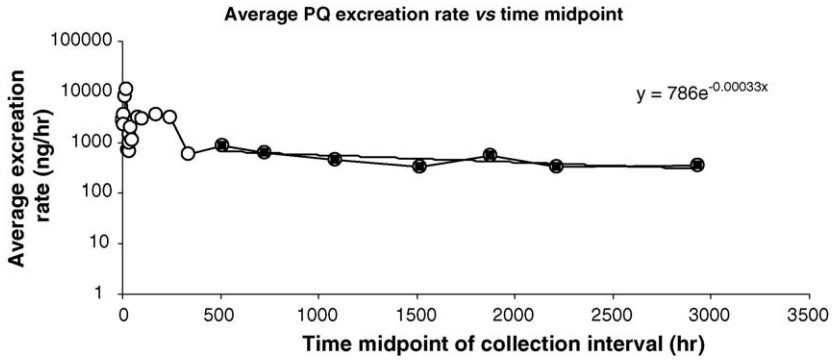

Fig. 3. Piperaquine urinary excretion rate vs. time (midpoint of collection interval) in one healthy Caucasian male volunteer after a single oral dose of Artekin® (three tablets each containing 320 mg piperaquine phosphate and 40 mg dihydroartemisinin) together with a fatty meal.

3.2. Clinical applicability

The total precision for all quality controls ($n = 3$ at each level) during the analysis of PQ in urine was within the limits (i.e. <15%) for routine drug analysis [17]. Urine samples up to 123 days after administration were analysed and evaluated to estimate the duration of detectable PQ in urine and the renal pharmacokinetics of PQ. Assuming that renal clearance is constant, the urinary excretion rate is proportional to plasma concentration [20]. A rough estimate of the biological half-life was obtained by regressing the exponential decline of excretion rates against the midpoint of the collection intervals (Fig. 3). The results suggest a terminal elimination half-life of 88 days (based on the seven last data points), which is several times longer than previously reported in plasma ($t_{1/2} = 20$–23 days) [10,11]. Recent findings in a healthy volunteer shows that the terminal elimination half-life in plasma could be as long as 80 days after a single oral dose of Artekin [21]. The present findings in urine support the supposition that the terminal elimination half-life might be underestimated. The presented data clearly demonstrates the possibility of following PQ for a longer period of time after administration compared to published studies [10,11,18]. The assay is not only a valuable tool for pharmacokinetic studies of PQ but can be of benefit when studying the development of resistance. For chloroquine (CQ), it has been proposed to determine the levels of CQ and its metabolite desethylchloroquine (DECQ) on the day of treatment failure to determine if treatment failure is due to recrudescence and emerging resistance to *P. vivax*. A minimum effective concentration (MEC) of 100 ng/mL (CQ + DECQ) in blood has been proposed to be an effective cut-off level to differentiate resistance from relapse [22–24]. For PQ, this has not yet been evaluated because of a high cure rate but will maybe be necessary in the future. The use of the presented assay could then be helpful in determining drug pressure in relapsing patients and monitoring of resistance. The analysis of clinical urine samples also disclosed large amounts of two unidentified peaks, probably metabolites using the reported assay settings. Pooled 24-h urine collected 5 days after administration suggests considerable amounts of both metabolites in urine (Chromatogram A; Fig. 4). The most polar of these metabolites (M1) is present in larger amounts than PQ and could be seen in all samples up to 123 days after administration (Chromatogram B; Fig. 4). This indicates the presence of metabolite(s) in the body for more than 123 days. The presence of M1 in plasma was confirmed during the validation of a new high-throughput method for analysing PQ in plasma, where it could be seen 7 days after administration in malaria patient samples [16]. Further studies will reveal if M1 can be correlated to PQ and used as a surrogate marker for PQ levels. Work is presently in progress to identify and characterize the metabolites.

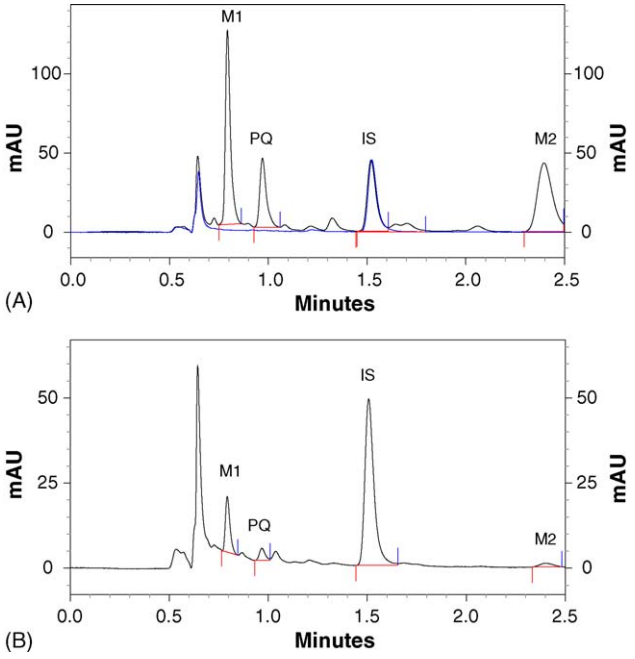

Fig. 4. Chromatograms of urine samples from one healthy Caucasian male volunteer after a single oral dose of Artekin® (three tablets each containing 320 mg PQ phosphate and 40 mg DHA) together with a fatty meal; (A) pooled 24 h urine sample collected 5 days after administration with day zero urine overlay and; (B) pooled 24 h urine sample collected 123 days after administration.

4. Conclusion

The developed assay presents a sensitive, reproducible, non-invasive and safe (patient and clinician) alternative to quantify PQ in the body. PQ remains stable during all method steps in

the assay. Urine from clinical studies can be stored temporarily at 8 °C but should be stored at −17 or −80 °C as soon as possible after collection and until analysis to avoid degradation. The present assay could also be an important tool for pharmacokinetic evaluation of combined urine and plasma data in clinical studies or to evaluate patient relapses with respect to drug resistance.

Acknowledgements

This study was part of the Wellcome Trust Mahidol University Oxford Tropical Medicine Research Programme funded by the Wellcome Trust of Great Britain. This research was also partly supported by the Swedish International Development Cooperation Agency (SIDA). This investigation received financial support from the Medicines for Malaria Venture.

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