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High throughput assay for the determination of piperaquine in plasma

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Abstract

A high throughput assay for the determination of the antimalarial piperaquine in plasma has been developed and validated. The assay utilises 96-wellplate formats throughout the whole procedure, and easily enables a throughput of 192 samples a day using a single LC system. Buffer (pH 2.0; 0.05 M) containing internal standard was added to 0.25 mL plasma in a 96-wellplate (2 mL wells). The samples were extracted on a MPC solid phase extraction deep well 96-wellplate (3 M Empore). Piperaquine and internal standard were analysed by liquid chromatography with UV detection on a Chromolith Performance (100 mm × 4.6 mm) column with a mobile phase containing acetonitrile–phosphate buffer (pH 2.5; 0.1 M) (8:92, v/v) at a flow rate of 3.0 mL/min. The within-day precisions for piperaquine were 3.3 and 2.3% at 40 and 1250 ng/mL, respectively. The between-day precisions for piperaquine were 5.8 and 1.3% at 40 and 1250 ng/mL, respectively. The total assay precisions using 29 replicates over 5 days were 6.7, 4.5 and 2.7% at 40, 200 and 1250 ng/mL, respectively. The lower limit of quantification (LLOQ) and the limit of detection (LOD) were 10 and 5 ng/mL, respectively using 0.25 mL plasma. Using 1 mL of plasma, it was possible to decrease LLOQ and LOD to 2.5 and 1.25 ng/mL, respectively.

Keywords: Antimalarial; High throughput; Liquid chromatography; Piperaquine; Solid phase extraction; 96-Well

1. Introduction

Malaria, caused by the mosquito-borne protozoan parasite *Plasmodium falciparum*, is the most important parasitic disease of man, infecting between 300 and 400 million people annually, and killing more people each year than any other infectious disease except AIDS and tuberculosis. Between 1 and 3 million die, mostly children younger than 5 years, and the majority of them in Africa [1,2]. Piperaquine (PQ), 1,3-bis-[4-(7-chloroquinolyl-4)-piperazinyl-1]-propane, is a bisquinoline antimalarial compound belonging to the 4-aminoquinoline group. PQ was first synthesized at Rhône-Poulenc in France in the 1950s as compound RP13228 but was not taken further into man. Shanghai Research Institute of Pharmaceutical Industry rediscovered PQ in the 1960s and in 1970s PQ rapidly replaced chloroquine as first-line mono-therapy in southern China [3]. It was deployed in large quantities in China until the 1980s when resistance emerged. PQ has recently received renewed interest as it has proved to be a suitable partner drug in artemisinin combined therapy (ACT) for the treatment of uncomplicated falciparum malaria. A combination of PQ and dihydroartemisinin (DHA) (Artekin®) has been used for a couple of years in China, Vietnam and Thailand with good results [4–9]. Recently, Holleykin, Sigma-Tau, Oxford University and the Medicines for Malaria Venture (MMV) have signed a joint agreement to develop and register this combination for worldwide use [10].

Although PQ has been used for decades in PR China, published pharmacokinetic data are still scarce. Only one population pharmacokinetic study has been published to date [7]. Likewise, very few methods that permit determination of PQ in biological fluids have been published. Two methods have been validated for the determination in plasma, one method for the determination in venous whole blood, and one for the

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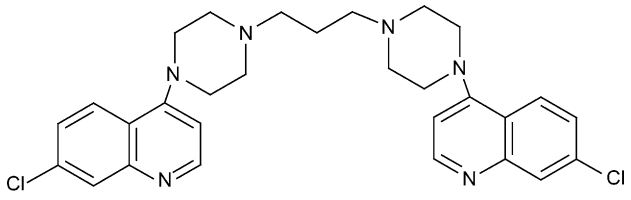
determination in capillary blood applied onto sampling paper [11–14].

The aim of this work was to develop a sensitive high throughput method suitable for determination of PQ in plasma during clinical studies. The presented assay aimed to utilise the best features from previous methods and combine these with 96-wellplate technology. An earlier method used protein precipitation with acidic acetonitrile prior to solid phase extraction (SPE) to achieve high recovery of the highly protein bound PQ [11]. This is very time consuming and impractical with a 96-wellplate assay. In the present assay the protein binding was instead disrupted by buffering plasma to a low pH (i.e. pH 2.0) prior to SPE which considerably facilitated a high throughput. An additional goal was to minimise the plasma volume needed to facilitate detailed pharmacokinetic studies with intense sampling schedules in children. The method has been validated according to published FDA guidelines [15].

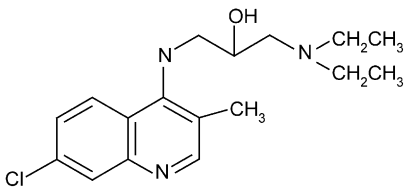
2. Materials and methods

2.1. Chemicals

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



PQ



IS

Fig. 1. Structures of PQ and IS.

2.2. Instrumentation

The LC system was a LaChrom Elite system consisting of a L2130 LC pump, a L2200 injector, a L2300 column oven set at 25 °C and a L2400 UV detector (Hitachi, Tokyo, Japan). The detector was set at 347 nm. Data acquisition was performed using LaChrom Elite software (VWR, Darmstadt, Germany). The compounds were analysed on a Chromolith Performance (100 mm × 4.6 mm i.d.) column protected by a Chromolith guard RP18 (10 mm × 4.6 mm i.d.) (VWR International, Darmstadt, Germany) using a mobile phase containing phosphate–acetonitrile buffer (pH 2.5; 0.1 M) (92:8, v/v) at a flow rate of 3 mL/min.

SPE was carried out using a 96-wellplate vacuum manifold (Agilent, Palo Alto, USA) and MPC-SD deep well SPE 96-wellplates (3 M Empore, Bracknell, UK).

2.3. Preparation of plasma standards

Concentrated stock solution of PQ (1000 μg/mL) was prepared in phosphoric acid (0.05 M). Working solutions of PQ ranging from 0.400 to 200 μg/mL were prepared by serial dilution of the stock solution in phosphoric acid (0.05 M). Two hundred microlitre of the working solutions was added to blank plasma (7.8 mL) to yield spiked calibration standards at eight different concentrations ranging from 10 to 5000 ng/mL. The calibration standards were divided into 250 μL aliquots and stored at −86 °C until analysis. A calibration curve was constructed using 250 μL plasma of each standard. Linear regression with peak-height ratio (PQ/IS response) against PQ concentration with 1/concentration^2 (x^2) weighting was used for quantification. Quality control (QC) samples for determination of accuracy and precision in plasma at three concentrations (40, 200 and 1250 ng/mL) were prepared in the same manner as the calibration standards and stored at −86 °C until analysis. The amount of stock solution in all spiked samples was kept lower than 2.5% of the total sample volume to minimise any systematic errors between real samples and standards.

2.4. Analytical procedure

An eppendorf multistepper was used to add 250 μL phosphate buffer (pH 2.0; 0.05 M) containing internal standard (3000 ng/mL) to 250 μL plasma in a 96-wellplate. The 96-wellplate was gently mixed on a vortex mixer for about 1 min. Using an eight-channel pipette 1000 μL phosphate buffer (pH 2.0; 0.05 M) was added to each well and the plate was left undisturbed for 10 min. Each well was mixed by aspiration and dispensation two times before the samples were loaded onto a conditioned MPC-SD deep well SPE 96-wellplate using an eight-channel pipette. All steps in the SPE procedure were conducted using an eight-channel pipette as follows:

methanol 1000 μL was added to each SPE well and vacuum at about 3–4 mmHg was applied for about 15–20 s. The plate was inspected to see that no wells contained any residual methanol. Phosphate buffer (pH 2.0; 0.05 M) 300 μL was added to each SPE well and vacuum at about 3–4 mmHg was applied for about 15–20 s. The plate was inspected again to see that no SPE wells contained any residual buffer. The samples (1500 μL) were loaded onto the SPE plate and vacuum at about 3–4 mmHg was applied for 5 min. The vacuum was thereafter increased with 1–2 mmHg every 3 min until all samples had passed through the SPE wells. Methanol–phosphate buffer (pH 2.0; 0.05 M) (80:20, v/v) 1000 μL was added to each SPE well and vacuum at about 3–4 mmHg was applied for 2 min. Full vacuum was applied for about 10 min where after the SPE column tips were wiped dry with paper. A 96-collection plate (1 mL) was inserted in the vacuum manifold and 500 μL methanol–triethylamine (98:2, v/v) was added to each SPE well. The SPE plate was left undisturbed for 2 min to let the elution solvent penetrate the membrane. Vacuum at about 3 mmHg was applied for about 20 s and an additional volume of 400 μL methanol–triethylamine (98:2, v/v) was added to each SPE well. The SPE plate was left undisturbed for 5 min and vacuum at about 3 mmHg was applied for 4 min. The vacuum was thereafter increased with 1–2 mmHg every 5 min until all elution solvent had passed through the SPE plate and into the collection plate.

The SPE eluates were evaporated in the 96-wellplate under a gentle stream of air at 80 °C. The samples were reconstituted in 100 μL acetonitrile–phosphate buffer (pH 2.5; 0.1 M) (5:95, v/v) using an eight-channel pipette and mixed on a vortex mixer for about 30 s. Fifty microlitre was injected into the LC system.

2.5. Validation

Linearity and calibration model were evaluated using eight calibration curves a day during 3 days. Precision and accuracy were evaluated by analysis of eight replicates at three different concentrations during 3 days. The concentrations were determined with 1/amount^2 weighted linear regression using a calibration curve prepared each day. Intra-, inter- and total assay precisions were calculated using a modified analysis of variance (ANOVA) approach. The approach was first proposed by Aronsson and Groth and is routinely used by Swedish hospital laboratories as recommended by the Swedish Board for Accreditation and Conformity Assessment (SWEDAC) [16,17]. Recovery was determined by comparing the peak-heights for the precision samples with direct injected solution containing the same nominal concentration of PQ as precision samples after SPE and reconstitution. Selectivity was evaluated by analysis of blank plasma from six different donors. The possibility of lowering the LLOQ by using 1 mL plasma was investigated by analysis of triplicates at 2.5, 80 and 700 ng/mL using 1 mL of plasma and triplicates at 80 and 700 ng/mL using 0.25 mL of plasma. A small modification was made to the method when 1 mL plasma was used. The final addition of phosphate buffer prior to SPE was 500 μL (pH 2.0; 0.1 M) instead of 1000 μL (pH 2.0; 0.05 M). A calibration curve prepared on the day of analysis using 0.25 mL plasma of each standard was used for quantification.

3. Results and discussion

3.1. Validation

Linear calibration curves were generated by 1/amount^2 (x^2) weighted linear regression analysis. The average calibration curve using ordinary linear regression, weighted $1/x$ and weighted $1/x^2$ can be seen in Fig. 2. It is clear from the figure that ordinary linear regression is unsuitable as it creates large errors at low concentrations. Ordinary linear regression requires a constant absolute error independent of concentration (i.e. homoscedastic data) [18]. For bioanalytical methods, it is normally the relative error (i.e. the relative standard deviation at each point) rather than the absolute error that is more or less constant throughout the range.

Both weightings seem to give comparable results from the graphical interpretation in Fig. 2. The distribution of the relative residuals is a good indicator of goodness of fit and should serve as guidance for choice of model. The average residual with spread (i.e. expressed as back calculated concentration) at each concentration can be seen in Table 1. The relative residuals should be randomly distributed around the nominal value with no obvious pattern [19,20]. With respect to the results in Table 1 a calibration model using $1/x^2$ weighting

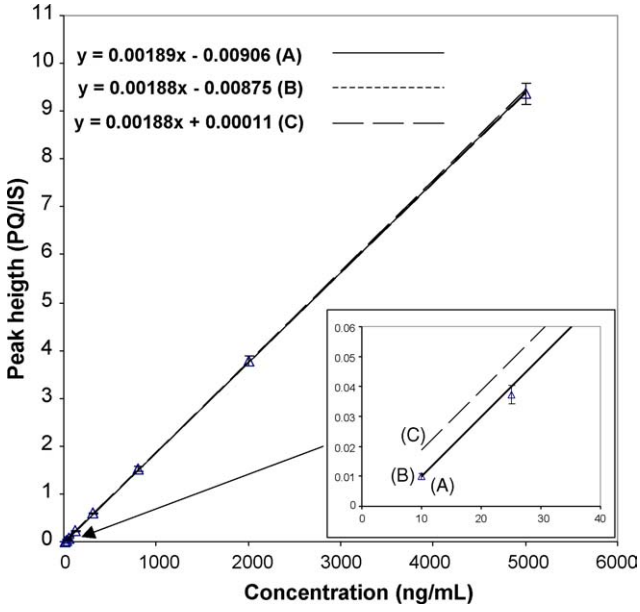


Fig. 2. Average calibration curves during validation ($n = 24$, mean response ± S.D.), weighted $1/x^2$ (A), weighted $1/x$ (B) and non-weighted linear regression (C).

Table 1
Average back calculated concentrations for the calibration standards expressed as percentage of nominal concentration ($n = 24$)

	10 ng/mL	25 ng/mL	50 ng/mL	128 ng/mL	320 ng/mL	800 ng/mL	2000 ng/mL	5000 ng/mL
$1/x$ weight								
Mean (%)	99.4	97.7	101.1	99.9	100.1	101.9	100.5	99.5
R.S.D.	8.0	3.9	2.9	3.4	1.4	1.5	1.4	0.8
$1/x^2$ weight								
Mean (%)	100.7	97.9	101.0	99.7	99.8	101.6	100.2	99.2
R.S.D.	2.2	5.1	2.8	2.7	1.3	1.8	1.9	2.1

Table 2
Accuracy and precision for the determination of piperaquine in plasma

Added (ng/mL)	Mean (%)	R.S.D.	Percentage of deviation (found vs. added)
Intra-assay ($n = 29$)			
20.0[a]	19.6	5.0	−2.1
40.0	41.2	3.3	3.1
200	202	3.6	1.1
1250	1288	2.3	3.0
Inter-assay ($n = 5$)			
20.0[b]		4.7	
40.0		5.8	
200		2.6	
1250		1.3	
Total assay ($n = 29$)			
20.0[a]		6.9	
40.0		6.7	
200		4.5	
1250		2.7	

[a] $n = 18$.
[b] $n = 6$.

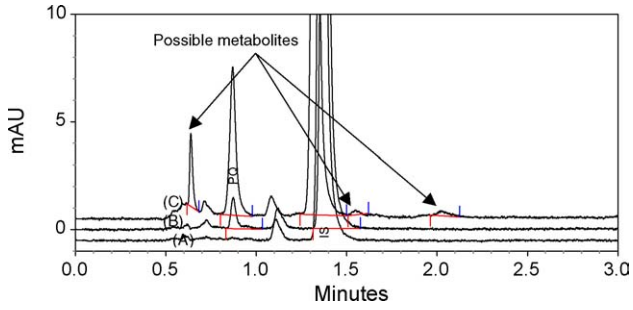


Fig. 3. Chromatograms for blank plasma (A), 20 ng/mL PQ (B) and patient sample 132 ng/mL PQ (C).

was chosen as this generates an evenly distributed low error over the whole range.

Precision and accuracy during the validation is shown in Table 2. The recovery of PQ was 85.8 ± 5.0, 87.4 ± 5.2 and 88.7 ± 3.4% (±S.D.) at 40, 200 and 1250 ng/mL, respectively. The recovery of IS was 91.3 ± 2.0% (±S.D.) and independent upon PQ concentration.

The lower limit of quantification (LLOQ) was determined to be 10 ng/mL with a R.S.D. and accuracy <20% [15,21]. The limit of detection (LOD) was 5 ng/mL. The LOD was chosen as the lowest concentration that could be reliably distinguished from the background noise (i.e. ≥3 times the S.D. of a blank plasma sample) [15,21]. The presented assay shows in general much lower variation, better sensitivity (i.e. LLOQ) and higher recoveries than the two previously published methods for quantification of PQ in plasma [11,14]. The average recovery with the present assay was 87% versus 75 and 55% with previous methods [11,14]. The intra-assay precision at 20 ng/mL (using 0.25 mL plasma) for the present assay was 5.0% versus 5.8% at 50 nM (using 0.50 mL plasma) and 9.9% at 20 ng/mL (using 1.0 mL plasma) [11,14]. Fig. 3 shows an overlay of chromatograms from a spiked plasma sample at 20 ng/mL, blank plasma and a patient sample containing 132 ng/mL. The patient sample was taken 7 days after a 3 days course with Artekin® (total 50.4 mg/kg PQ). The patient sample indicates the presence of one or more metabolites and work is underway to identify these.

No interference from the other related antimalarials has earlier been observed using the current LC setup [12]. No endogenous peaks that would interfere with the quantification of PQ or IS were observed from the different plasma sources which is a direct result of the very selective SPE procedure. PQ has earlier been reported to be stable in plasma stored at −80 and −20 °C. Using 1 mL plasma it was possible to lower the LLOQ to 2.5 ng/mL. The mean concentration found at LLOQ was 2.68 ± 0.31 ng/mL (mean ± S.D.) which meets the requirements according to international guidelines. The mean concentrations found at 80 and 700 ng/mL using 1 mL of plasma were 72.5 ± 6.1 and 672 ± 12 ng/mL (mean ± S.D.), respectively. The mean concentrations found at 80 and 700 ng/mL using 0.25 mL of plasma were 78.1 ± 0.3 and 705 ± 16 ng/mL (mean ± S.D.), respectively. All results complied well with the generally accepted limits for R.S.D. and accuracy (<15%).

4. Conclusion

A high throughput method for the determination of piperaquine in plasma has been developed and validated. The assay has been proven sensitive and reproducible and uses only 0.25 mL of plasma. The total analysis time for one batch (96 samples) is only approximately 6 h. The assay will be suitable for the analysis of samples from clinical studies and for larger population therapeutic drug monitoring studies at for instance day 7.

Acknowledgements

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