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Short communication

High throughput assay for the determination of lumefantrine in plasma

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Abstract

A high throughput bioanalytical assay for the determination of lumefantrine in plasma has been developed and validated extensively. The within-day precisions for lumefantrine were 5.2, 3.5 and 2.5% at 200, 2000 and 15000 ng/mL, respectively. The between-day precisions were 4.0, 2.8 and 3.1% at 200, 2000 and 15000 ng/mL, respectively. The lower limits of quantification (LLOQ) and the limits of detection (LOD) were 25 and 10 ng/mL, respectively using 0.250 mL plasma. The average recovery of lumefantrine was 85% and independent upon concentration. The use of 96-well plate format and short chromatographic run has increased the daily sample throughput four times. The assay is particularly suitable for large therapeutic drug monitoring studies using day 7 sampling.
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1. Introduction

The antimalarial lumefantrine/benflumetol (LF) was first synthesised and registered in China and is now commercially available only in a coformulated product with artemether as Co-artemether®/Riamet®. This combination has proved very well tolerated and highly efficacious in children and adults, even against multi-drug resistant strains of *Plasmodium falciparum* [1–5]. LF is a highly lipophilic compound which is more than 99.9% bound to plasma proteins [6]. Absorption is very variable. The day 7 plasma lumefantrine level has been shown to be the most important determinant of cure following treatment with the coformulation [7]. To date only three methods for the determination of LF in plasma have been published. The two older methods used liquid–liquid extraction techniques to achieve 25 and 13 ng/mL, respectively as the lower limits of quantification using a 1 mL plasma sample [8,9]. Both methods have practical drawbacks relating to the use of liquid–liquid extraction (i.e. the methods are time consuming, labour intensive and require large volumes of hazardous solvents). A method allowing simultaneous determination of LF and its desbutyl-metabolite by automated solid phase extraction (SPE) and liquid chromatography was developed recently [10]. This method is as sensitive as the two previous methods but uses only 0.250 mL plasma, important as the majority of malaria-affected patients are small children. The aim of this work was to develop a sensitive high throughput bioanalytical assay for single determinations of LF in plasma. This would be especially suitable for therapeutic drug monitoring and analysis of day 7 samples where a rapid result is required. The method has been validated according to published guidelines [11–13].

2. Materials and methods

2.1. Chemicals

LF and the internal standard (IS) (Fig. 1) were obtained from Novartis Pharma AG (Basel, Switzerland). Acetonitrile (HPLC-grade), methanol (pro analysis) and HPLC-water

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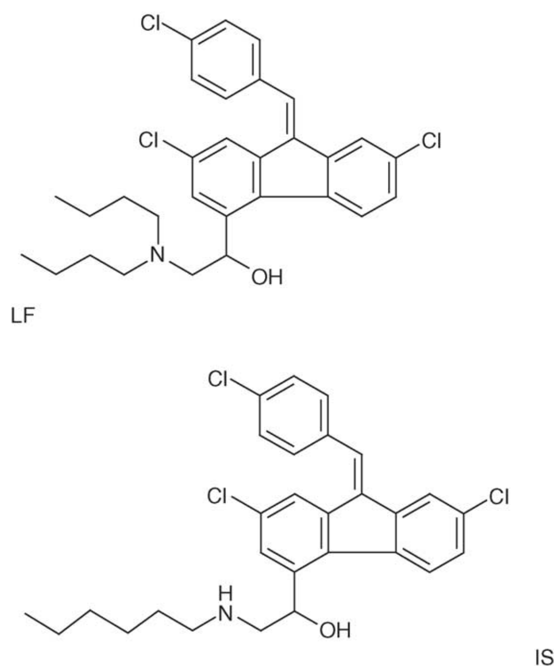

Fig. 1. Structures of LF and IS.

were obtained from JT Baker (Phillipsburg, USA). Glacial acetic acid (GR for analysis) was obtained from Merck (Darmstadt, Germany). Trifluoroacetic acid (HPLC grade) and sodium perchlorate (HPLC grade) were obtained from BDH (Poole, England). The phosphate buffer solutions were prepared by mixing appropriate amounts of sodium hydroxide and ortho-phosphoric acid, obtained from Merck (Darmstadt, Germany), with HPLC water.

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 335 nm. Data acquisition was performed using LaChrom Elite software (VWR, Darmstadt, Germany). The compounds were analysed on a SB-CN (250 mm × 4.6 mm, 5 μm) column (Agilent, Palo Alto, USA) protected by a short guard column SecurityGuard CN (4 mm × 3 mm i.d.) (Phenomenex Inc., Cheshire, UK) using a mobile phase containing acetonitrile–phosphate buffer (pH 2.0; 0.1 M) (58:42, v/v) and sodium perchlorate 0.01 M (i.e. mol/L mobile phase) at a flow rate of 1.2 mL/min. SPE was carried out using a 96-wellplate vacuum manifold (Agilent, Palo Alto, USA) and C8-SD deep well SPE 96-wellplates (3M Empore, Bracknell, UK).

2.3. Preparation of plasma standards

Stock solutions of LF 1 mg/mL were prepared freshly in methanol-acetic acid (99.8:0.2, v/v). Working solutions ranging from 1.25 to 750 μg/mL were prepared by serial dilution of LF stock solution in acidic methanol. Stock solution of IS (0.25 mg/mL) was prepared and stored in an amber glass bottle at about 8 °C protected from light. A consistent volume of 100 μL of working solution or stock solution was added to blank plasma (4900 μL) to obtain eight calibration standards in the range 25–20000 ng/mL. Quality control (QC) samples for determination of accuracy and precision in plasma, at three concentrations (200, 2000 and 15000 ng/mL), were prepared by addition of 150 μL working solution to blank plasma (7350 μL). The calibration standards and QC samples were stored as 250 μL aliquots at −86 °C until use.

2.4. Analytical procedure

All samples were divided initially into 250 μL aliquots in 2 mL eppendorf tubes. Plasma proteins were precipitated with 500 μL acetonitrile-acetic acid (99:1, v/v) containing internal standard (2.50 μg/mL) and immediately mixed at least 10 s. The tubes were left to settle for a minimum of 5 min before spun in a micro centrifuge for 5 min at 15000 × *g*. The supernatants from each eppendorf tube were transferred to a polypropylene 96-wellplate containing 900 μL HPLC water. All steps in the SPE procedure were conducted using an 8-channel pipette as follows: The SPE plate was conditioned with 500 μL methanol followed by 300 μL acetonitrile–water–acetic acid (30:69.5:0.5, v/v). The samples (∼1650 μL) were loaded onto the SPE plate at a low vacuum which was continuously increased until all samples had passed through the SPE wells. The SPE plate was washed with 1000 μL acetonitrile–water–acetic acid (30:69.5:0.5, v/v), dried under full vacuum for about 10 min and wiped dry with paper. A glass 96-well collection plate (1 mL) was inserted into the vacuum manifold and the SPE plate was eluted in two steps using 500 + 400 μL methanol–trifluoroacetic acid (99.9:0.1, v/v). The SPE plate was left to equilibrate for 2 min between the two elution steps in order to let the elution solvent penetrate the membranes. The SPE eluates in the collection plate were immersed partly into water at 65 °C and evaporated until dry, under a gentle stream of air. The samples were reconstituted in 200 μL methanol–hydrochloric acid 0.01 M (70:30, v/v). The plate was sealed, mixed for 5 min and 50 μL was injected into the LC-system.

2.5. Validation

Linearity and calibration models were evaluated using back-calculated values from nine calibration curves and prediction of eight replicate QC samples at three different concentrations. Accuracy and precision were evaluated by analysis of triplicate QC samples at three different concentrations over 7 days, using a freshly prepared calibration curve each day. Intra-, inter- and total-assay precisions were calculated using experimental design (i.e. single factor ANOVA) as recommended by ICH guidelines [12,13]. Method performance was evaluated further by investigating within day accuracy

and precision for a full 96-well plate. One calibration curve was generated and used to predict eight replicates of each calibration point and eight replicates of QC samples at three different concentrations. Recovery for LF and IS was determined by comparing the peak area for the QC samples with that of direct injected solution, containing the same nominal concentration as extracted and reconstituted samples, over 5 and 3 days, respectively. Selectivity was evaluated by analysis of blank plasma from ten different sources and direct injection of related lipophilic antimalarials (i.e. atovaquone, halofantrine, mefloquine, quinine) into the LC-system.

3. Results and discussion

In order to derive a benefit in terms of throughput from using 96-well plate technology the LC run time must be relative short (i.e. total run time for the whole plate, 96 samples, should be less than 24 h).

Single determination of LF allowed a shorter chromatographic run time per sample (i.e. 12 min) as it was not necessary to seek separation between the more hydrophilic desbutyl-lumefantrine and endogenous peaks in the front of the chromatogram. Manipulation of the sodium perchlorate concentration in the mobile phase allowed alteration of retention for LF and IS whilst endogenous compound remained unaffected [10]. It was found that weighted $1/x^2$ regression was the most appropriate choice of calibration model considering the result from back calculated values, predicted QC samples and the FDA requirement to use the simplest calibration model that fits the data adequately. Accuracy, intra-, inter- and total-assay precisions are summarised in Table 1. The assay precision using three replicates over seven days is further illustrated in Fig. 2. Only one sample (i.e. 1 out of 63) was outside 15% deviation from the nominal value. Accuracy and precision using one calibration curve to analyse a full 96 wellplate is shown in Table 2. The recovery of LF ($n = 15$) was 83.0 ± 3.7, 84.4 ± 4.2 and 86.5 ± 2.5 (% ± S.D.) at 200, 2000 and 15000 ng/mL, respectively. The recovery of IS was 85.8 ± 2.6 (% ± S.D.) and independent upon LF concentration.

Table 1
Accuracy and precision for the determination of lumefantrine in plasma

Added (ng/mL)	Mean	R.S.D. (%)	%Deviation (found vs. added)
Intra-assay ($n = 21$)			
200.0	206.1	5.2	3.0
2000	2057	3.5	2.9
15000	14590	2.5	−2.8
Inter-assay ($n = 7$)			
200	207.2	4.0	3.6
2000	2069	2.8	3.5
15000	14553	3.1	−3.0
Total-assay ($n = 21$)			
200		4.9	
2000		3.3	
15000		3.0	

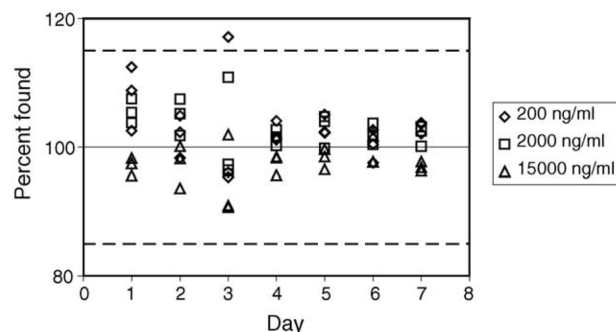

Fig. 2. Variation for replicate QC samples analysed during 7 days. Broken lines represents 85 and 115% found.

Table 2
Within day accuracy and precision ($n = 8$) for a full 96-well plate

Concentration (ng/mL)	Mean found (%)	R.S.D. (%)
25	113.4	16.77
100	108.2	2.67
200	104.7	2.85
300	107.1	4.18
1000	103.7	4.89
2000	97.8	2.45
3000	98.8	3.42
10000	95.4	2.41
15000	93.9	3.51
20000	91.5	3.05

The lower limits of quantification (LLOQ) was determined as 25 ng/mL with R.S.D. and accuracy <20% [12,13]. The limit of detection (LOD) was 10 ng/mL. The LOD was chosen as the lowest concentration that could be distinguished reliably from the background noise (i.e. ≥3 times the S.D. of a blank plasma sample) [12,13]. This should be adequate since therapeutic lumefantrine concentrations at day 7 typically are around 500 ng/mL [14,15]. The presented assay shows comparable sensitivity, accuracy and precision to previously published methods for quantification of LF in plasma [8–10]. The main advantage with the presented assay is the increased throughput of samples and that only 0.25 mL plasma is required for analysis. No interference from the

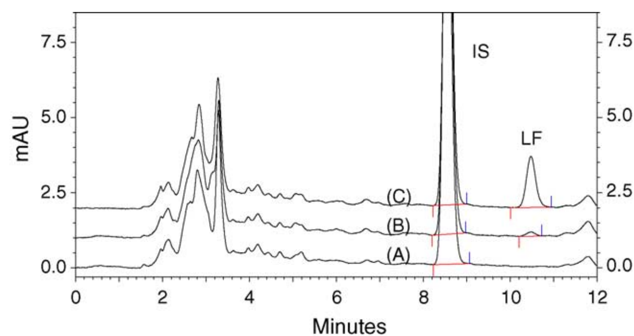

Fig. 3. Overlay of chromatograms from blank plasma (A), spiked plasma sample at 25 ng/mL (LLOQ) (B) and 300 ng/mL (C).

related antimalarials investigated was found in the LC system. Blank plasma from ten different sources were evaluated for selectivity and no endogenous peaks interfered with the quantification of LF. Fig. 3 shows an overlay of chromatograms from blank plasma (A), spiked plasma sample at 25 ng/mL (LLOQ) (B) and 300 ng/mL (C).

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