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Development and validation of an automated solid-phase extraction and liquid chromatographic method for determination of lumefantrine in capillary blood on sampling paper

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Abstract

A bioanalytical method for the determination of lumefantrine in 100 μl blood applied onto sampling paper, by solid-phase extraction and liquid chromatography, has been developed and validated. Whatman 31 ET Chr sampling paper was pre-treated with 0.75 M tartaric acid before sampling capillary blood to enable a high recovery of lumefantrine. Lumefantrine was extracted from the sampling paper, then further purified using solid-phase extraction and finally quantified with HPLC. The between-day variation was below 10% over the range 0.4–25 μM. The lower limit of quantification was 0.25 μM in 100 μl capillary blood. No decrease in lumefantrine concentration in dried blood spot is seen after 4 months storage at 22 °C. The method was also evaluated in field samples from patients in Tanzania after treatment with lumefantrine/artemether. Lumefantrine could be estimated accurately enough to assess bioavailability and treatment compliance on day 7 (i.e. 4 days after the last dose) after a standard regimen with the lumefantrine/artemether combination.

Keywords: Lumefantrine; Sampling paper; Dried blood spots; Capillary blood; Antimalarial drugs; Solid-phase extraction; Liquid chromatography

1. Introduction

Malaria is caused by the mosquito transmitted protozoan parasite *Plasmodium falciparum*. More than 300 million people are infected every year and between 1 and 3 million people die, the majority of them being children below the age of five in Africa [1,2].

The antimalarial lumefantrine (LF) was first synthesised and registered in China and is now commercially available only as a coformulated product together with artemether (Coartem®/Riamet®). This combination is well tolerated and has proven highly efficacious for treatment of uncomplicated *falciparum* malaria [3–7]. This drug combination is now becoming the most recommended first line treatment for uncomplicated malaria in African countries as a replacement for previously used monotherapies due to the increasing resistance to these drugs. LF is a highly lipophilic compound with an extensive protein binding (99.9%) [8]. The absorption of the drug is very variable and influenced by concomitant food intake [9,10]. After standard treatment, the day 7 plasma LF level has been shown to be the most important determinant of bioavailability and compliance with the coformulation [11].

To date a few methods for the determination of LF in plasma have been published, none in blood [12–15]. The two oldest methods use liquid–liquid extraction while the two newer methods have adopted solid-phase extraction for sample preparation. The sensitivity for all methods in terms of LLOQ is

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approximately 47 nM. The earlier methods require as much as 1 ml of plasma (i.e. approximately 2.5 ml blood). The most recent method has been adapted to a lower volume (i.e. 0.25 ml plasma) to facilitate clinical studies in children [12]. The majority of malaria-affected patients are small children and venous blood sampling reduces the chances that the parents will let their children enrol in clinical studies on uncomplicated malaria. Capillary sampling from a finger prick is much less invasive and generally well accepted. The requirement on facilities (i.e. centrifuge, freezer, etc.) at the clinic/field site is also reduced since the blood spot can be dried at ambient temperature without the need for centrifugation and storage in a freezer. A previous paper [16] describes the relationship between lumefantrine plasma concentrations of simultaneously sampled capillary and venous samples. They concluded that although there was a good correlation between the two types of samples, there was not complete agreement. They proposed that the difference between the two measurements was due to the variance of the drug assay and the variable admixture of interstitial fluid in capillary blood samples. This survey was comparing plasma and does not necessarily apply for blood since absorption in blood cells may have an influence on the result. For the most reliable interpretation of study results, a mix of capillary and venous samples should be avoided.

The aim of this work was to develop a method with adequate sensitivity for quantification of LF in capillary blood sampled onto sampling paper, to be applied especially in drug efficacy monitoring studies in small children. The method is validated according to the current FDA guideline for bioanalytical method validation [17].

2. Materials and methods

2.1. Chemicals and materials

LF and internal standard TA 3039 (IS) were obtained from Novartis Pharma AG (Basel, Switzerland). The structures are shown in Fig. 1. Trifluoroacetic acid, tartaric acid (Fluka), acetonitrile (CHROMASOLV®) and methanol (CHROMASOLV®) were all purchased from Sigma–Aldrich (St. Louis, MO, USA). Hydrochloric acid, sodium perchlorate and other chemicals of pro analysi grade were from Merck kGaA (Darmstadt, Germany). Deionised water was obtained from a Milli-Q deionised water system (Millipore, Bedford, MA, USA). Chromatographic sampling paper 31 ET Chr was from Whatman (Whatman International, Maidstone, UK). Drug-free blood with heparin as an anticoagulant was obtained from the Department of Blood Transfusion, Falun Central Hospital, Sweden. The phosphate buffer solutions were prepared by mixing sodium hydroxide, *ortho*-phosphoric acid and Milli-Q water.

2.2. Instrumentation and chromatographic conditions

The chromatographic HPLC system consisted of a Waters Alliance 2695 separations module (Milford, MA, USA) and a Spectra Physics Analytical UV 2000 detector (San José, CA, USA) set at 335 nm. Data acquisition was performed using Chromatography station for WINDOWS 1.7 (DataApex Ltd., Prague, The Czech Republic). The mobile phase was acetonitril:phosphate buffer (0.1 M, pH 2) (55:45 v/v) with 0.03 M sodium perchlorate with a flow rate of 0.4 ml/min through a Zorbax SB-CN column, 3.5 μm (3.0 mm × 150 mm) (Zorbax Inc., Wilmington, NC, USA).

Solid-phase extraction (SPE) was carried out on an automated SPE system, ASPEC XL (Gilson, Middleton, WI, USA) using a C8 standard density disk SPE column (3 M Empore, Bracknell, UK). The ASPEC system uses positive air pressure instead of vacuum to force liquids through the columns.

2.3. Preparation of sampling paper, stability samples and calibration standards

The chromatographic sampling paper Whatman 31 ET Chr was completely soaked for a few seconds in a solution of 0.75 M tartaric acid and dried on a wire-net in a horizontal position. Concentrated stock solution of LF and IS was prepared in methanol–acetic acid (99.8:0.2 v/v). Stock solution of LF was diluted in methanol–water (50:50 v/v) to prepare working solutions. Stock solution was stored in cryo tubes in −20 °C until first time use, and there after refrigerated at 6 °C. Working solutions was prepared freshly before use and added to blank whole blood to produce calibration standards and quality control samples. The volume of working solution was less than 1% in blood.

Samples for stability studies were prepared by adding appropriate amount of working solution to blank whole blood to a final concentration of 5 μM and then 100 μl was applied to the sampling paper. Three different variation of sampling paper treatment were evaluated, untreated, with 0.25 and 0.75 M tartaric acid. Stability samples were stored in zip-lock bags at 22 and 37 °C up to 4 months. At every stability measurement, triplicate samples was extracted by SPE and analysed. Recovery

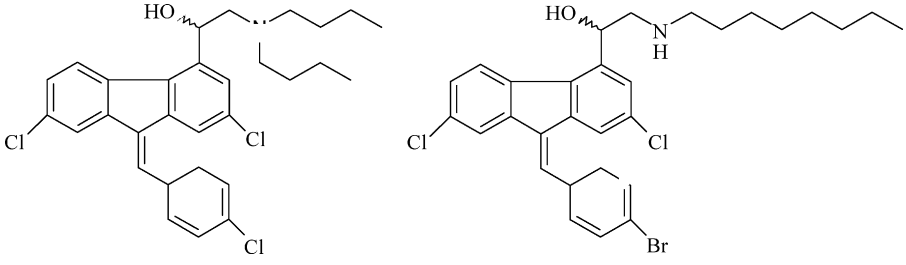

Fig. 1. Structure of lumefantrine and IS.

Table 1
SPE procedure on ASPEC XL robot

SPE-step	Solvent	Volume (ml)	Flowrate (ml/min)	Airpush (ml)
Activation	Methanol	0.5	3	–
Conditioning	Acetonitrile–water–acetic acid (30:69.5:0.5 v/v/v)	0.3	3	–
Loading	Sample	2.6	0.8	1.7
Washing	Acetonitrile–water–acetic acid (30:69.5:0.5 v/v/v)	0.5	2	2
Elution 1	Methanol–trifluoroacetic acid (99.9:0.1 v/v)	0.5	0.5	–
Elution 2	Methanol–trifluoroacetic acid (99.9:0.1 v/v)	0.5	0.5	1.5

was determined against reference samples (spiked reconstitution solvent) stored in −20 °C.

Calibration standards were prepared in batches of 1.5 ml by adding appropriate amount of working solution to blank whole blood to obtain a calibration range with seven standards from 0.25 to 30 μM. Precision and accuracy samples (QC samples) were prepared in batches of 3.8 ml in four different concentrations (0.4, 1.0, 10 and 25 μM). One hundred microliters spots of spiked blood were applied onto pre-treated sampling paper.

2.4. Analytical procedure

Blood spot (100 μl) was cut out from the sampling paper and then cut in three pieces and placed in a 5 ml borosilicate glass tube. One milliliter acetic acid 0.5 M followed by 1 ml acetonitrile with IS (1 μM) was added. Tubes were vortex mixed for a few seconds and placed on a sample tube rocker for 1 h, thereafter centrifuged for 10 min at 2500 × *g* and the liquid was decanted into new tubes. Nine hundred and forty microliters of 0.5 M acetic acid was added and the samples were then loaded onto the SPE column. The extraction procedure is shown in Table 1. The eluates were evaporated under a gentle stream of air at 65 °C and reconstituted in 100 μl methanol–hydrochloric acid (0.01 M) (70:30 v/v). Fifty microliters was injected into the LC-system.

2.5. Validation

The accuracy and precision of the method were estimated by analysis of four replicates at four different concentrations during 6 days. The concentrations were determined with 1 amount-weighted linear regression using a calibration curve analysed at each run and within- and between run precisions were calculated. Lower limit of quantification (LLOQ) was selected to a concentration where the precision was within 15% and the signal to noise ratio (s/n) exceeded 10:1. The extraction recovery at each concentration was determined by comparing the peak height for the precision samples with a direct injected solution containing the same nominal concentration of LF diluted in reconstitution solvent. Freeze-thaw stability was determined at low and high concentration (QC level 0.4 and 10 μM) simulating sampling handling and then refreezing in three cycles. Short term stability/robustness testing was performed, with the purpose to simulate what would happen with samples during equipment failure, etc. Samples were evaluated for 20 h during different stages in the method (i.e. in solution prior SPE loading, on SPE sorbent bed, in elution solvent, as evaporated elution solvent and in the LC-injector). Selectivity was evaluated by analysis of six blank samples from six different blood donors. Other antimalarial drugs were evaluated for interference (i.e. Atovaquone, halofantrine, mefloquine, sulfadoxine, and quinine) and some other common drugs (i.e. paracetamol, acetylsalicylic acid, salicylic acid and amoxicillin). The drugs were injected into the LC-system.

2.6. Analysis of samples from patients

The method was tested with real samples collected from nine patients with uncomplicated malaria undergoing standard 3 day treatment with Coartem™ in Mlandizi, Tanzania. Patients, both male and female, between 25 and 72 years of age were included after receiving informed consent. One hundred microliters capillary blood was collected 40 and 135 h after last treatment dose.

3. Results and discussion

3.1. Method development

The method used similar LC and SPE settings, with some adjustments, as in the previous developed method [13] for LF. The LC column was however changed to a "solvent saver" model (150 mm × 3 mm, 3.5 μm) with shorter retention time and equally good efficiency as the previous column and only consumed about one-third of the amount of mobile phase. The metabolite desbutyl-lumefantrine was not included in this study since it was considered to be in too low concentrations to be measurable [18]. The internal standard was also changed to a slightly more hydrophobic compound with a longer retention time than LF, which is more suitable for the method.

3.2. Solubility and adsorption

Both LF and IS have poor solubility in water and need an organic modifier, e.g. acetonitrile to stay in solution. A solution of acetonitrile–H_2O (v/v) shows a breakpoint in solubility at about 35% acetonitrile content (at typical working concentrations of LF). In an acidified environment, acetonitrile–acetic acid (0.5 M), the breakpoint is around 25–30% acetonitrile content for LF and around 30–35% for the IS. There are also some indications of adsorption of LF to plastic test tubes during evaporation, mainly when residues from blood/plasma is present, but

this effect has not been seen for glass tubes of borosilicate type [13].

3.3. Development of sampling paper extraction step

One of the most difficult parts during the method development was to achieve a high and reproducible recovery from the sampling paper. A variety of different organic and inorganic solvent combinations were evaluated in different combinations to improve the extraction recovery of LF. There was also an attempt to modify the paper to become more hydrophobic and minimize ion bonding as described in an earlier publication [19]. The paper was soaked in 10 mM dodecylethyldimethylammonium bromide and left to dry at room temperature. All experiments failed to improve the recovery and the modification of the sampling paper had no effect. It was then discovered that LF exhibited a time dependent adsorption when applied as a bloodspot to the sampling paper. Initially, just when the blood had dried on the sampling paper, the recovery is about 60%. But after 4–7 days, the recovery had continuously been dropping down to about 15–20% recovery where it levelled out. Another article describing analysis of retinol in dried blood spots has documented the same effect [20]. They reported a decline in concentration in freshly prepared samples for 6–10 days and thereafter the concentration remained constant for at least 80 days. For LF, this effect was seen then blood was applied onto sampling paper but not when plasma was applied onto sampling paper. Blood was also applied to microscope slides to dry at room temperature. A recovery of 60–70% was achieved at day 0 but as for sampling paper it continuously decreased down to about 40% after a few days. The cause for this time dependent absorption is not known, but a method to prevent it was needed.

Different approaches to disrupt LF binding from the dried blood were tested. Addition of 10 M urea (to denaturise the proteins) or addition of pepsin in HCl solution (to digest the blood components) were unsuccessful. Several modifications to the sampling paper were evaluated. Acetic acid had earlier shown to improve the solubility of LF in different solutions, although acetic acid would evaporate too quickly to be of any use. The approach was to find other, larger acids, which would not evaporate and would be suitable for treatment of the sampling paper. Malonic acid, tartaric acid and oxalic acid were selected and shorter stability tests were performed. The final choice was L-(+)-tartaric acid, since it gave relative high recovery, was easy to handle and was considered safe to use.

A stability study was performed on dried blood spots during 4 months comparing untreated, 0.25, and 0.75 M paper treatment with tartaric acid which can be seen in Fig. 2. The peak heights of LF in extracted dried blood spots were compared against reference samples (LF spiked in reconstitution solvent and stored at −20 °C) to calculate the % recovery. LF was considered stable for at least 4 months on paper treated with 0.75 M tartaric acid. LF was only stable for 2 months on paper treated with 0.25 M tartaric acid. Samples stored at 6 °C (only with 0.75 M tartaric treatment) and −20 °C and was analysed on day 120. The 0.75 M paper treatment at 6 °C showed good stability for LF. Both paper treated with 0.25 and 0.75 M tartaric acid showed good stability

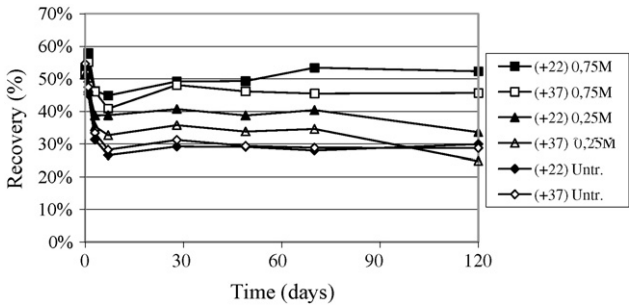

Fig. 2. Stability of lumefantrine in whole blood applied onto sampling paper that was treated with 0.75, 0.25 M tartaric acid or untreated paper and stored at 22 and 37 °C. Recovery (%) is against spiked reconstitution solvent. At day "0" the blood spots was extracted after only 20 min showing the recovery of not fully dried blood spots.

when stored at −20 °C. These results indicate that the samples should be refrigerated or frozen if the expected storage time is longer than a few months.

3.4. Validation

To determine the most appropriate calibration curve, several calibration models were evaluated, e.g. non-weighted, $1/x$ weighted, $1/x^2$ and log–log. After evaluating back calculated values for calibration standards and predicted concentrations for the QC samples, $1/x$ linear regression model was considered to be the simplest model that adequately described the concentration–response relationship. The response was linear over the calibration range. The mean slopes for the calibration curve ($n = 6$) were 0.1028 (S.D. 0.0059) and the mean intercept were 0.0032 (S.D. 0.0021) with a mean correlation of 0.999 (S.D. 0.0006). Table 2 shows a summary of the precision and accuracy of the validated method and Fig. 3 shows an overlay of some of the calibration standards. The precision at LLOQ 0.25 μM was 6% R.S.D. with a signal to noise ratio (s/n) greater than 10:1. Limit of detection was estimated to about 0.1 μM with a s/n 3:1. Recovery of the method was determined to 60–65% for all QC levels.

Short time stability was evaluated during the validation process. The three freeze and thaw cycles showed good stability indicating no problem refreezing samples. During robustness

Table 2
Accuracy and precision for the determination of lumefantrine on sampling paper

Added (μM)	Mean (μM)	R.S.D. (%)	% deviation (found vs. added)
Within-day ($n = 24$)			
0.4	0.42	5.0	4.7
1.0	1.07	4.2	6.9
10	10.7	2.7	7.1
25	24.6	4.6	−1.8
Between-day ($n = 6$)			
0.4	0.42	9.7	
1.0	1.07	3.2	
10	10.7	1.7	
25	24.6	3.7	

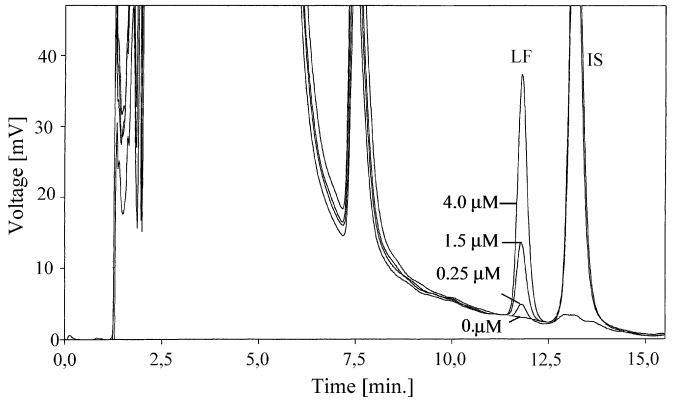

Fig. 3. An overlay view of a blank whole blood, 0.25, 1.5 and 4.0 μM lumefantrine calibration standards.

testing, no problems were observed during the 20 h samples were in the solution prior SPE or extracted in the elution solvent or stored evaporated for 20 h. However, when adsorbed on the SPE column bed, a decrease in recovery (15–30%) was seen after 20 h, but this would rarely be a problem since the sample typically is adsorbed on the SPE bed for only 10–15 min during extraction. Samples stored in the LC-injector for 36 h injecting a sample every 2 h showed no change and was considered stable.

Selectivity evaluated from six blank samples from six different blood donors showed no interfering peaks. No interferences from other related antimalarials were observed when they were injected into the LC-system (UV 335 nm).

3.5. Analysis of samples from patients

The clinical applicability of the method was evaluated using samples collected from nine malaria patients in Tanzania about 40 h and 135 h after the last oral dose of Coartem™. The first dose was supervised while the last doses were self-administered. One hundred microliters capillary blood was collected on sampling paper pre-treated with 0.75 M tartaric acid. The measured concentrations for the nine patients ranged from 0.8 to 9 μM after 40 h and from 0.2 to 2 μM after 135 h. An example is shown in Fig. 4. The high inter-individual variation in bioavailability has been described previously and is probably most influenced by concomitant food intake [10,21–23]. LF is a lipophilic compound and the absorption is greatly increased when administered together with food with high content of fat. The concentration levels were of the same order of magnitude as previously described in venous plasma samples. Median values in the present study were 3.3 and 0.8 μM on days 4 and 7 compared to 7.3 and 0.7 μM in one of the previous studies [22]. In laboratory environment, the blood spots are dry after about 1 h on pre-treated sampling paper (compared to about 30 min on non-treated 31 ET Chr paper). However, the drying time may increase when humidity is high and this was also reported for the Tanzanian samples.

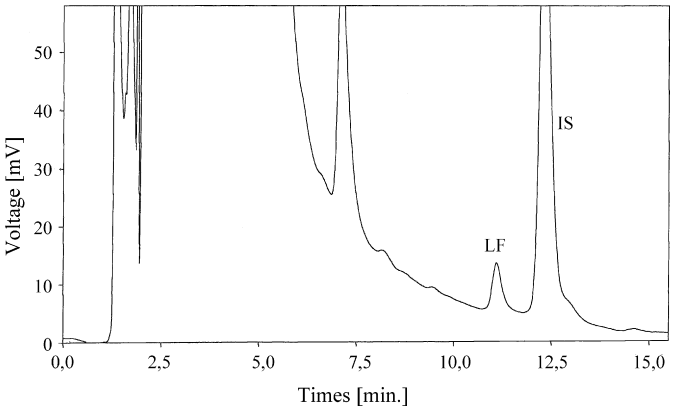

Fig. 4. Patient sample (1.0 μM) withdrawn 135 h after last dose of lumefantrine.

4. Conclusions

Capillary blood collection on sampling paper is the most feasible and applicable way to collect and store samples from patients in clinical field studies. With the treatment of 0.75 M tartaric acid, the recovery is increased to enable accurate day 7 level determinations (the main determinant for efficacy of Coartem™) of LF [11]. The method will provide a valuable tool for follow up of compliance and efficacy after administration of Coartem™. Samples can be stored at room temperature for a few months but if longer storage time is expected, the samples should be frozen.

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