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Measurement of lumefantrine and its metabolite in plasma by high performance liquid chromatography with ultraviolet detection

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ABSTRACT

Artemether-lumefantrine (ARM-LUM) has in recent years become the first-line treatment for uncomplicated malaria in many Sub-Saharan African countries. Vigorous monitoring of the therapeutic efficacy of this treatment is needed. This requires high-quality studies following standard protocols; ideally, such studies should incorporate measurement of drug levels in the study patients to exclude the possibility that insufficient drug levels explain an observed treatment failure. Several methods for measuring lumefantrine (LUM) in plasma by HPLC are available; however, several of these methods have some limitations in terms of high costs and limited feasibility arising from large required sample volumes and demanding sample preparation. Therefore, we set out to develop a simpler reversed phase high performance liquid chromatography (RP-HPLC) method based on UV detection for simultaneous measurement of LUM and its major metabolite the desbutyl LUM (DL) in plasma. Halofantrine was used as an internal standard. Liquid–liquid extraction of samples was carried out using hexane–ethyl acetate (70:30, v/v). Chromatographic separation was carried out on a Synergi Polar-RP column (250 mm × 300 mm, particle size 4 μm). The mobile phase consisted of acetonitrile–0.1 M ammonium acetate buffer adjusted to pH 4.9 (85:15%, v/v). Absorbance of the compounds was monitored at 335 nm using a reference wavelength of 360 nm. Absolute extraction recovery for LUM and DL were 88% and 90%, respectively. Inter- and intraday coefficients of variation for LUM and DL were ≤10%. The lower limits of quantification for LUM and DL were 12.5 and 6.5 ng/ml, respectively. After validation, the methodology was transferred to a local laboratory in Tanga Tanzania and samples from a small subset of malaria patients were analysed for LUM. The method appears to be applicable in settings with limited facilities.

1. Introduction

Lumefantrine (LUM) in combination with artemether (Coartem) is the first fixed-dose artemisinin-based combination therapy currently available to treat uncomplicated malaria. The total supplies of this combination increased substantially, from 11.2 million treatment courses in 2005 to 62 million in 2006 and 66.3 million in 2007, with procurement of more than 78 million treatment courses in 2008 [1]. Coartem is highly efficacious and generally well tolerated [2]. The advantage of current wide-scale use of Coartem in various regions of the malaria-endemic world is that LUM has only been available in co-formulation with artemether and thus no apparent development of resistance to LUM *in vivo* can be expected. Combination therapy with LUM decreases the likelihood of development of resistance to artemether; therefore it is important to monitor the therapeutic efficacy of the combination and thus provide advance warning even in case of minor changes in efficacy. Pharmacokinetic and pharmacodynamic studies of Coartem have shown that plasma LUM concentration on day 7 is the principal determinant of treatment outcome, where values >500 ng/ml being associated

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with >90% cure rates and values <280 ng/ml are predictive of treatment failures [3].

Four high performance liquid chromatography (HPLC) methods have been developed for the quantitative determination of LUM in plasma [4–7]. Two of these methods were based on liquid–liquid extraction (LLE) but a sample volume of 1 ml plasma has been used to achieve low quantification limits of 25 and 12 ng/ml, respectively [4,5]. Since the majority of malaria patients are small children, using such a large sample volume will not be practical when studying LUM pharmacokinetics in uncomplicated malaria. In addition, the calibration curves used in those studies (25–800 and 12–4000 ng/ml, respectively) do not cover the therapeutic concentrations of the drug as LUM plasma levels might reach 12 μg/ml during a 4-day regimen [6]. The other two methods [6,7] used solid phase extraction (SPE), which is an attractive alternative to the liquid–liquid extraction (LLE), since it is faster and the extraction is more complete. They also used 250 μl plasma and a wider calibration range, 0.024–20 and 0.021–10.10 μg/ml for LUM and DL, respectively. However, toxic organic solvents have been used for protein precipitation pre-SPE and in the elution step. Moreover, the SPE columns and the automated SPE/HPLC system are expensive and unaffordable for the malarial endemic countries. The aim of the present study was thus to develop a LLE-based HPLC–UV method for the simultaneous determination of LUM and DL in plasma. The method has been validated according to published FDA-guidelines [8]. After validation, the methodology was transferred to a local laboratory in Tanga Tanzania and to ensure the ultimate success of the transfer, the method has been fully evaluated following the guidance of FDA and the International Society of Pharmaceutical Engineering [9,10]. The transfer was controlled by a procedure that included: development of a transfer plan, definition of transfer tests and acceptance criteria (validation experiments, sample analysis: sample type and replication), training of local staff, full method validation and testing of few samples.

2. Materials and methods

2.1. Chemicals and glassware

LUM and DL were obtained from Novartis (Basel, Switzerland) and Dafra pharma International (Belgium). Halofantrine was purchased from Sigma–Aldrich (Denmark). All chemicals and solvents used in this study were of analytical grade. HPLC-grade acetonitrile, methanol, potassium phthalate and ammonium acetate were purchased from Sigma (Denmark). Glacial acetic acid (purity 99.8%) was obtained from Merck (Darmstadt, Germany). Ammonium acetate buffer solution (0.1 M, pH 4.9) was prepared by mixing appropriate amounts of ammonium acetate and acetic acid.

2.2. HPLC instrumentation and chromatographic conditions

The system was Agilent 1100 series liquid chromatography system consisting of Binary pump, 96 well-plate autosampler, thermostatted column compartment and Agilent 1100 Series diode-array detector (Agilent Technologies, Denmark). Absorbance of the compounds was monitored at 335 nm using a reference wavelength of 360 nm. Data acquisition was performed using Agilent ChemStation software. The compounds were analysed on a Synergi Polar-RP column (250 mm × 300 mm, particle size 4 μm; Phenomenex, Denmark). The column was protected by a short Security Guard Cartridge POLAR-RP (4 mm × 2.0 mm) (Phenomenex Inc., Denmark). Column temperature was adjusted to 25 °C. The flow rate of the mobile phase was set at 1.0 ml/min.

2.3. Preparation of calibration standards

Stock solutions of LUM and DL (1 mg/ml) were prepared in a mixture of methanol–acetic acid (100:2, v/v, respectively) and stored in cryo tubes at −80 °C until first use, and there after stored at 4 °C. Stock solution of LUM and DL was diluted with the same solvent to prepare working solutions. Working solutions were freshly prepared and added to blank plasma to obtain 10 calibration standards in the range of 12–12000 ng/ml. The volume of working solutions was less than 1% in the spiked plasma samples. The calibration points were used to cover the therapeutic concentrations achieved by LUM *in vivo*. The calibration standards were stored as 250 μl aliquots at −80 °C until use. Stock solution of the internal standard (0.25 mg/ml) was prepared in the same solvent used for the drugs and stored in glass tubes at 4 °C protected from light.

2.4. Analytical procedure

Borosilicate glass tubes were used in all extraction steps to minimise the adhesion of the drugs. To adjust the pH of the samples 200 μl of phthalate buffer (pH 2) were added to 200 μl spiked plasma standards/samples containing 50 μl of the IS (2500 ng/ml). Samples were mixed on a vortex for few seconds. The spiked samples were then extracted with 3 ml of hexane–ethyl acetate mixture (70:30, v/v, respectively). To obtain optimum recovery, samples were shaken vigorously for 15 min and then centrifuged at 4000 rpm for 10 min. The organic phase was transferred into new borosilicate tubes and evaporated to dryness under a stream of nitrogen. To get good retention and clean chromatograms, the samples were reconstituted in 200 μl methanol–water–acetic acid (93:6:1, v/v, respectively). The tubes were vortexed vigorously and the contents transferred to HPLC vials. 100 μl was injected into the LC system.

2.5. Validation

2.5.1. Quality testing of the method

Within-day and day-to-day accuracy and precision were evaluated by analysing four replicates of spiked plasma at three concentrations (75, 1500 and 7000 ng/ml), over 7 days, using a freshly prepared calibration curve each day. The analytical recovery of the extraction procedure for LUM and DL was determined by comparing the peak height for the quality samples with that of direct injection of the drugs dissolved in reconstitution liquid and containing the same nominal concentration of each quality sample, over 5 days.

2.5.2. Limits of quantification and detection

The limit of quantification (LOQ) was defined as the lowest concentration that can be determined with acceptable accuracy (from 88% to 120%) and precision with a relative standard deviation (RSD) of ≤20%. The limit of detection (LOD) was defined as the concentration with a signal-to-baseline noise ratio of 2.5.

2.5.3. Selectivity

Interference by endogenous constituents in plasma was assessed by analysis of blank plasma samples from 10 different subjects. A range of antimalarials (mefloquine, quinine, artemether, amodiaquine, and chloroquine), anti-HIV drugs (lamivudine, stavudine, nevirapine, zidovudine, and efavirenz) and paracetamol, were also tested for interference in the assay by direct injection of methanol-based standards onto the HPLC system.

2.5.4. Storage stability and freeze–thaw stability

Stability of LUM and DL stocks stored at 4 °C was determined over periods of up to 60 days. Stability of LUM and DL in plasma

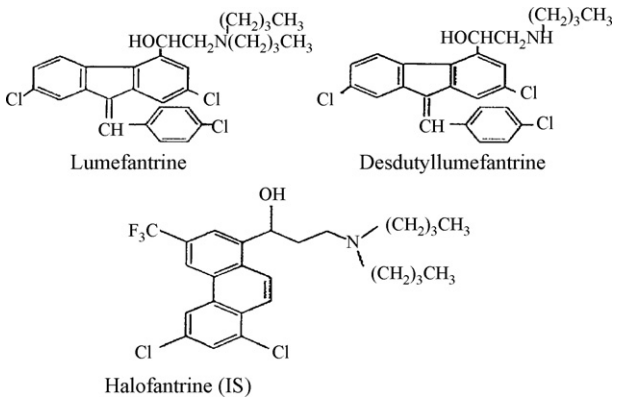

Fig. 1. Chemical structures of lumefantrine, desbutyl lumefantrine and the internal standard halofantrine.

was evaluated at 200 and 4000 ng/ml. Triplicates were assayed, when freshly prepared and after storage at −20 and −80 °C, after 9 months. Stability of LUM/DL after extraction was tested using triplicate plasma samples containing 200 and 4000 ng/ml. Reconstituted samples were left at room temperature in the autosampler and aliquots were injected onto the HPLC after 24 h.

Stability of LUM/DL in plasma subjected to three freeze–thaw cycles was also tested. Triplicate samples containing 100 and 500 ng/ml were assayed and the recovery LUM/DL concentrations were compared to freshly spiked plasma samples.

2.5.5. Method transfer and clinical application in a malaria-endemic setting

The method was transferred to the Pharmacology Laboratory of the National Institute of Medical Research, Tanga Centre, NE Tanzania. The method setup was identical with regard to glass tubes, mobile phase, HPLC column, extraction procedure and UV wavelength. The HPLC system was different and some minor modifications to the laboratory procedures had to be made in order to adapt it to the local conditions. The method was fully evaluated as recommended by FAD [8]. The clinical application of the transferred method was assessed by measuring LUF and DL levels in plasma samples from four patients, who participated in an on-going clinical trial of drug efficacy and pharmacokinetic interactions of artemether-lumefantrine and antiretroviral drugs in adults HIV/AIDS patients treated for uncomplicated *Plasmodium falciparum* malaria in Tanga district (Clinical Trials.gov identifier NCT 00885287). To reduce variability in absorption, a standard meal is given at each dosing. Blood samples employed in the present assay were collected at days 3, 7, 14, 21, 28 and day 42 post first dose. Two millilitres of blood was collected in EDTA tubes and plasma was separated by centrifugation. Plasma samples were stored in cryo tubes at −80 °C and analysed within a maximum of 50 days after sample collection. On the day of analysis, the samples were thawed at room temperature. An aliquot of 200 μl plasma was added to a borosilicate tube together with HF internal standard before proceeding with the extraction procedure. Duplicates of quality control samples at three levels were analysed in each run to ensure satisfactory method performance in accordance with guidelines of routine drug analysis [9,10] (Fig. 1).

3. Results and discussion

3.1. Method development

Baseline separation of LUM, DL and the IS was achieved when the pH of the mobile phase was 4.9 and when column temperature was adjusted to 25 °C. The pH of the mobile phase affects LUM

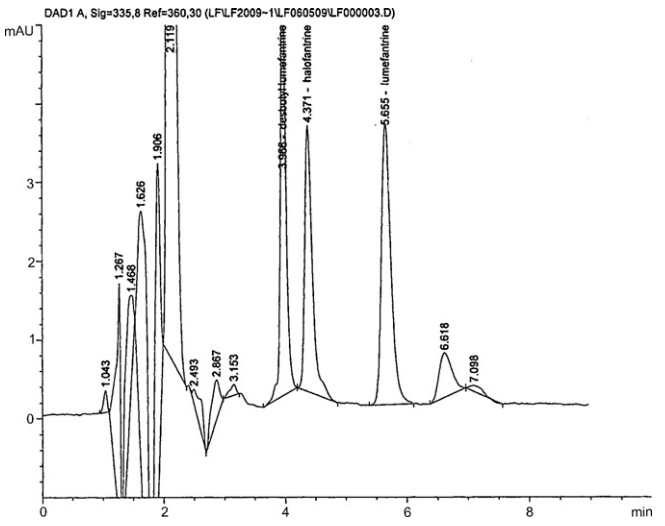

Fig. 2. A chromatogram of a plasma sample spiked with 1000 ng/ml lumefantrine, desbutyl lumefantrine and halofantrine.

ionisation and solvation, and therefore it had a major impact on selectivity and retention of the ionisable LUM. In addition the presence of acetic acid in the mobile phase and in the injection solvent contributed to the separation of the analytes and for the sharp peaks obtained as acetic acid enhances the elution strength of the mobile phase by forming ion-pair with LUM, this increases the polarity of LUM in the mobile phase by keeping the capacity factor (k') of the analyte between 2 and 2.5 The effect of acetic acid in reducing the k' by forming an ion-pair with LUM has been experimented and confirmed in the paper's of Zeng et al. [5]. Enhancing the elution strength of the mobile phase affected also selectivity (α) by improving interaction of the analytes with the stationary and the mobile phases. Acetic acid has earlier been shown to improve solubility of LUM in different solutions and for that reason we decided to reconstitute the extracted samples in a solvent that contains acetic acid [5]. It was noticed that increasing acetonitrile concentration in the mobile phase and setting column temperature at 25 °C decreased the retention times of the analytes more than the retention of the endogenous interfering compounds. Chromatograms of drug free plasma and spiked plasma sample containing 1000 ng/ml LUM, DL and 2500 ng/ml halofantrine are shown in Fig. 2. The retention time of DF, LUM and halofantrine was 4.0, 4. 5 and 5.7 min, respectively.

3.2. Extraction behaviour of LUM, DF and halofantrine

To minimise drugs adsorption to test tubes before extraction, borosilicate tubes were used. Four neutral organic phases (hexane/ethyl acetate (70:30%, v/v), hexane/tertbutylmethylether (70:30%, v/v), 100% tertbutylmethylether, and 100% diisopropylether were evaluated for their extraction from human plasma. Recovery of LUM and DL increased from less than 56–88% and 90%, respectively, upon using hexane/ethyl acetate (70:30%, v/v) as the extracting phase and borosilicate tubes throughout the extraction steps as compared to using the other mentioned organic phases and untreated glass tubes. However, the extraction efficiency of LUM, but not DF, into hexane/ethyl acetate was pH dependent. Sample extraction was facilitated by a strong acidic pH well below the pK_a of LUM (pK_a 9) where most of the compound exists in the ionised form. Therefore, we decided to treat the plasma samples with phthalate buffer to adjust pH of the sample to 2.0 before extraction.

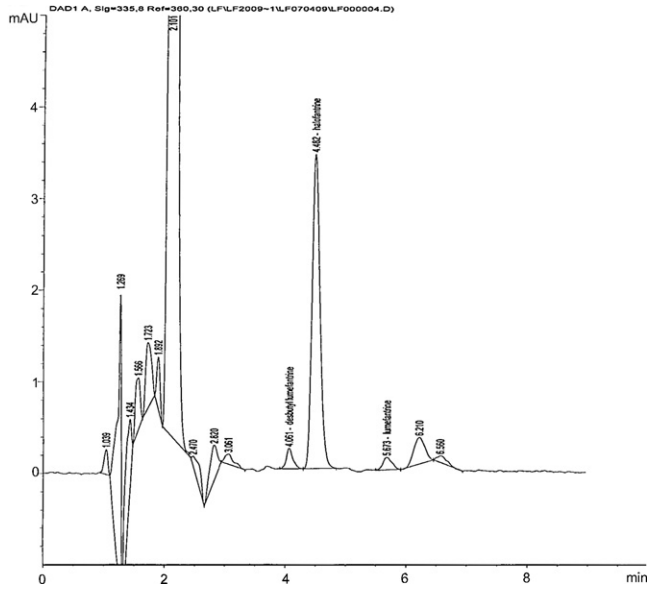

Fig. 3. A chromatogram of a spiked plasma sample at the lower limit of quantification of LUM and DF (12.5 ng/ml).

3.3. Validation

Since ordinary linear regression was found to be unsuitable as it created large errors at low concentrations other calibration models (non-weighted, log–log, $1/x$ weighted and quadratic) were evaluated. Quadratic regression model was considered the best model to describe the concentration–response relationship as an evenly distributed low error was observed over the whole range. The response was linear over the calibration range (the mean r value for 8 calibration curves was = 0.99). The equation of the calibration curves (number of calibration curves = 8) ($y = ax^2 + bx + c$) was: $y = 1.13x^2 + 1.62x + 5.32$ for DF and $8.6x^2 + 1.8x + 2.07$ for LUM, where the mean slopes for the calibration curves were 1.13 (SD 0.02) and 8.6 (SD 0.003) and the mean intercepts were 1.62 (SD 0.001) and 1.8 (SD 0.006) for LUM and DF, respectively.

Table 1 shows a summary of the precision and accuracy of the validated method. Residual standard deviation (RSD) was <8 at all tested levels. The absolute recovery of LUM and DL over the concentration range 12–12,000 ng/ml in spiked plasma was 88% (range 88–102%) and 90%, (range 89–103), respectively. The LOQ was 12.5 ng/ml for both LUF and DL with an accuracy <20% and a signal to noise ratio of 9. The LOD was 10 and 6.5 ng/ml, for LUM and DF, respectively. Fig. 3 shows a chromatogram from a spiked plasma sample at the LOQ (12.5 ng/ml) for both LUM and DF. The presented assay showed comparable sensitivity, accuracy, that ranged from 96% to 100% at all tested levels, and precision to

Table 1
Accuracy and precision for determination of lumefantrine and desbutyl lumefantrine in plasma.

Added (ng/ml)	Mean (%)		RSD	
	LUM	DF	LUM	DF
Intra-assay (N = 20)				
75	72.0	72.5	4.0	6.2
1500	1492	1489	5.2	6.6
7000	6989	6991	3.4	7.1
Inter-assay (N = 12)				
1500	1502	1498	3.3	3.2

LUM: lumefantrine. DL: desbutyl lumefantrine. N: number. RSD: residual standard deviation.

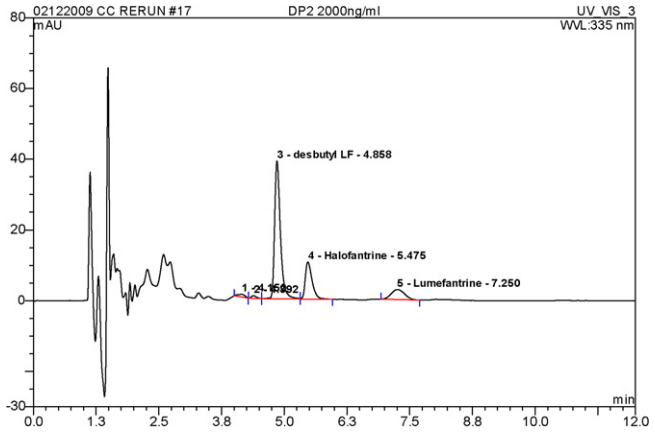

Fig. 4. A chromatogram of a plasma sample spiked with 25 ng/ml lumefantrine and 2000 ng/ml desbutyl lumefantrine.

previously published methods for quantification of LUM and DF in plasma [4–6].

3.4. Stability and selectivity

Both LUM and DF were stable during the freeze/thaw cycles. Long term storage stability showed that 75% of the drugs was depleted when plasma samples were stored at −20 °C, but not at −80 °C for 9 months (recovery after 9 months was only 17.5). Samples stored at 4 °C for a month had almost the same content of both LUM and DF, but more degradation products were found in the stored samples. It is thus recommended that plasma samples should be stored at −80 °C and that drug measurements should be

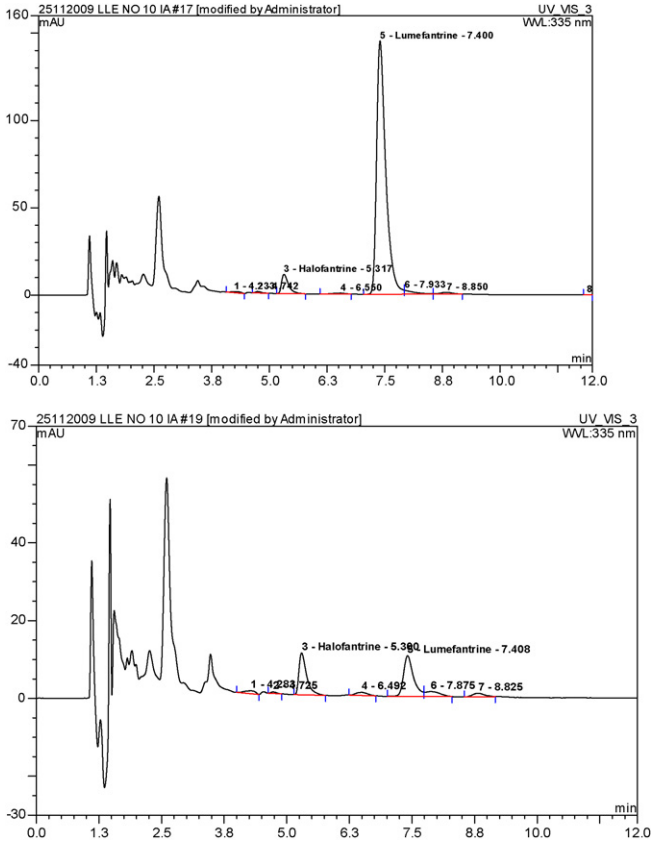

Fig. 5. A patient sample showing lumefantrine concentration 3 days post-treatment.

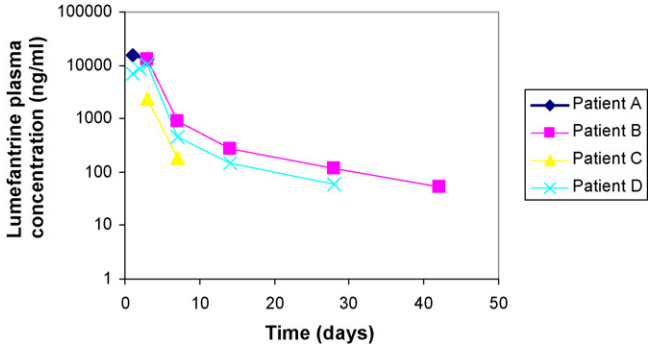

Fig. 6. Lumefantrine concentrations during the follow-up period of four HIV patients treated for uncomplicated malaria.

conducted within maximum 9 months to get reliable results. The reconstituted samples left at room temperature in the autosampler were found to contain the same amount of LUM and DF after 24 and 36 h ($P = 0.2$ and 0.5, respectively).

No interference from the chemically related or the commonly used antimalarials (quinine, mefloquine, artemether, chloroquine, and amodiaquine) or paracetamol were observed. Blank plasma from 10 different individuals was evaluated for selectivity and no endogenous peaks interfered with quantification of LUM and DL.

3.5. Application of the method

The method was adapted to the HPLC system and laboratory conditions existing in NIMR Tanga, Tanzania, as described above. Based on the validation performed here, the extraction procedure as well as the HPLC method seemed to be robust and amenable for adaptation for routine use in a developing country. Accuracy at all tested levels was <12% and recovery of LF and DF ranged from 88% to 112%. The total precision for all quality control samples ($n = 2$ at each level) during the analysis was 5.0%, 4.0% and 2.0% at 100, 500 and 2000 ng/ml, respectively. A chromatogram of spiked plasma sample containing 25 ng/ml LUM and 2000 ng/ml DF is shown in Fig. 4. The retention time of DF, halofantrine and LUF was 4.8, 5. 4 and 7.2 min, respectively.

In order to evaluate the performance in clinical studies, a few samples from an on-going study were analysed for concentration of LUF and DL. The results of the clinical study will be published elsewhere. A chromatogram showing a patient plasma sample at day 3 post-treatment is presented in Fig. 5. In all tested plasma samples DL concentration was below the detection limit (12.5) and thus could not been quantified. Fig. 6 shows LUF concentrations in the four patients during the follow-up days. Range of LUF plasma concentration and variability in patients A, B and D is comparable to the published literature [11,12]. The low plasma concentrations in patient C might be due to possible interactions between LUM and anti-HIV drugs or other things that will be discussed in details in another publication.

4. Conclusion

A method for the determination of LF and DF has been developed and evaluated. The assay proved sensitive and reproducible, and only requires the small amount of 200 μl of plasma. The assay was linear in the wide calibration range 12.5–12,000 ng/ml by the use of quadratic regression. The achievements with the present assay are the simple extraction procedure using standard organic liquids, a short run time (less than 10 min/sample) and that only 200 μl plasma is required for the analysis. The assay is suitable for the analysis of samples from clinical studies and for larger population therapeutic drug monitoring studies at for instance day 7. In addition, it seems to be robust for adaptation between laboratories so that e.g. drug monitoring may be performed locally.

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