Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Pharmaceutical and Biomedical Analysis 37 (2005) 1081–1088
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Development and validation of a bioanalytical method using automated solid-phase extraction and LC-UV for the simultaneous determination of lumefantrine and its desbutyl metabolite in plasma

N. Lindegårdh[a,b,*], A. Annerberg[a], D. Blessborn[c], Y. Bergqvist[c], N. Day[a,b], N.J. White[a,b]

[a] *Faculty of Tropical Medicine, Mahidol University, Bangkok 10400, Thailand*
[b] *Nuffield Department of Clinical Medicine, Centre for Tropical Medicine, University of Oxford, Oxford, UK*
[c] *Dalarna University College, S-781 88 Borlänge, Sweden*

Received 20 May 2004; received in revised form 22 July 2004; accepted 24 July 2004
Available online 21 September 2004

Abstract

A bioanalytical method for the determination of lumefantrine (LF) and its metabolite desbutyl-lumefantrine (DLF) in plasma by solid-phase extraction (SPE) and liquid chromatography has been developed. Plasma proteins were precipitated with acetonitrile:acetic acid (99:1, v/v) containing a DLF analogue internal standard before being loaded onto a octylsilica (3 M Empore) SPE column. Two different DLF analogues were evaluated as internal standards. The compounds were analysed by liquid chromatography UV detection on a SB-CN (250 mm × 4.6 mm) column with a mobile phase containing acetonitrile–sodium phosphate buffer pH (2.0; 0.1 M) (55:45, v/v) and sodium perchlorate 0.05 M. Different SPE columns were evaluated during method development to optimise reproducibility and recovery for LF, DLF and the two different DLF analogues. The within-day precisions for LF were 6.6 and 2.1% at 0.042 and 8.02 μg/mL, respectively, and for DLF 4.5 and 1.5% at 0.039 and 0.777 μg/mL, respectively. The between-day precisions for LF were 12.0 and 2.9% at 0.042 and 8.02 μg/mL, respectively, while for DLF 0.7 and 1.2% at 0.039 and 0.777 μg/mL, respectively. The limit of quantification was 0.024 and 0.021 μg/mL for LF and DLF, respectively. Different amounts of lipids in plasma did not affect the absolute recovery of LF or DLF.

Keywords: Antimalarial; Lumefantrine; Lipophilic; Liquid chromatography; Solid-phase extraction; Stability; Validation

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 one and three million die, mostly children younger than 5 years and the majority of them in Africa [1,2].

Lumefantrine (LF) is a highly lipophilic antimalarial compound, which is more than 99.9% bound to plasma proteins [3]. LF (originally called Benflumetol) was first synthesised and registered in China and is now commercially available as Co-artemether®/Riamet® (LF + artemether). This combination when given as a four-dose regimen (a total of LF 1920 mg and artemether 320 mg) over 48 h has proven to be very effective for the treatment of falciparum malaria in both Africa and Asia [4]. Desbutyl-lumefantrine (DLF) is a putative metabolite of LF. However, LF is likely to be metabolised to a low extent, as to date there have been no reports of metabolite formation in vivo [4,5]. In vitro studies have shown that DLF actually has a higher efficacy than LF, though DLF has not yet been evaluated in vivo [5]. To the best of our knowledge there are neither published articles regarding the pharmacokinetics of DLF nor any methods to quantify DLF in biological fluids. To date only two methods for the determination of LF in plasma have been published,

* Corresponding author. Tel.: +66 2 3541395x122.
E-mail address: niklas@tropmedres.ac (N. Lindegårdh).

0731-7085/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2004.07.041

both using liquid–liquid extraction. Both methods used 1 mL plasma to achieve 0.025 and 0.012 μg/mL, respectively, as the lower limit of quantification [6,7]. In addition, the calibration ranges (i.e. 0.025–0.800 and 0.012–4.00 μg/mL) used in the previous methods have proven to be insufficient. Extensive clinical trials have shown that therapeutic LF concentrations as high as 12 μg/mL can be found during a four-dose regimen over 48 h [4]. A method using a wider calibration range used divided calibration curves, which can lead to erroneous results if concentrations in clinical samples fall near the break point between the two curves [7]. The aim of this work was to develop a solid-phase extraction (SPE) method that permits sensitive simultaneous determination of both DLF and LF in plasma over the necessary calibration range. A secondary goal was to re-investigate the stability of LF at −20 °C after earlier reports of limited stability at this commonly used field storage temperature. The method has been validated according to published FDA-guidelines [8].

2. Materials and methods

2.1. Chemicals

LF, DLF, IS-1 and IS-2 were obtained from Novartis pharma AG (Basel, Switzerland). The structures are shown in Fig. 1. Acetonitrile (HPLC-grade), methanol (pro-analysis) and HPLC-water were obtained from JT Baker (Phillipsburg, USA). Trifluoroacetic acid, sodium perchlorate and triethylamine were obtained from BDH (Poole, UK).

2.2. Instrumentation

The LC-system was a LaChrom Elite system consisting of a L2130 LC pump, L2200 injector, L2300 column oven set at 25 °C and 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) column (Agilent, Palo Alto, USA) protected by a short guard column security guard 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) (55:45, v/v) and sodium perchlorate 0.05 M at a flow rate of 1.2 mL/min. SPE was carried out on an automated SPE system consisting of an ASPEC XL (Gilson, Middleton, WI, USA) using a C8 standard density disk SPE column (3 M Empore, Bracknell, UK). The ASPEC system uses a positive air pressure instead of vacuum to force liquids through the SPE columns.

2.3. Preparation of stability samples and calibration standards

Concentrated stock solutions of LF, DLF, IS-1 and IS-2 (1 mg/mL) were prepared in methanol–acetic acid (99.8:0.2, v/v). Stock solution of LF and DLF were diluted with methanol to prepare working solutions. The stock solutions were stored in amber glass bottles at about 8 °C in the dark, and the working solutions were prepared freshly before use. Appropriate amounts of the working solutions were added

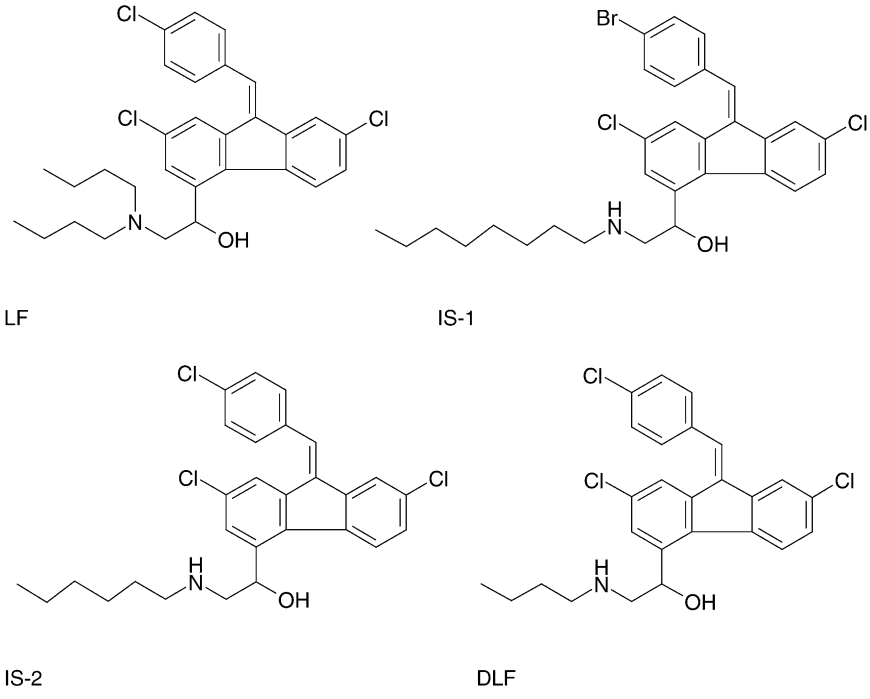

Fig. 1. Structures of LF, DLF, IS-1 and IS-2.

to blank plasma to obtain calibration standards in the range 0.024–20.0 and 0.021–1.01 μg/mL for LF and DLF, respectively. QC samples were prepared at four different levels (Table 2). The calibration standards and QC samples were prepared in batches of 10 and 20 mL, respectively, divided into 250-μL aliquots and stored at −86 °C until use. 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

Plasma (0.250 mL) was precipitated using 0.5 mL acetonitrile–acetic acid glacial (99:1, v/v) containing IS-2 (2.5 μg/mL). The microtubes were placed on a vortex mixer for approximately 10 s, left undisturbed for approximately 10 min and finally centrifuged at 15,000 × *g* for 5 min. The supernatant was decanted into a 5 mL glass tube, and 960 μL of HPLC-water was added. The samples were then loaded onto the SPE columns using an automated SPE system. The SPE procedure can be seen in Table 1. The SPE eluates in glass tubes were evaporated under a gentle stream of air at 65 °C and reconstituted in 100 μL of methanol–sodium phosphate buffer (pH 2.0; 0.05 M) (70:30, v/v). Fifty microliters was injected into the LC-system.

2.5. Solubility and adsorption

Solubility was evaluated by addition of equal amounts of stock solution of the analytes to seven amber glass autosampler vials. Acetonitrile or methanol and water were thereafter, added in different ratios to produce 2 mL sample with final concentrations of organic solvent in the vials ranging from 10 to 70%. The concentration of the analytes was chosen to the same nominal concentrations, as the highest calibration standards after SPE and reconstitution. The vials were left undisturbed for approximately 24 h whereafter the upper 0.5 mL was transferred to an injector vial. Fifty microliters of each sample was injected into the LC-system. Aliquots of a spiked analyte solution (∼0.1 μg/mL) were transferred into glass, silanised glass and polypropylene inserts and left undisturbed for approximately 24 h before being injected into the LC-system. The solution contained the minimum required amount of organic solvent as determined in the solubility experiment. Spiked plasma samples and spiked precipitation solvent at a low concentration were processed as described above, and the eluates were collected in Eppendorf microtubes, 5 mL polypropylene tubes and 5 mL glass tubes. The eluates were evaporated, reconstituted and injected into the LC-system.

2.6. Influence of lipid content on method accuracy

Plasma and serum samples are sometimes cloudy due to an increased content of lipoproteins. This is mainly caused by an increased triglyceride concentration. As LF is more than 99.9% protein bounded, mainly to high-density lipoproteins (HDL), it is important that the accuracy is independent of food intake [3]. The absolute recovery of halofantrine (HF) using liquid–liquid extraction has been reported to be highly dependent on the amount of lipoproteins in the sample [9]. The accuracy of the extraction method was evaluated for samples with different contents of fed and fasted plasma to ensure robust results independent of food intake prior to the time of sampling. Pre- and post-prandial (taken 1 h after a fatty meal) plasma was used to make a series of samples with different pre- and post-prandial plasma content. All samples were spiked to the same concentration using spiked post-prandial plasma. Additionally, two sets of calibration curves were prepared in pre- and post-prandial plasma, respectively. Pre-spiked plasma was used for preparation of the samples to ensure a very low content of organic solvent in the final samples. The samples were divided into 250-μL aliquots and stored at −86 °C until analysis. All samples were quantified with a calibration curve prepared on the day of analysis.

2.7. Validation

Precision and accuracy were evaluated by analysis of five replicates at four different concentrations during five days (Table 2). The concentrations were determined with 1/amount-weighted linear regression using a calibration curve prepared each day. Recovery was determined by comparing the peak areas for the precision samples with direct

Table 1
SPE procedure

SPE-step	Solvent	Volume (mL)	Flow rate (mL/min)	EQ time (min)	Airpush (mL)	Flow rate (mL/min)	EQ time (min)
Condition	Methanol	0.5	3	0.2	–	–	–
	Acetonitrile–water–acetic acid (30:69.5:0.5)	0.3	3	0.25	–	–	–
Load	Sample	1.66	1	0.3	0.7	1	0.2
Wash	Acetonitrile–water–acetic acid (30:69.5:0.5)	0.5	2	0.25	1.5	6	0.25
Elute	Methanol-trifluoroacetic acid (99.9:0.1)	0.5	0.5	0.15	–	–	–
		0.25	0.5	0.15	–	–	–

Table 2
Validation performance

Concentration	LF (μg/mL)				DLF (μg/mL)			
	0.042	0.419	0.725	8.02	0.039	0.335	0.467	0.777
Within day $n = 25$ (%)	6.6	3.0	3.0	2.1	4.5	2.4	2.4	1.5
Between day $n = 5$ (%)	12.0	2.6	3.5	2.9	0.7	1.3	2.4	1.2
Total (%)	13.6	4.0	4.6	3.6	4.6	2.7	3.4	1.9
Recovery (%)	75	75	63	70	61	70	69	64
Accuracy (%)	−19.0	−8.1	−5.4	−6.8	−7.6	−2.0	−6.6	4.4

injected solution containing the same nominal concentration of DLF and LF as precision samples after SPE and reconstitution. LF has earlier been reported to be stable in plasma stored at −80 °C but not at –20 °C [6]. Plasma was spiked at a concentration of 1.3 μg/mL and stored at −86, −20 and about 8 °C in both glass vials and Eppendorf microtubes. The samples were analysed after 4 months. Freeze-thaw stability was determined at a low and high concentration (QC level two and four, Table 2) after three cycles. Short-term stability was evaluated during 24 h for all steps in the method (i.e. injector vials, loading prior SPE and elution solvent). Selectivity was evaluated by analysis of blank plasma from six different donors and direct injection of related lipophilic antimalarials (i.e. atovaquone, halofantrine, mefloquine, mefloquine carboxyl metabolite and quinine) into the LC-system.

2.8. Method performance during a clinical study

The presented method was used to analyse lumefantrine plasma samples taken during a three-arm, open, randomised clinical trial of artemether–lumefantrine versus artesunate–mefloquine versus chloroquine-sulphadoxine–pyrimethamine in patients with uncomplicated falciparum malaria in southern Laos (Phalanxay). The patients had 42-day follow-up and those in the artemether–lumefantrine arm had a day 7 plasma sample taken for lumefantrine concentration. Day 7 plasma concentrations of LF have proved an important determinant of therapeutic response [4]. Duplicates of quality control samples at three levels were analysed in each analytical run to ensure satisfactory method performance in accordance with guidelines for routine drug analysis [8].

3. Results and discussion

3.1. LC method development

Several problems were encountered during the method development of this assay.

The internal standard IS-2 chosen for this assay is very similar to LF and separation in the LC-system was not readily achieved. LF and IS-2 co-eluted using methanol but were successfully separated using acetonitrile in the mobile phase on a CN column. Addition of sodium perchlorate to the mobile phase was found to be very efficient for altering the separation between the analytes and interfering endogenous compounds. The perchlorate-ion forms an ion pair with the charged analytes and thereby increases their retention while the neutral endogenous compounds remains unaffected. It was found that increased acetonitrile content as well as increased column temperature decreased the retention of the analytes more than the retention of interfering endogenous compounds. Last but not least, it was crucial that the guard column contained the same phase (i.e. CN) as the analytical column. The endogenous compounds were strongly retained by a 4-mm-long C18 guard column and co-eluted with the analytes. This effect was surprisingly strong considering that a 250-mm analytical column (i.e. 63 times longer than the guard column) was used. Fig. 2 shows chromatograms obtained with the different guard columns. This effect could be used to alter selectivity for other assays.

3.2. SPE method development

The extent of protein binding has a strong correlation with lipophilicity of the analyte and tends to be almost 100% for very lipophilic drugs [3,9–11]. However, extremely lipophilic drugs such as atovaquone, LF and HF also have extremely limited solubility in a water phase and would need the association with proteins or organic solvent to stay in solution. The recovery of LF from plasma in this assay was increased from less than 5 to ∼75% when the proteins were precipitated prior to SPE instead of sample dilution 1:5 with acidic buffer.

The initial approach was to use reversed-phase SPE on a C8 column to extract DLF, LF and the first synthesised internal standard IS-1 after precipitation with acetonitrile–acetic acid (99:1, v/v). This proved to be problematic due to the difference in lipophilicity between the analytes. In order to avoid breakthrough of DLF, the load and wash step could not contain more than 30% acetonitrile. However, IS-1 needed approximately 40% acetonitrile to be fully dissolved at the studied concentration. Using only 30% acetonitrile led to a lower recovery and higher variation for IS-1, and using 40% led to a lower recovery and higher variation for DLF. Different cation exchangers were evaluated (i.e. C2, CBA, SCX-1, and SCX-2) to find a suitable SPE sorbent retaining all analytes. The weak cation exchangers had a high breakthrough in the SPE loading step while the strong cation exchangers gave a low and non-reproducible recovery for DLF and IS-1 using a variety of elution compositions. A possible explanation is

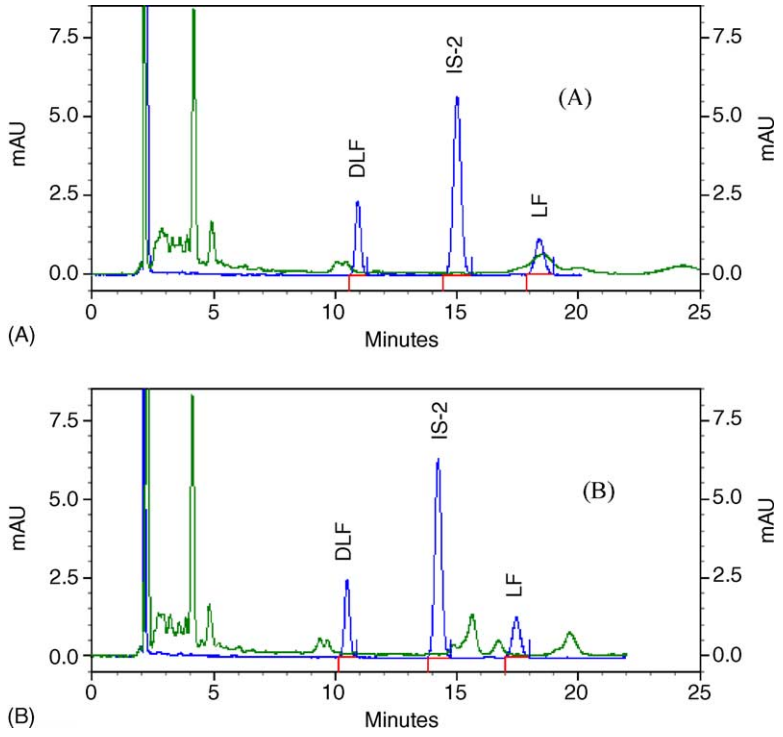

Fig. 2. Chromatograms of blank plasma and direct injection of LF, DLF and IS-2 using a C18 guard column (A) and a CN guard column (B).

that DLF and IS-1 were degrading at high pH even though no additional peaks were seen in the chromatograms. The same phenomena have been reported for HF that has identical structure to LF around the amine group. The metabolite desbutyl-HF degraded at alkaline pH but not at HF. The secondary amine desbutyl-HF degraded and formed a ring structure linking the nitrogen and the oxygen atoms [12]. A new internal standard IS-2 was synthesised as a compromise between DLF and LF with respect to lipophilicity. Using this internal standard, it was possible to use the C8 SPE column and 30% acetonitrile in the SPE loading and washing step.

3.3. Solubility and adsorption

The peak heights and areas of each analyte in the 14 vials were compared to evaluate the minimum required amount of organic solvent in the samples. There were lower analyte responses for LF/IS and DLF in samples containing less than 50 and 20% acetonitrile, respectively. Slightly higher percentage of methanol than acetonitrile was required to ensure complete solubility. The reconstitution solvent was chosen to methanol–phosphate buffer (pH 2.0; 0.05 M) (70:30, v/v) as this ensured complete solubility without causing any significant band broadening in the LC-system.

No difference in analyte response was observed using the different inserts. A significant and variable adsorption of the analytes to both Eppendorf microtubes and polypropylene tubes was observed when the processed plasma eluates were evaporated. The responses for the analytes were 0–50% lower when evaporated in plastic tubes compared to evaporation in glass tubes. There were no differences between glass and plastic for the SPE-processed spiked precipitation solvent. It was most likely lipophilic plasma residues that caused the variable adsorption to the plastic surface during evaporation.

3.4. Influence of lipid content on method accuracy

No effect on the accuracy as a function of different content of pre- and post-prandial plasma was detected. The calibration curves prepared in pre- and post-prandial plasma can be seen in Fig. 3. A fresh calibration curve prepared on the day of analysis was used to predict the concentration in the different samples. The intercept and slope were not significantly different from zero and one, respectively.

3.5. Validation

Linear calibration curves were generated by 1/amount-weighted linear regression analysis. It was necessary to use weighting, since the highest calibration point was 2000 times the lowest calibration point. It was advantageous to use 1/amount-weighted regression instead of dividing the calibration range or using any other weighting. Precision, accuracy and recovery during the validation are shown in Table 2. The recovery of the internal standard was 88.5 ± 2.5% (±S.D.) and independent of LF concentration. The assay was linear in the range 0.024–20.0 and 0.021–1.01 μg/mL for LF and

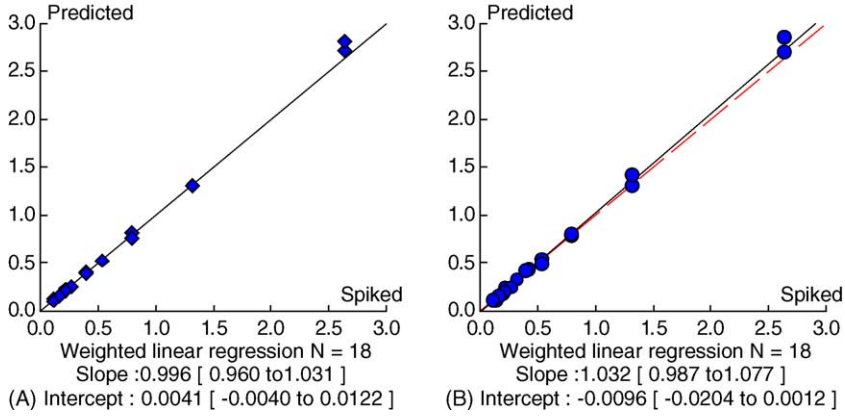

Fig. 3. Mean plots of spiked pre-prandial (A) and post-prandial (B) plasma. Predicted concentration (μg/mL) using a freshly prepared calibration curve (*y*) vs. spiked nominal concentration (μg/mL) (*x*).

DLF, respectively, with correlation coefficients $r > 0.99$ using eight standards. Back-calculated values for the calibration standards were used to verify linearity. The mean slopes for the calibration curves ($n = 5$) were 0.4417 (S.D. 0.0084) and 0.4500 (S.D. 0.0075), and the mean intercepts were 0.0033 (S.D. 0.0052) and −0.0026 (S.D. 0.0015) for LF and DLF, respectively. The lower limit of quantification (LLOQ) was determined to 0.024 and 0.021 μg/mL for LF and DLF, respectively, with a R.S.D. and accuracy <20%, and a signal-to-noise ratio of about 10 [8]. The limit of detection (LOD) was 0.010 μg/mL for both analytes. 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) [8,13]. Fig. 4 shows an overlay of chromatograms from a spiked plasma sample at the lower limit of quantification (LF 0.024 μg/mL and DLF 0.021 μg/mL) and blank plasma.

The analytes were stable during the freeze/thaw cycles and for at least 24 h in each step of the method. Long-term storage stability showed that LF was stable in plasma at both −86 and −20 °C for at least 4 months (i.e. >88% recovered). This is in disagreement with the previous report stating that about 25% of LF had degraded when stored at −20 °C for 3 months [6]. The samples stored at 8 °C contained approximately the same average content but the variation was significantly higher. The beginning of the chromatograms also contained much more endogenous peaks than what was found for the samples stored at −86 and −20 °C. It is recommended that plasma samples are stored frozen at −86 or −20 °C.

No interference from the other related antimalarials were observed when they were injected into the LC-system (Fig. 5). All compounds were detected at 335 nm except mefloquine and mefloquine carboxyl metabolite that were detected at 220 nm because of poor absorbance at 335 nm. No endogenous peaks that would interfere with the quantification of DLF and LF were observed from the different plasma sources.

3.6. Method performance during a clinical study

The total precision for all quality controls ($n = 23$ at each level) during the analysis of LF was 13.8, 6.4 and 5.7% at 0.042, 0.419 and 8.02 μg/mL, respectively. DLF was not quantified in this day 7 studies. The results from the clinical study will be published elsewhere. A chromatogram showing a patient plasma sample is shown in Fig. 6.

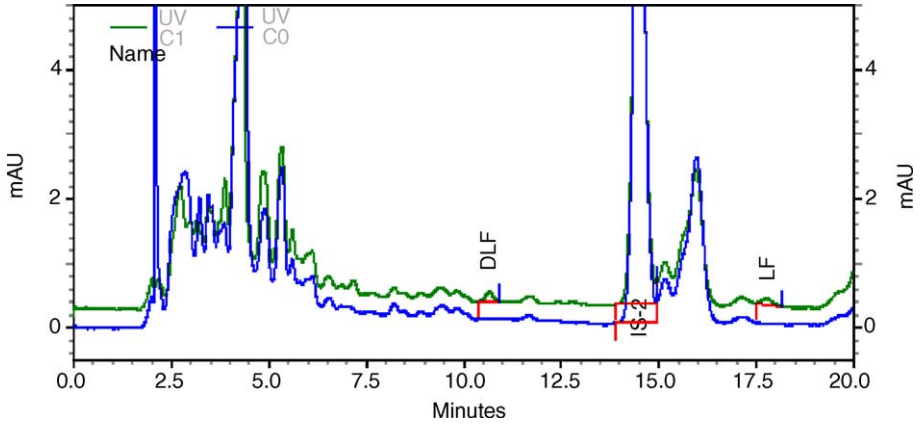

Fig. 4. Spiked plasma sample (LF 0.024 μg/mL and DLF 0.021 μg/mL) and zero-plasma.

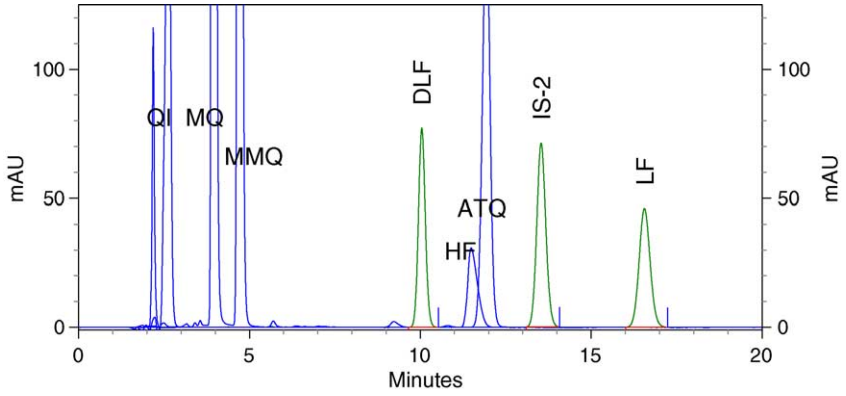

Fig. 5. Chromatograms demonstrating the selectivity of the LC-system. Direct injections of quinine (QI), mefloquine (MQ), mefloquine carboxyl metabolite (MMQ), halofantrine (HF), atovaquone (ATQ), DLF, IS-2 and LF.

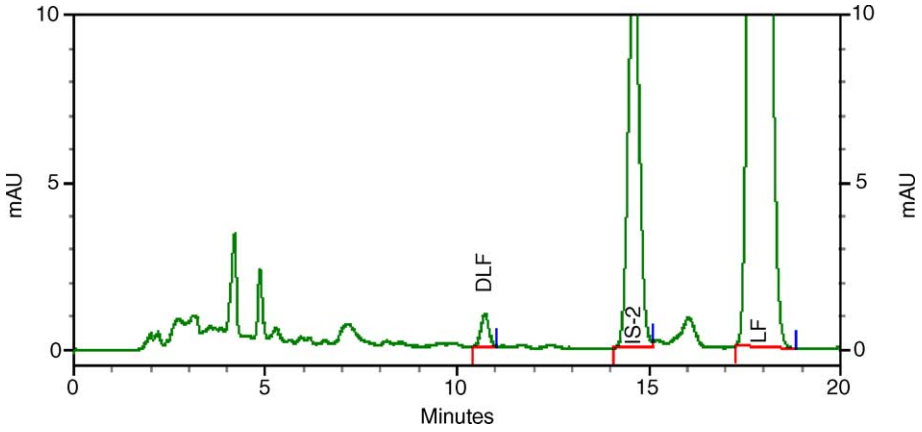

Fig. 6. Patient sample containing DLF.

4. Conclusion

A bioanalytical method for the simultaneous determination of the putative metabolite desbutyl-lumefantrine and lumefantrine in plasma has been developed and validated. The assay has been proven sensitive and reproducible and uses only 0.25 mL of plasma. The assay was linear in the wide calibration range 0.024–20.0 μg/mL by the use of 1/amount-weighted regression. The assay has been implemented for the analysis of clinical samples with satisfying performance data for the daily control samples. Lumefantrine was found to be stable at −20 °C for at least 4 months and can consequently be stored temporarily at this temperature during field-based clinical studies.

Acknowledgements

We are grateful to Novartis Pharma AG for providing lumefantrine, desbutyl-lumefantrine and the two internal standards. This study was funded by the Welcome Trust of Great Britain.

References

[1] World Health Organisation Expert Committee on Malaria, 20th report, WHO, Geneva, 2000.
[2] N.J. White, F. Nosten, S. Looareesuwan, W.M. Watkins, K. Marsh, R.W. Snow, G. Kokwaro, J. Ouma, T.T. Hien, M.E. Molyneux, T.E. Taylor, C.I. Newbold, T.K. Ruebush II, M. Danis, B.M. Greenwood, R.M. Anderson, P. Olliaro, Lancet 353 (1999) 1767–1965.
[3] D. Colu, C. Parisot, F. Leg, G. Lefevre, Eur. J. Pharm. Sci. 9 (1999) 9–16.
[4] N.J. White, M. van Vugt, F. Ezzet, Clin. Pharmacokinet. 37 (1999) 105–125.
[5] H. Noedl, T. Allmendinger, S. Prajakwong, G. Wernsdorfer, W.H. Wernsdorfer, Antimicrob. Agents Chemother. 45 (2001) 2106–2109.
[6] S.M. Mansor, V. Navaratnam, N. Yahaya, N.K. Nair, W.H. Wernsdorfer, P.H. Degen, J. Chromatogr. B. Biomed. Appl. 682 (1996) 321–325.
[7] M.Y. Zeng, Z.L. Lu, S.C. Yang, M. Zhang, J. Liao, S.L. Liu, X.H. Teng, J. Chromatogr. B. Biomed. Appl. 681 (1996) 299–306.
[8] Guidance for Industry Bioanalytical Method Validation, Department of Health and Human Services, Food and Drug Administration, Rockville, US, 2001.
[9] C.J. Porter, S.M. Caliph, W.N. Charman, J. Pharm. Biomed. Anal. 16 (1997) 175–180.

[10] R.J. Artymowicz, V.E. James, Clin. Pharm. 12 (1993) 563–570.
[11] G. Colmenarejo, A. Alvarez-Pedraglio, J.L. Lavandera, J. Med. Chem. 44 (2001) 4370–4378.
[12] A.J. Humberstone, G.J. Currie, C.J. Porter, M.J. Scanlon, W.N. Charman, J. Pharm. Biomed. Anal. 13 (1995) 265–272.
[13] C. Hartmann, J. Smeyers-Verbeke, D.L. Massart, R.D. McDowall, J. Pharm. Biomed. Anal. 17 (1998) 193–218.