Journal of Chromatography B, 876 (2008) 261–265

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

A field-adapted sampling and HPLC quantification method for lumefantrine and its desbutyl metabolite in whole blood spotted on filter paper

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ARTICLE INFO

Article history:
Received 24 June 2008
Received in revised form 6 October 2008
Accepted 2 November 2008
Available online 7 November 2008

Keywords:
Lumefantrine
Desbutyllumefantrine
Filter paper

ABSTRACT

A quantitative reverse-phase HPLC method with UV detection, for lumefantrine (LF) and desbutyllumefantrine (DLF) in whole blood spotted on filter paper was developed. The analytes were stabilized on filter paper by treatment of blood with phosphoric acid (1.6 mol/L). Halofantrine was used as internal standard and the analytes were extracted from filter paper using methanol. The methanolic extract was extracted with di-isopropylether after addition of acidic phosphate buffer (pH 2). Chromatographic separation was carried out on a Zorbax Eclipse XDB-phenyl column (4.6 mm × 150 mm, particle size 5 μm) at a flow rate of 1 mL/min using a mobile phase of acetonitrile–ammonium acetate buffer (0.1 M ammonium acetate and 0.01 M acetic acid, pH 6.5) (10:90). The absorbance of the compounds was monitored at 335 nm. The average extraction recovery from filter paper ranged between 45–51% for LF and 25–33% for DLF for a concentration range between 300 and 3000 nM. Inter- and intra-assay coefficients of variation for LF and DLF were ≤9.2. Limits of quantification for LF and DLF were 300 nM. The method has been applied in malaria patients. In conclusion, a simple procedure for blood sampling and quantitative measurement of lumefantrine and desbutyllumefantrine suitable for field studies in resource-limited laboratories was developed.

1. Introduction

With about 300 million cases per year, malaria remains the world's most important infectious disease in terms of human suffering and death [1–3]. The burden of malaria disease continues to increase as the countries in which it is endemic face the risk of widespread resistance of the parasite to conventional antimalarial drugs and increasing resistance of the vector to insecticide [4,5]. Emerging resistance and problematic side-effects to known antimalarial drugs have stimulated the search for new therapies such as Coartem® [6].

Coartem® is a well-tolerated, fast-acting and effective blood schizontocidal drug that serves primarily to treat uncomplicated falciparum malaria that is resistant to other antimalarials and has generally been adopted as a first-line treatment of uncomplicated falciparum malaria in most countries [2,7–10]. It combines the fast onset of action of artemether (an artemisinin derivative) in terms of prompt parasite clearance with the long duration of action and high cure rate of lumefantrine (LF) [10–12].

Lumefantrine, 2-(dibutylamino)-1-[(9E)-2,7-dichloro-9-[(4-chlorophenyl)methylidene] fluoren-4-yl]ethanol (Fig. 1) is an arylamino alcohol [13]. It is a lipophillic compound with low intrinsic clearance and erratic oral variability and therapeutic levels are more reliably achieved by co-administration with a fatty meal [13–18].

Studies indicate that the area under the plasma concentration–time curve is the most important pharmacokinetic determinant of the therapeutic response following artemether plus lumefantrine treatment and day 7 levels below 280 ng/mL (529 nM) are a useful predictor for cure [16,19,20]. The main metabolite, desbutyllumefantrine (DLF) (Fig. 1) has been found to have significantly higher antiplasmodial activity as compared with the parent compound [2,15].

Clinical studies with Coartem® under field conditions require suitable methods for sampling and quantitative determination of LF and DLF. Several HPLC-based methods have been published for determination of LF in plasma [21–24]. These are not suitable for field studies in resource-limited countries because of their immediate requirement of electricity during collection of plasma. Sampling of whole blood on filter paper is a suitable approach but problematic as lumefantrine is unstable on filter paper [25].

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doi:10.1016/j.jchromb.2008.11.002

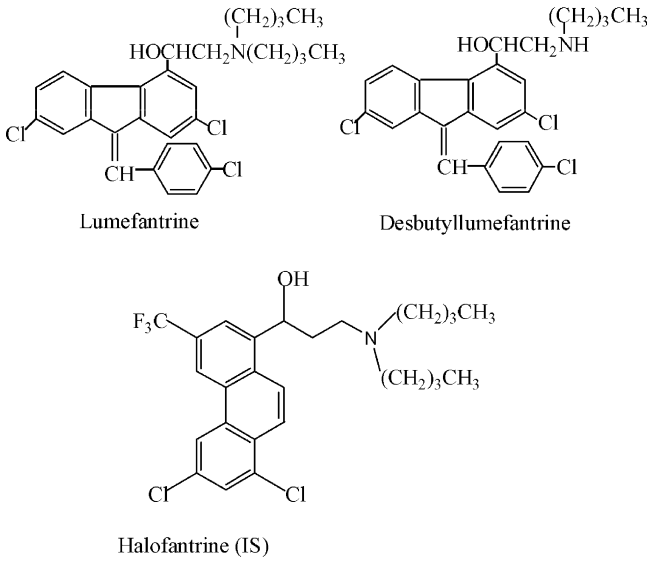

Fig. 1. Structures of lumefantrine, desbutyllumefantrine and halofantrine (IS).

We recently developed a filter paper sampling procedure for amodiaquine, which also had stability problems when whole blood was sampled on filter paper [26]. The aim of this work was to develop and validate a sampling method for whole blood samples, and an affordable HPLC method for the simultaneous quantitative determination of LF and DLF. Our method involves liquid–liquid extraction that is cheaper than solid phase extraction as reported by Blessborn et al. [25]. It is therefore suitable for resource-limited settings.

2. Materials and methods

2.1. Chemicals and reagents

LF, DLF (Fig. 1) were obtained from Novartis Pharma AG (Basel, Switzerland). Halofantrine, which was used as internal standard (IS) (Fig. 1) was a gift from Mr. Byamugisha Tarsis from the Uganda National Drug Authority. Acetonitrile (HPLC-grade), methanol (pro analysis), acetic acid (pro analysis), sodium dihydrogen phosphate and ortho-phosphoric acid were obtained from Merck gmbH (Darmstadt, Germany). Perchloric acid (HPLC-grade) was obtained from BDH (Poole, England). The phosphate buffer solutions were prepared by mixing appropriate amounts of sodium dihydrogen phosphate and ortho-phosphoric acid. All other chemicals were analytical grade and were used without purification. The filter paper used was Whatman No. 3. However, other types of paper can be used as long as the same papers are used for the standard curve.

2.2. Instrumentation and chromatographic conditions

The chromatographic analysis was carried out using an Agilent Technologies Chemstation LC System (1100 series, Brookside, Pkwy, Alpharetta, USA) equipped with a photometric detector, a standard flow cell, a binary pump with a degasser and an auto-sampler. The analytical column was a Zorbax Eclipse XDB-Phenyl column (150 mm × 4.6 mm I.D.), 5 μm (Chromtech, Hargersten, Sweden), connected to a short Zorbax guard column EXT C18 (4 mm × 3 mm I.D.) (Agilent, Palo Alto, USA) using a mobile phase containing 10% acetonitrile in ammonium acetate buffer (0.1 M ammonium acetate and 0.01 M acetic acid, pH 6.5). The absorbance was monitored at 335 nm and elution was carried out at room temperature using a flow rate of 1.00 mL/min.

2.3. Sampling of whole blood on filter paper

Concentrated stock solutions of LF, DLF, or IS were prepared by dissolution of each compound in methanol with about five drops of acetic acid and stored at −70 °C. During method development and validation, there was need to have enough blood. Thus, fresh venous whole blood as opposed to capillary blood was collected from drug-free healthy volunteers in vacuum tubes with EDTA as anticoagulant. Phosphoric acid (1.6 mol/L) was fortified with LF and DLF using fresh stock solutions to achieve 300–3000 nM concentrations. Aliquots of collected blood (100 μL) were mixed with equal amounts of the fortified phosphoric acid solutions and 100 μL of the mixture was spotted onto a filter paper and allowed to dry for 2–3 days. It was necessary to spike phosphoric acid instead of whole blood to avoid hemolysing the blood and therefore cause rapid breakdown of the analytes before drying on filter paper. Unknown samples were prepared by mixing whole blood with an equal amount of phosphoric acid and 100 μL of the mixture was spotted on filter paper as described. The prepared filter paper samples were stored at room temperature between 20 and 25 °C until assay.

2.4. Extraction procedure

Filter paper containing dried blood spots was cut into four to six small pieces and put into 12 mL polypropylene test tubes. Subsequently, 50 μL of IS solution (144 μM in methanol) and 1 mL of methanol were added followed by sonication for 15 min. Thereafter, 2 mL of sodium phosphate buffer (0.4 M, pH 2), 120 μL of KOH (1 M) and 8 mL of di-isopropylether were added. Addition of KOH was necessary so as to cater for the acid in the filter paper. The test tubes were shaken for 20 min and centrifuged for 10 min at 3500 × *g*. The organic (upper) phase was transferred to a new polypropylene test tube and gently evaporated with dry nitrogen and left to stand overnight in a fume hood (circulating dry air). The residue was reconstituted in 150 μL of mobile phase and 130 μL was injected the chromatograph.

2.5. Standards and calibration procedure

Calibrators were prepared as described above and peak height ratios of the analytes to the IS were plotted against concentrations. In our experience we preferred to use peak heights instead of peak areas. This was particularly advantageous during analysis of patient samples where some interfering peaks became evident. For method validation, a standard curve with four to five calibration points was run on each day of analysis and a regression equation with the slope, intercept and correlation coefficient (r^2) was generated automatically with the ChemStation software.

2.6. Method validation

2.6.1. Recovery

The recovery of LF and DLF after application and storage of blood onto filter paper and during extraction was documented at three different concentrations using 18 dry filter paper spots prepared on the same day. The filter papers were worked up as described and the peak heights were compared with those from a direct injection of a reference solution containing comparable amount of each analyte.

2.6.2. Accuracy and precision

The accuracy and precision were documented using prepared controls at three different concentrations of LF and DLF in blood dried on filter paper. The controls were assayed as described before both in one series (intra-day) and on different occasions (inter-day).

Table 1
Recovery for LF and DLF from filter paper to final extract.

Nominal conc. (nM)	Absolute recovery (mean ± S.D.) (%)		
	Lumefantrine	Desbutyllumefantrine	*n*
500	45 ± 2	29 ± 3	6
1000	51 ± 4	28 ± 4	6
3000	45 ± 3	25 ± 2	6

The variability in the measurement of the analyte was expressed as a coefficient of variation (C.V.).

2.6.3. Limit of quantification

Our limit of quantification (LOQ) was taken as the smallest point on the calibration curve. In our case, 300 nM is reported as the LOQ because with this concentration, we were able to quantify both analytes with a C.V. not more than 15%. Our method in real terms utilizes only 50 μL of whole blood that is mixed with an equal volume of phosphoric acid to make a blood spot of 100 μL but higher sensitivity can be achieved if all the 100 μL of whole blood is used.

2.6.4. Storage stability

The stability of the compounds was investigated by analyzing the spots over a period of 5 months. The calibrators used during the analysis were always prepared using a fresh stock solution of LF and DLF.

2.6.5. Interference

Interference of commonly used antimalarials such as chloroquine, amodiaquine, desethylamodiaquine, quinine, mefloquine and paracetamol with the analytes of our method was studied by adding the different compounds to phosphoric acid (1.6 mol/L), mixing with drug-free blood and analyzing the samples according to the method. The drug-free whole blood was obtained from eight individuals.

2.6.6. Clinical application

The method was used to analyze for lumefantrine from whole blood spotted on filter paper. Day 3 and day 7 venous whole blood samples were taken from four malaria patients who had been treated with a standard dose of Coartem® (four tablets containing 20 mg artemether and 120 mg lumefantrine, twice daily for 3 days). The samples were treated according to the described procedure and were kept at room temperature until they dried. They were later analyzed at the Department of Laboratory Medicine, Division of Clinical Pharmacology, Karolinska University Hospital, Huddinge, Sweden. However, Patient D did not return for follow up on day 7 but day 3 values for the same patient were available and were therefore included.

Table 2
Accuracy, intra- and inter-assay imprecision for LF and DLF.

Analyte	Nominal conc. (nM)	Amount measured (mean ± S.D.)	C.V. (%)		
			Intra-day	Inter-day	*n*
LF	500	489 ± 17	9.2	3.4	6
	1000	931 ± 33	4.0	3.6	6
	3000	3068 ± 114	3.9	3.7	6
DLF	500	560 ± 38	6.2	6.7	6
	1000	977 ± 78	5.1	8.0	6
	3000	3301 ± 138	1.9	4.2	6

3. Results and discussion

The present study demonstrates that the sampling procedure using filter paper recently developed for amodiaquine and its metabolite [26] could also be used for stabilizing lumefantrine and its metabolite. Studies on amodiaquine suggested that inac-

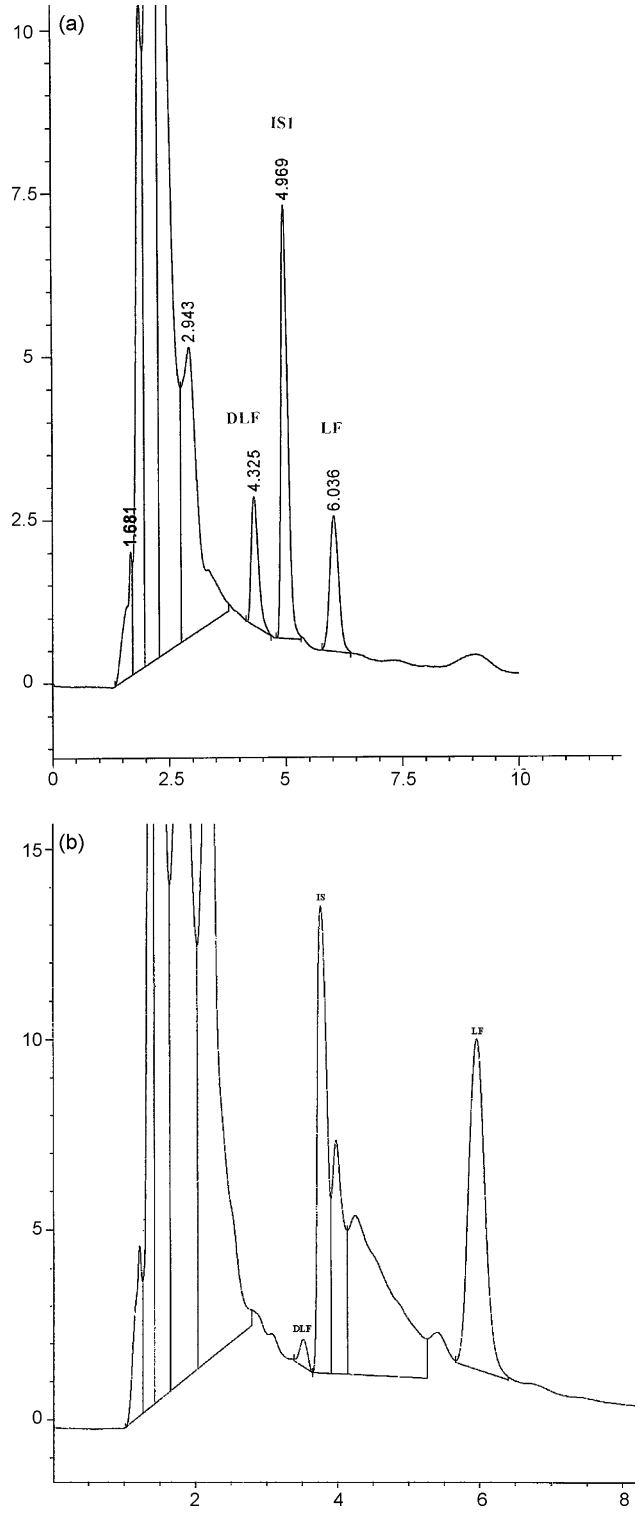

Fig. 2. Chromatograms showing a processed sample containing lumefantrine (LF, 1000 nM), desbutyllumefantrine (DLF, 1000 nM), internal standard (IS) (a) and a day 3 patient sample spiked with IS (b).

Table 3
Whole blood concentrations of LF and DLF for patients on standard medication with Coartem®.

Analyte		Concentration (nM)			
		A	B	C	D
LF	Day 3	3918	978	3786	9250
	Day 7	704	576	1084	NS
DLF	Day 3	678	<300	640	888
	Day 7	<300	<300	<300	NS

NS: no sample.

tivation of iron from hemoglobin was essential for recovery of the analytes. This is most likely due to inhibition of oxidation of the analytes when hemoglobin is released from the red blood cells after application on the filter paper. Phosphoric acid is effective in binding with iron (II) and enabled a high recovery of the analytes.

Stability of the analytes was demonstrated over the entire concentration range (300–3000 nM), as the measured values for both LF and DLF were between 93 and 112% of the nominal values for a period of 5 months. In this work, the filter paper spots were stored under two different conditions, that is, at about 20 °C in Sweden for 2 months and at room temperature (25 °C) in Uganda for 3 months before they were analyzed. No significant changes in concentration (<10%) were observed during storage at both conditions.

A somewhat similar procedure using tartaric acid for stabilizing LF on filter paper was published recently [25]. The practical procedure used was based on drying filter paper with tartaric acid solution and then applying the blood directly onto the paper. In our experience with phosphoric acid, it was essential to mix the blood with the acid before applying it to filter paper. The authors discussed that the lipophillic nature of LF caused it to adsorb to the surface. We think also that a chemical breakdown due to oxidation should be considered, and that phosphoric acid is an essential component in the procedure.

Using the described conditions, the chromatograms of lumefantrine, desbutyllumefantrine and internal standard obtained from spiked and patient blood samples are shown in Fig. 2(a) and (b). The blank blood ($n = 10$) was free from chromatographic interference from the background. The LF, DLF and internal standard eluted as independent peaks.

The overall recovery of LF and DLF including extraction from filter paper, extraction into the organic phase and back to the phosphate buffer was low (Table 1) but comparable to that reported earlier [25]. The low recovery values may potentially lead to variability in the method performance. This however, was not the case as adequate accuracy and precision was achieved with our method. Both the accuracy and precision are reported in Table 2 and fulfill acceptance criteria for a quantitative method. As reported earlier in a similar method published in our laboratory [26], some potassium hydroxide was added to the filter paper samples during the extraction process to give an optimum pH for simultaneous extraction of LF and DLF.

The linearity of the method as measured by the correlation coefficient of inter-assay linear regression curves (r^2) was better than 0.99 for both LF and DLF in all cases in the measured range of 300–3000 nM. The (mean ± S.D.) regression equation for the calibration curves prepared from calibrators was ($y = 0.0002x + (0.0239 \pm 0.0095)$, $r^2 = 0.9950 \pm 0.0024$) ($n = 5$) for LF (y = peak height ratio LF/I.S., x = concentration of LF in nmol/L and $y = 0.0003x + (0.0303 \pm 0.0147)$, $r^2 = 0.9948 \pm 0.0036$) ($n = 5$) for DLF (y = peak height ratio DLF/I.S., x = concentration of DLF in nmol/L). The limit of quantification was 300 nM for both LF and DLF.

The method was applied to four malaria patients A, B, C and D aged 12, 12, 13 and 43 years, respectively, who had been treated with the recommended dose of Coartem® (four tablets containing 20 mg artemether and 120 mg lumefantrine, twice daily for 3 days). With our method it was possible to accurately quantify LF on day 7 and the metabolite too, up to day 3 as shown in Table 3. However, no DLF was detected on day 7 for all the patients.

4. Conclusion

A quantitative HPLC method for lumefantrine and its major metabolite in whole blood applied on filter paper was successfully developed. The sampling procedure is simple and we consider the method appropriate for measuring therapeutic drug concentrations of lumefantrine and the metabolite in field studies.

Acknowledgements

This study was funded by SIDA/SAREC through Makerere University/Karolinska Institute Research Collaboration and by grants SIDA/SAREC (SWE-1999-260, 99-266 Bil. Th. 106 and SWE-2002-063). We are grateful to Mr. Byamugisha Tarsis of Uganda National Drug Authority for providing halofantrine, Hajjat Kafuuma Safiina of Kawempe Muslim Secondary School for the technical support offered during collection of blood samples from the patients, Dr. Per Andersson from Karolinska Institute for all the initiative he undertook to have us obtain the reference materials and to Novartis Pharma AG for providing lumefantrine and desbutyllumefantrine.

References

[1] M. Ashton, T. Gordi, T.N. Hai, N.V. Huong, N.D. Sy, N.T. Nieu, D.X. Huong, M. Johansson, L.D. Cong, Biopharm. Drug Dispos. 19 (1998) 245.
[2] W.H. Wernsdorfer, Expert Rev. Anti-Infect. Ther. 2 (April) (2004) 181.
[3] M. Rajankanth, K.P. Madhusudanan, R.C. Gupta, J. Chromatogr. B 783 (2003) 391.
[4] T.M. Davis, H.A. Karunajeewa, K.F. Ilett, Med. J. Aust. 182 (2005) 181.
[5] D. Webster, A.V.S. Hill, Bull. World Health Organ. 81 (2003) 902.
[6] V. Agtmael, A. Michiel, J.J. Butter, E.J.G. Els, V. Boxtel, J. Chris, Ther. Drug Monit. 20 (1998) 109.
[7] W.H. Wernsdorfer, B. Landgraf, V.A. Kilimali, G. Wernsdorfer, Acta Trop. 70 (1998) 9.
[8] A.A. Omari, C. Gamble, P. Garner, Trop. Med. Int. Health 9 (2004) 192.
[9] A.A. Omari, C. Gamble, P. Garner, Cochrane Database Syst. Rev. (2005), CD005564.
[10] G. Lefevre, P. Carpenter, C. Souppart, M.S.H. Schmidli, J.M. Martin, A. Lane, C. Ward, D. Amakye, J. Clin. Pharmacol. 42 (2002) 1147.
[11] M. Bindschedler, G. Lefevre, F. Ezzet, N. Schaeffer, I. Meyer, M.S. Thomsen, Eur. J. Clin. Pharmacol. 56 (2000) 375.
[12] R. Bakshi, I. Hermeling-Fritz, I. Gathmann, E. Alteri, Trans. R Soc. Trop. Med. Hyg. 94 (2000) 419.
[13] N.J. White, M.V. Vugt, F. Ezzet, Clin. Pharmacokinet. 37 (1999) 105.
[14] F. Ezzet, M.V. Vugt, F. Nosten, S. Looareesuwan, N.J. White, Antimicrob. Agents Chemother. (2000) 697.
[15] H. Noedl, T. Allmendinger, S. Prajakwong, G. Wernsdorfer, W.H. Wernsdorfer, Antimicrob. Agents Chemother. 45 (2001) 2106.
[16] M.V. Vugt, F. Ezzet, L. Phaipun, F. Nosten, N.J. White, Trans. R Soc. Trop. Med. Hyg. 92 (1998) 564.
[17] E.A. Ashley, K. Stepniewska, N. Lindegardh, A.A.A. Kham, A. Brockman, P. Singhasivanon, N.J. White, F. Nosten, Trop. Med. Int. Health 12 (2007) 195.
[18] A.A. Ashley, K. Stepniewska, N. Lindegardh, R. McGready, A.A.R. Hutagalung, T. Singtoroj, G. Hla, A. Brockman, S. Proux, J. Wilahphaingern, P. Singhasivanon, N.J. White, F. Nosten, Trop. Med. Int. Health 12 (2007) 201.
[19] R.N. Price, A.-C. Uhlemann, M.V. Vugt, A. Brockmann, R. Hutagalung, S. Nair, D. Nash, P. Singhasivanon, T.J.C. Anderson, S. Krishna, N.J. White, F. Nosten, Clini. Infect. Dis. 42 (2006) 570.
[20] F. Ezzet, Proceedings of Conference on European co-operation in the field of Scientific and technical research Geneva, European Commission, Brussels, 1997, p. 319.
[21] M.-Y. Zeng, Z.-L. Lu, S.C. Yang, M. Zhang, J. Liao, S.-L. Liu, X.-H. Teng, J. Chromatogr. B 681 (1996) 299.

[22] S.M. Mansor, V. Nvaratnam, N. Yahaya, N.K. Nair, W.H. Wernsdorfer, P.H. Degen, J. Chromatogr. B 682 (1996) 321.
[23] A. Annenberg, T. Singtoroj, P. Tipmanee, N.J. White, N.P. Day, N. Lindegardh, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 822 (2005) 330.
[24] N. Lindegårdh, A. Annerberg, D. Blessborn, Y. Bergqvist, N. Day, N.J. White, J. Pharm. Biomed. Anal. 37 (2005) 1081.
[25] D. Blessborn, S. Romsing, A. Annerberg, D. Sundquist, A. Bjorkman, N. Lindegårdh, Y. Bergqvist, J. Pharm. Biomed. Anal. (2007), doi:10.1016/j.jpba.2007.015.
[26] M. Ntale, M. Mahindi, J.W. Ogwal-Okeng, L.L. Gustafsson, O. Beck, J. Chromatogr. B 859 (2007) 137.