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Quantification of cationic anti-malaria agent methylene blue in different human biological matrices using cation exchange chromatography coupled to tandem mass spectrometry

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Abstract

Selective and sensitive methods for the determination of the cationic dye and anti-malarial methylene blue in human liquid whole blood, dried whole blood (paper spot), and plasma depending on protein precipitation and cation exchange chromatography coupled to electrospray ionisation (ESI) tandem mass spectrometry (MS/MS) have been developed, validated according to FDA standards, and applied to samples of healthy individuals and malaria patients within clinical studies. Acidic protein precipitation with acetonitrile and trifluoroacetic acid was used for liquid whole blood and plasma. For the extraction of methylene blue from paper spots aqueous acetonitrile was used. Sample extracts were chromatographed on a mixed mode column (cation exchange/reversed phase, Uptisphere MM1) using an aqueous ammonium acetate/acetonitrile gradient. Methylene blue was quantified with MS/MS in the selected reaction monitoring mode using ESI and methylene violet 3RAX as internal standard. Depending on the sample volume (whole blood and plasma 250 μL, and 100 μL on paper spots) the method was linear at least within 75 and 10,000 ng/mL and the limit of quantification in all matrices was 75 ng/mL. Batch-to-batch accuracies of the whole blood, plasma, and paper spot methods varied between −4.5 and +6.6%, −3.7 and +7.5%, and −5.8 and +11.1%, respectively, with corresponding precision ranging from 3.8 to 11.8% CV. After a single oral dose (500 mg) methylene blue concentrations were detectable for 72 h in plasma. The methods were applied within clinical studies to samples from healthy individuals and malaria patients from Burkina Faso.

Keywords: Methylene blue; Cation exchange chromatography; Tandem mass spectrometry; Whole blood; Plasma; Paper spots

1. Introduction

The use of the thiazine dye methylene blue (MB) for the treatment of malaria has been described over 100 years ago [1,2]. The mechanism of its anti-malarial activity is not fully understood, but MB has been shown to inhibit the glutathione reductase of *Plasmodium falciparum* more effectively than human glutathione reductase [3]. Inhibition of glutathione reductase leads to a decrease in intracellular levels of reduced glutathione, which is essential for the degradation of haematin [4,5]. Reduced degradation might result in an accumulation of haematin, which leads to poisoning of the parasites. In vitro experiments have confirmed the anti-malarial potency of MB alone and in combination with other anti-malarials [6]. Compared with other thiazine dyes MB yielded the highest activity and selectivity against different isolates of *P. falciparum* with IC50 values of approximately 4 nmol/L [7]. Whole blood concentrations of up to 100 nmol/L (=28.4 ng/mL) were reached in healthy individuals after oral administration of 100 mg MB [8], which is a dose usually applied for the oral treatment of methaemoglobinaemia [9]. On the basis of the in vitro results this dosage is expected to yield anti-malarial effectiveness. For dose finding, the evaluation of pharmacokinetics, and the safety of MB co-administered to a typical chloroquine regimen clinical stud-

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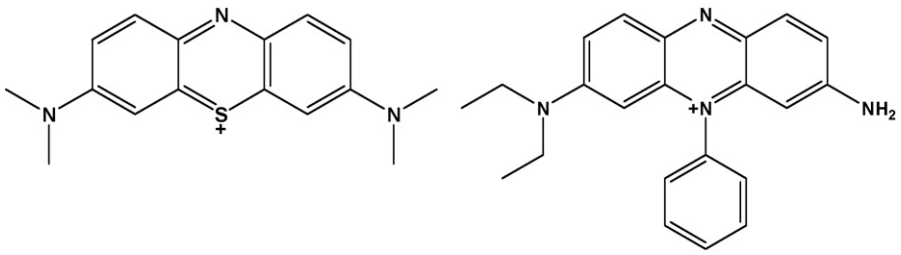

methylene blue
(analyte)
CAS 61-73-4
$[C_{16}H_{18}N_3S]^+$
Exact mass: 284.12
Mol. Wt.: 284.40

methylene violet 3RAX
(internal standard)
CAS 4569-86-2
$[C_{22}H_{23}N_4]^+$
Exact mass: 343.19
Mol. Wt.: 343.33

Fig. 1. Chemical structures and characteristics of the cations methylene blue (analyte) and methylene violet 3RAX (internal standard).

ies were performed [10–13]. Within these and further studies analytical quantifications of MB were necessary for pharmacokinetic assessment and documentation of compliance. Because the assay was also to be used in blood samples of children, high sensitivity and selectivity in combination with fast quantification in low sample volumes of whole blood and plasma were required. Another demand was MB quantification from paper spots, which were prepared for safe and easy transfer of originally infectious blood samples from Burkina Faso (Africa) to the laboratory in Heidelberg (Germany). For this purpose whole blood (100 μL) was spotted on filter paper and dried, which is a usual procedure [14–16]. The analytical methods had to ensure quantitative extraction and selective determination separated from potential artefacts caused by extraction from paper and biological matrix. Previous studies in which MB had to be analysed in biological matrices used high-performance liquid chromatography (HPLC) with UV-detection at high wavelength (>600 nm) and liquid/liquid extraction with chlorinated organic solvent [8,17]. Quantification in urine has been performed using capillary electrophoresis with UV-detection and liquid/liquid extraction with chlorinated organic solvent [18]. To our knowledge liquid chromatography coupled to tandem mass spectrometry (LC/MS/MS) has never been applied to MB quantification in biological matrices apart from our previous studies [6,10–13]. Chromatography of ionic analytes in general including MB (Fig. 1) should usually be performed using ion pair chromatography or ion exchange chromatography. Both do not really fit the demands of ESI-MS/MS which excludes non-volatile ingredients from the liquid phase. On the other hand reversed phase chromatography of polar ionic analytes without ion pair reagent will lead to dead volume elution. Therefore, in this paper we describe the development, validation [19,20], and application of ion exchange chromatography using a mixed mode phase onto cationic methylene blue. Although highly concentrated volatile buffers in gradient mode and extracts from biological matrices were applied to the ESI-MS/MS system no contamination of the ion source occurred. Quantification of MB is also challenging because of the dye's adsorptive properties during the sample preparation process which causes binding to different surfaces (e.g. glass, plastic, and endogenous substances like proteins). In previous studies [8,10] very low concentrations of MB in plasma were determined. We hypothesised that a large proportion of available MB will build up bound residues which will not be released with common liquid/liquid extraction procedures. In this paper we describe extraction procedures which led to more concentrated extracts by liberating more MB from the biological matrices. This was achieved by addition of acids or cationic reagents to displace MB from its counter ion. Hence it can be assumed that the use of former methods resulted mainly in the determination of the free fraction of MB in the respective matrix.

2. Clinical studies, participants, materials, and methods

2.1. Clinical studies and participants

The study protocols were approved by the Ethics Committee of the Medical Faculty of Heidelberg. The studies were conducted by the Departments of Internal Medicine VI, Clinical Pharmacology and Pharmacoepidemiology, and of Tropical Hygiene and Public Health in accordance with good clinical practice guidelines, the Declaration of Helsinki, and local legal requirements. For the determination of pharmacokinetic parameters healthy individuals received MB orally (500 mg) and intravenously (50 mg). The pharmacokinetic profiles of MB in plasma of one individual after oral and intravenous administration are shown in this paper; the pharmacokinetic results will be published elsewhere.

Within a clinical study in Burkina Faso, 20 paediatric patients received MB 20 mg/kg body weight MB orally for malaria therapy. Whole blood samples (100 μL) 1 h after administration were spotted onto filter paper, dried, stored darkly at room temperature, and transferred to Heidelberg for analysis.

2.2. Materials

The aqueous solution of MB containing 25 mg/mL was supplied by Mayrhofer Pharmazeutika GmbH (Linz, Austria) under contract of DSM Fine Chemicals Austria (Linz,

Austria). For analytical purposes methylene blue chloride certified reference standard (CAS-No. 61-73-4; 87% content pure dye) originated from Calbiochem-Novabiochem GmbH (Bad Soden/Germany) and methylene violet 3RAX reference standard (internal standard, CAS-No. 4569-86-2; 90.8% content pure dye) was supplied by Sigma–Aldrich Fine Chemicals (Taufkirchen/Germany). Whatman filter paper (Cat-No. 10535097) for whole blood paper spots was supplied by Schleicher & Schüll (Dassel/Germany). Blank whole blood and plasma for calibration and validation procedures was donated by healthy individuals. All other reagents and solvents used for chromatographic, spectroscopic, and sample preparation were of analytical or higher quality and were purchased from E. Merck (Darmstadt, Germany). Water was deionised and filtered by a TKA system HP 6UV/UF (Niederelbert, Germany).

2.3. Human blood samples

EDTA whole blood samples (2.5 mL each) were immediately frozen and stored at −20 °C. Heparinised blood samples (7.5 mL each) were immediately centrifuged (3000 × *g* for 10 min at 4 °C) and separated plasma samples were stored at −20 °C until analysis.

2.4. Standard solutions

The internal standard methylene violet 3RAX was weighed (9.90 mg) into a volumetric flask (10 mL) and filled up with acetonitrile/water (1/1, v/v). For whole blood and plasma 50.6 μL were diluted to a final volume of 20 mL and for paper spot analysis 10.1 μL to 10 mL using acetonitrile/water (1/1, v/v).

For calibration 10.02 mg of MB reference standard was weighed into 10 mL volumetric flasks that were filled up with acetonitrile/water (1/1, v/v). From this stock solution the highest calibration solution for whole blood and plasma was prepared by transferring 1147 μL of the stock solution into a 10 mL volumetric flask and filled up with acetonitrile/water (1/1, v/v). For paper spot calibration 4588 μL of the stock solution were diluted to 10 mL resulting in the highest paper spot calibration solution. All other calibration solutions were prepared by diluting this solution with acetonitrile/water (1/1, v/v).

For quality control (QC) of whole blood, plasma, and paper spots, the standard solution was prepared as described for calibration; the amount of MB reference standard weighed for this solution was 9.20 mg. From this main solution a QC stock solution was prepared in the same way as the highest calibration solution. QC solutions in three concentrations were prepared by diluting this QC stock solution.

2.4.1. Calibration samples

Blank whole blood and plasma (250 μL each) was spiked with 25 μL of respective calibration solutions, yielding whole blood and plasma MB concentrations of 0.00, 75.00, 375.0, 975.0, 3500, 6750, and 10,000 ng/mL. For paper spot calibration whole blood (1.0 mL) was spiked with 25 μL of calibration solutions, yielding the same concentrations for calibration. From this volume paper spots were prepared by pipetting 100 μL spots onto the filter paper which were subsequently air dried at room temperature.

2.4.2. QC samples

QC samples were prepared as described for calibration samples. The MB concentrations represented in whole blood, plasma, and paper spots were 229.5 ng/mL (QC A), 3214 ng/mL (QC B), and 6198 ng/mL (QC C).

2.5. Instrumental analysis parameters

The LC/MS/MS system (Thermo Electron, Dreieich, Germany) consisted of a Surveyor HPLC (quaternary Surveyor LC pump plus, Surveyor autosampler plus with integrated column heater and cooled sample tray) and a triple stage quadrupole mass spectrometer (Model TSQ 7000 with API-2 ion source and performance kit).

The ionic characteristics of MB in combination with reversed phase chromatography using buffer and acetonitrile led to elutions shortly after the dead volume [10]. Usual ion pair chromatography depends on the addition of ion pair reagents that are not volatile leading to residues in the ion source and reducing the robustness of mass spectrometric determination. Therefore a mixed mode ion exchange column based on benzylsulfonic acid groups supplemented with reversed phase properties was chosen. For ion exchange chromatographic separation an Uptisphere mixed mode column (MM1, 5 μm particle size, 120 Å pore size, 100 mm length, 2 mm inner diameter) from Interchim S.A. (Montlucon Cedex, France) with integrated guard column was used at 40 °C. The solvents consisted of 0.1% acetic acid including 100 mM ammonium acetate (solvent A) and 2.5% formic acid/acetonitrile (1/1) including 500 mM ammonium acetate (solvent B). The fast gradient elution started isocratic with 95%/5% (solvent A/B) for 1 min reaching 5%/95% (solvent A/B) at 5 min which was then kept isocratic until 12 min total elution time. The flow rate was 0.45 mL/min. During the first 5 min the eluting mobile phase was switched into the waste, and subsequently the eluent was introduced without splitting into the electrospray ion source of the mass spectrometer. ESI interface parameters were as follows: middle position, spray voltage 4.5 kV, sheath gas (N_2) 90 psi, aux gas (N_2) 10 scales, capillary heater temperature 350 °C. Selected reaction monitoring measurements were performed at 1.5 kV multiplier voltage. MS/MS transitions monitored in the positive ion mode were *m/z* 284.0 → *m/z* 268.0 at 42 V for MB and *m/z* 343.0 → *m/z* 299.0 at 46 V for methylene violet 3RAX. Source CID collision energy was set to 10 V to degrade solvent–analyte clusters. The mass spectrometer was tuned automatically using a solution of myoglobin and Xcalibur 1.3 MS system software standard procedures. The voltages responsible for the ion beam focus (heated capillary, skimmer lens, tube lens, lens stacks) were optimised during flow injection (syringe pump; 10 μL/min) of standard solutions (MB and methylene violet 3RAX in methanol) into the LC mobile phase (50%/50% solvent A/B; 0.45 mL/min). The intensity of each base peak was monitored and adjusted to

maximum. The parameters influencing these transitions were optimised: the Ar pressure in the collision quadrupole was set to 2.0 mbar and the offset voltage was adjusted for MB and methylene violet 3RAX.

2.6. Liberation of methylene blue from biological matrix

The test on possibly increased liberation of MB in the presence of acid (displacement from counter ions) was performed with 10 selected plasma samples (1 mL) from one volunteer after oral administration of 500 mg MB by spiking with aqueous hydrochloric acid (1 M, 100 μL). In response to this test giving evidence for increased MB release, these plasma samples were processed as previously published [10] using protein precipitation with pure acetonitrile. Subsequently the extracts were reanalysed according to Section 2.7 using protein precipitation in presence of $CaCl_2$ solution and acetonitrile containing trifluoroacetic acid.

2.7. Extraction procedures

Whole blood and plasma calibration, QC, and study samples (250 μL) were spiked with internal standard solution (25 μL) and $CaCl_2$ solution (100 mM, 25 μL). Study samples additionally received 25 μL acetonitrile/water (1/1, v/v) for volume compensation. For protein precipitation acetonitrile including 1% trifluoroacetic acid (1 mL) was added and vortexed for 5 min. Subsequently samples were centrifuged (10 min, 16,000 × *g*, 10 °C), and from the clear supernatant 100 μL were transferred to 900 μL LC mobile phase in an autosampler vial.

Paper spot samples (calibration, QC, and study samples) were cut from the paper sheet, soaked in demineralised water (500 μL), spiked with internal standard solution (25 μL), treated with ultrasound (15 min), and finally ice cold acetonitrile (1 mL) was added for protein precipitation. After vortexing for 5 min the samples were centrifuged (10 min, 16,000 × *g*, 10 °C), and from the clear supernatant 150 μL were transferred to 600 μL LC mobile phase in an autosampler vial.

Sample extracts were kept in the autosampler at 15 °C for a maximum of 12 h and always 15 μL were injected into the LC/MS/MS system.

2.8. Ion suppression

When extracts from biological sample processing are injected, co-eluting sample matrix may influence the chromatograms by baseline variation or unexpected peaks, may alter the ionisation process by ion suppression, or may contaminate the ion source, resulting in decreased accuracy and precision, particularly at lower level of quantification (LLOQ). The influence of ion suppression on ESI and the quantification of MB was monitored using the post column infusion method [21,22]. Therefore MB and internal standard (∼2000 ng/mL, each) were introduced by a syringe pump at a flow of 10 μL/min post column into the eluent flow. Blank samples from each matrix were prepared according to the sample preparation procedures and 15 μL of the respective supernatants were injected under the described chromatographic and spectroscopic conditions to analyse a potential influence of eluting matrix compounds onto analyte responses.

2.9. Validation of the analytical methods

Analytical method validation for whole blood, plasma, and paper spots was performed in three analytical batches according to the recommendations published by the U.S. Food and Drug Administration (FDA) [19,20].

Accuracy was calculated as the ratio of the measurements averaged concentrations for individual batches divided by the nominal value and expressed in percent. Precision was defined as the ratio of standard deviation and mean calculated value in percent. These values are reported within-batch and batch-to-batch. For this purpose validation batches ($n = 3$) each containing 7 calibration samples and 18 QC samples at three different concentrations (QC A, B, and C) were analysed, and from these values accuracy and precision of the method were calculated and expressed as mean values ± standard deviation (S.D.). Additionally accuracy and precision at the LLOQ were determined from one validation batch.

Extraction recovery rates for MB and the internal standard were calculated for the three matrices (whole blood, plasma, and paper spots) within the validation procedure in duplicate determination at three concentrations. Therefore resulting peak areas of MB and internal standard after extraction from the respective matrices were compared to the peak areas of pure solutions containing 100% amount. This FDA conform procedure accounts for all influences on recovery, e.g. extraction rate, matrix and suppression effects, and is often addressed as process efficiency.

Selectivity for each matrix was measured using blank matrix from six different individuals. These samples were processed according to the method described without addition of analytes and internal standard and signals at the analytes retention times were evaluated.

Stability of the drugs was tested in three freeze-and-thaw cycles using whole blood and plasma matrix. Stability of MB on paper spots was tested over time under different stress conditions: paper spots at QC A, B, and C concentration levels ($n = 6$, each) were stored for 30 days in the dark at 4 °C, room temperature, 50 °C, and at room temperature exposed to daylight. Subsequently the accuracies for MB were calculated.

2.10. Calculations and statistical methods

Calibration curves were determined for MB using the respective calibration samples for each matrix. Peak area ratios of the analyte and internal standard were calculated and weighted linear regressions ($1/x$) were performed for each analytical batch using the procedures of Thermo Electron's software Xcalibur LCQuan 1.3. Pharmacokinetic parameters were calculated with WinNonlin Professional Version 5.2 (Pharsight Corp., Mountain View, USA).

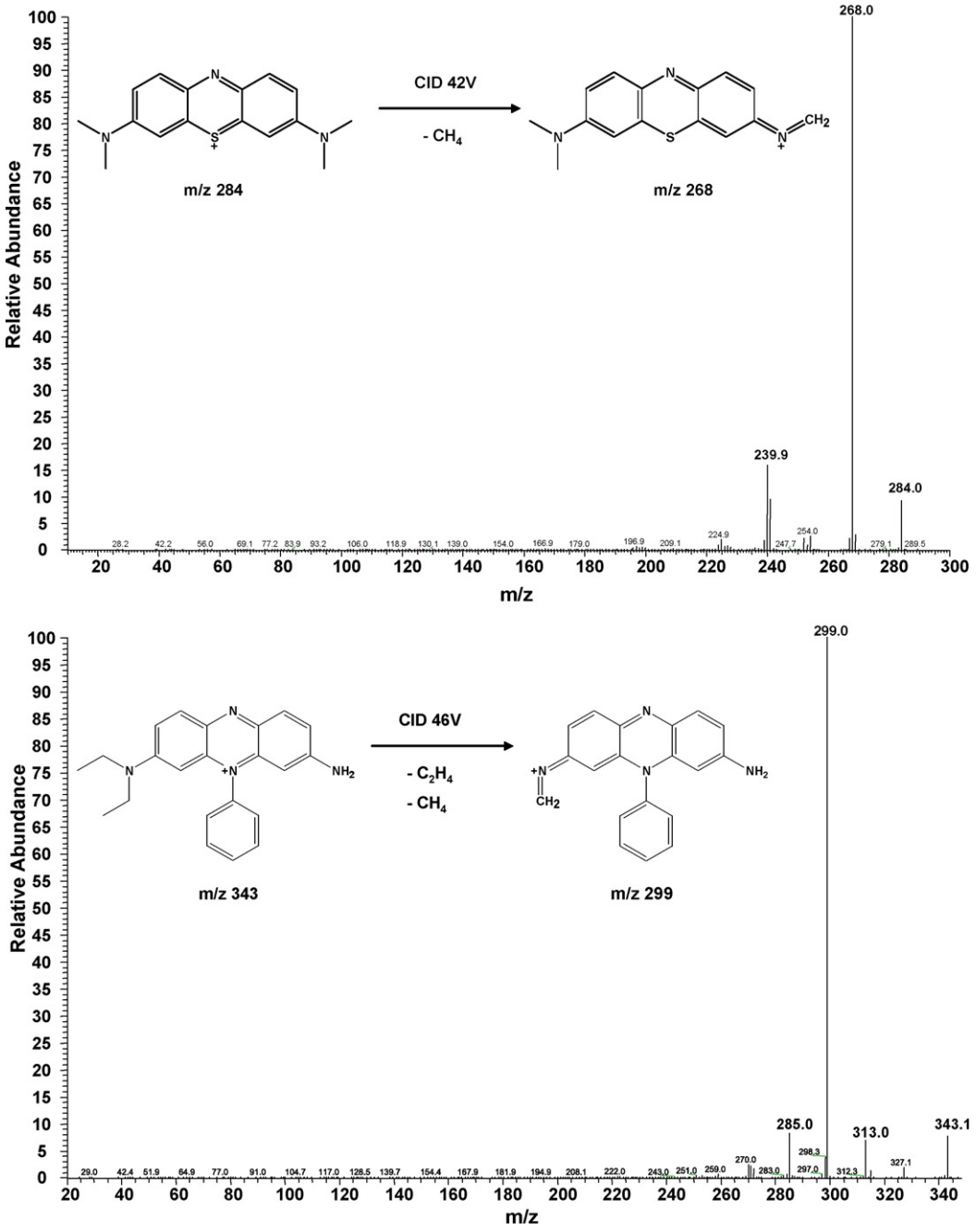

Fig. 2. Tandem mass spectra (product ion scan using ESI and CID) of methylene blue precursor ion *m/z* 284.0 and methylene violet 3RAX *m/z* 343.0 with the proposed fragmentation pathway according to Mass Frontier^{TM} 2.0 (Thermo Electron).

3. Results and discussion

3.1. Performance of the laboratory methods

3.1.1. Chromatographical and mass spectrometric characteristics

Optimal coupling of LC to an MS-ESI source is achieved using common reversed phase chromatography with acetonitrile and water or buffers containing volatile additives (e.g. ammonium acetate). Interchims Uptishere MM1 column in combination with an ammonium acetate gradient from 100 to 500 mM and a parallel acetonitrile gradient from 0 to 50% resulted in short chromatograms (12 min). MB and its internal standard were separated very well and the large amount of ammonium acetate could be volatilised by maximum nitrogen spray of the ESI source efficiently preventing any contamination. The common ionisation by ESI adding H^+ to the analyte could not happen, because MB and methylene violet 3RAX are already positively charged, but both compounds could be analysed specifically because they build up intensive $[M]^+$ ions. Fragmentation in the product ion scan mode produced intensive daughter ions (*m/z* 284 → *m/z* 268 for MB and *m/z* 343 → *m/z* 299 for methylene violet 3RAX) at 42 and 46 V CID energy. Fig. 2 shows the respective mass spectra and the expected

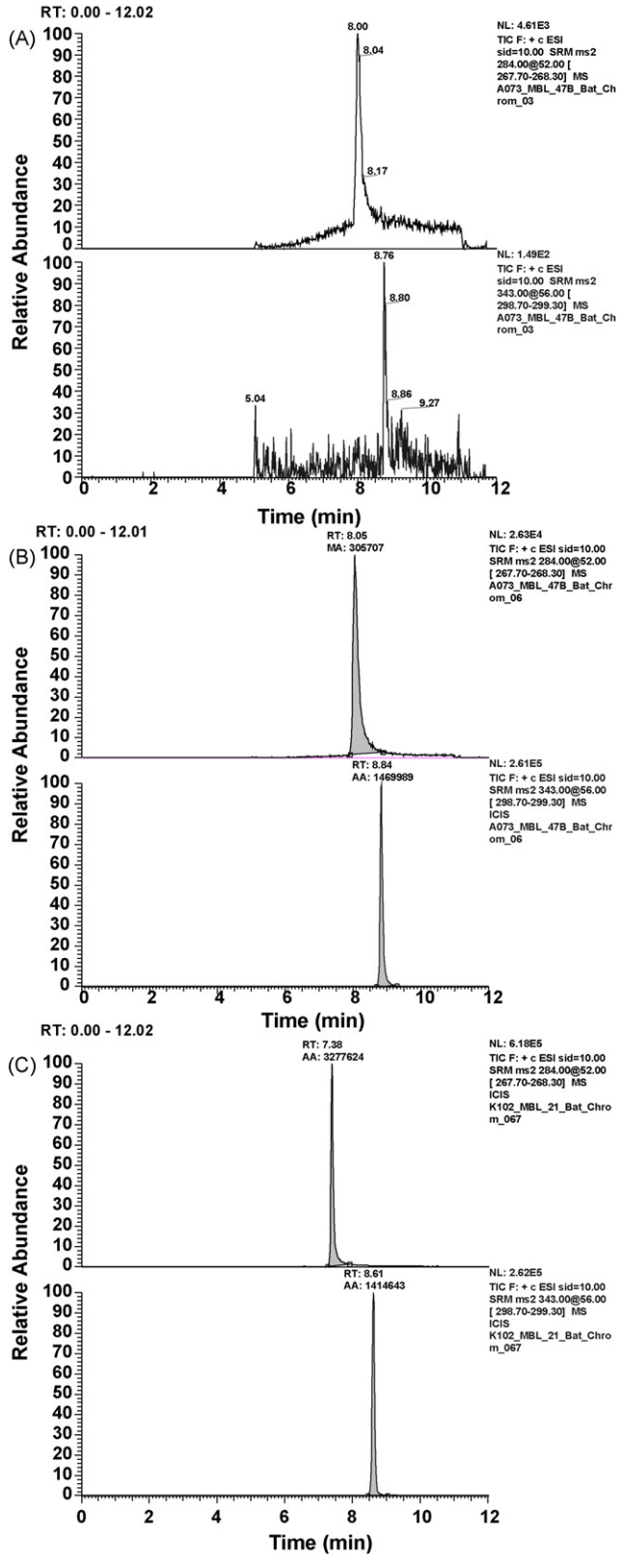

Fig. 4. LC/MS/MS chromatograms of processed plasma samples. Upper traces: methylene blue 284.0 → 268.0, CID 42 V, Ar 2.0 mbar. Lower traces: methylene violet 3RAX 343.0 → 299.0, CID 46 V, Ar 2.0 mbar. (A) Blank plasma sample, (B) plasma quality control sample at LLOQ (75 ng/mL), and (C) plasma blood sample 10 h after oral administration (calculated concentration 919.6 ng/mL).

fragmentation reaction. These mass transitions in the selected monitoring mode were used for specific and sensitive quantification.

3.1.2. Performance and optimisation of the extraction procedures

Extensive adsorption of MB to different surfaces is well known [23]. After MB drug intake it generates bound residues depending on electrostatic interaction, which are not readily broken down by extraction with usual ion pair reagents. Likely for this reason MB concentrations in whole blood and plasma appeared rather low in earlier studies [8,10] suggesting that mainly the unbound dye (free fraction) had been extracted from the biological matrix. These statements have been supported by the tests described in Section 2.6. The observation that addition of acid to 10 plasma samples containing MB (after ingestion by the volunteer, not spiked) coloured the plasma blue by releasing the drug from its binding site led us to optimise earlier extraction methods [10] by adding different acids and cations. The tests using 10 plasma samples of a volunteer revealed concentrations between 45.4 and 81.0 ng/mL after protein precipitation with pure acetonitrile. Addition of $CaCl_2$ solution and acetonitrile containing trifluoroacetic acid resulted in concentrations between 3471 and 7921 ng/mL in the same samples, which was an increase of a mean factor 79.2 (±18.9 S.D.). Indeed, addition of trifluoroacetic acid (1% in acetonitrile) and $CaCl_2$ (100 mM) to acetonitrile were most efficient in displacing MB from its counter ions. Drug recovery rates after addition of acetonitrile (fourfold surplus: 1 mL incl. 1% F_3C–COOH to 0.25 mL whole blood or plasma) were assessed in duplicate determination at concentration levels of QC A, B, and C. From whole blood the recoveries of MB/internal standard were 111.6%/108.6% (QC A), 99.4%/100.1% (QC B), and 106.7%/95.0% (QC C). The respective values in plasma were 74.4%/90.5% (QC A), 87.1%/82.8% (QC B), and 76.3%/82.6% (QC C). Addition of water to Whatman paper spots already resulted in weak acidic solutions (pH 5.5) and further addition of acid resulted in destruction of the paper with precipitation and extracts blocking LC column and MS ion source. Therefore after addition of pure water to paper spots, pure acetonitrile was added, resulting in recovery rates of MB/internal standard of 48.6%/93.9% (QC A), 49.4%/94.6% (QC B), and 50.3%/95.6% (QC C). Compared to earlier methods [8,10] these methods revealed high MB concentrations in biological samples from study participants and the calibration range had to be extended towards higher concentrations.

3.1.3. Ion suppression effects

With these methods no matrix interference or drug interference resulting in unexpected peaks were observed in the blank matrices (whole blood, plasma, and paper spots) from six individuals apart from slight memory effects, which were far below the LLOQ (Figs. 3–5, chromatograms A and B). For both compounds ion suppression was most intensive around 7 min likely caused by the ammonium acetate/acetonitrile gradient. As confirmed by the results of QC determination quantification of MB and the internal standard are thus only slightly affected because

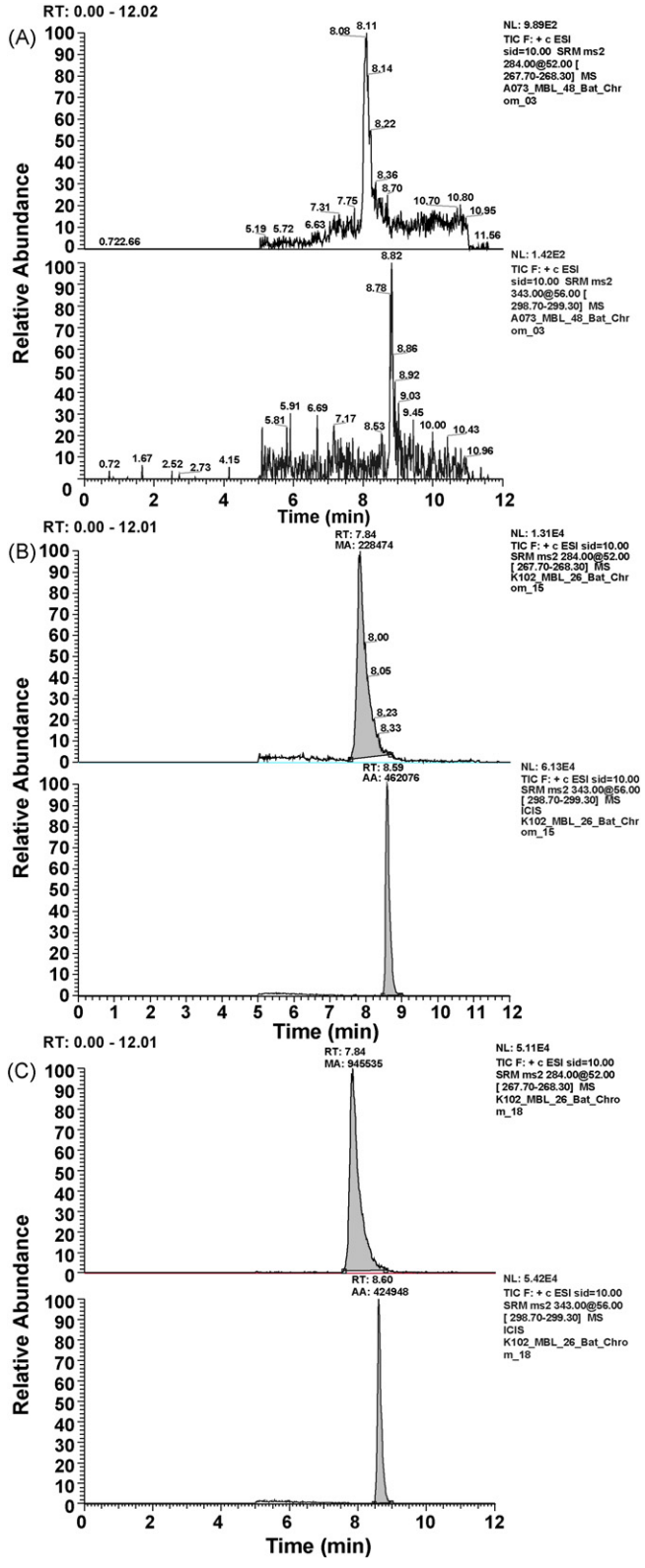

Fig. 3. LC/MS/MS chromatograms of processed whole blood samples. Upper traces: methylene blue 284.0 → 268.0, CID 42 V, Ar 2.0 mbar. Lower traces: methylene violet 3RAX 343.0 → 299.0, CID 46 V, Ar 2.0 mbar. (A) Blank whole blood sample, (B) whole blood quality control sample at LLOQ (75 ng/mL), and (C) whole blood sample 0.5 h after oral administration (calculated concentration 982.7 ng/mL).

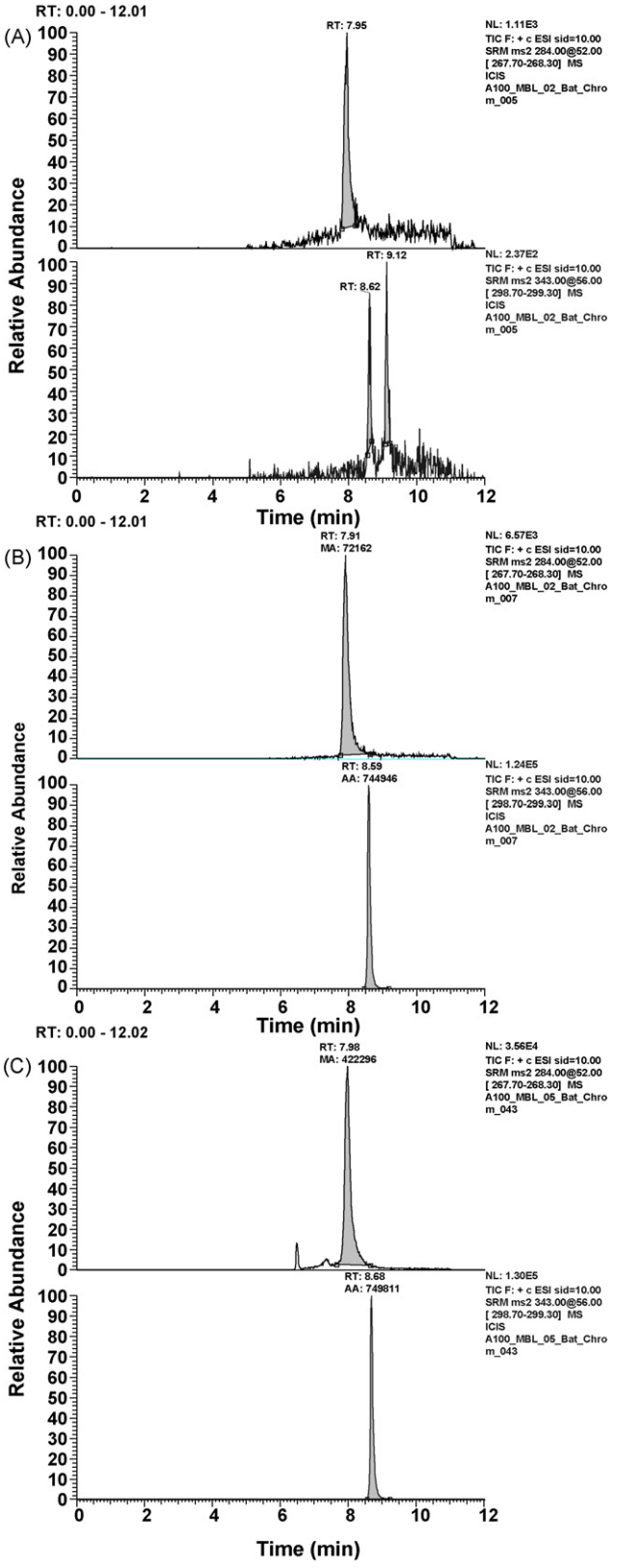

Fig. 5. LC/MS/MS chromatograms of processed paper spot samples. Upper traces: methylene blue 284.0 → 268.0, CID 42 V, Ar 2.0 mbar. Lower traces: methylene violet 3RAX 343.0 → 299.0, CID 46 V, Ar 2.0 mbar. (A) Blank paper spot sample, (B) paper spot quality control sample at LLOQ (75 ng/mL), and (C) paper spot blood sample 1 h after oral administration (calculated concentration 1089 ng/mL).

they elute later and because the intensity of the ion suppression effect is low (Fig. 6).

3.1.4. Validation results

The extraction methods combined with the LC/MS/MS quantification methods entirely met FDA's requirements for procedure validation. Within-batch the accuracies varied between −12.8 and +13.7% and batch-to-batch accuracies ranged from −5.8 to +11.1%. Drug concentrations in study samples were measured within the calibration range of 75–10,000 ng/mL (linearity always $r^2 \geq 0.9900$) for all three matrices and could be quantified with appropriate precision and accuracy (Table 1). LLOQ for MB in all matrices was 75 ng/mL (lowest calibration point) with accuracy/precision of −3.5%/6.7% (whole blood), +12.5%/7.1% (plasma), and +4.8%/10.1% (paper spots). Stability in whole blood and plasma was tested by three freeze and thaw cycles in whole blood at QC B level (3214 ng/mL) and no concentration change was observed (accuracy ranged between +7.4 and +15.0%). Storing of MB containing paper spots in the darkness at any temperature did not result in degradation within 30 days. No concentration change was observed and accuracies were within ±15%. However, 30 days storing at daylight resulted in extensive degradation of MB. The resulting accuracies were always below 50%. Therefore study paper spots were stored in the dark at room temperature.

3.2. Whole blood, plasma, and paper spot concentrations in study participants and patients

MB quantification in different biological matrices is necessary, because nearly no data is available concerning pharmacokinetics and optimum therapeutic concentrations. Whole blood and respective plasma samples need to be processed, because the drug penetrates the membrane of erythrocytes [6]. Moreover, blood sampling on paper spots which is necessary to allow handling, storage, and transport of biological material in developing countries, requires different steps of sample processing. The methods described in this paper were applied to blood samples of healthy individuals in a bioavailability study and to patients treated with MB against malaria. Fig. 7 shows MB plasma concentration time curves in a healthy individual after oral (500 mg) and intravenous administration (50 mg) of MB. After oral intake C_{max} was reached after 2 h (T_{max}), with MB concentrations detectable for 72 h. After intravenous administration MB was detectable for 24 h. In an earlier study maximum whole blood concentrations of up to 28.4 ng/mL were reached in healthy individuals after oral administration of 100 mg methylene blue [8]. Even after adjustment for differences in dose and taking into account differences in plasma and whole blood concentrations it is obvious that the concentrations obtained with the optimised analytical methods were much higher than those found in former studies likely because of the more efficient (acidic) extraction procedure.

In 20 patients from Burkina Faso, 1 h after MB administration (20 mg/kg) the concentrations on paper spots ranged between 531 and 2645 ng/mL. Figs. 3C, 4C, and 5C show chromatograms

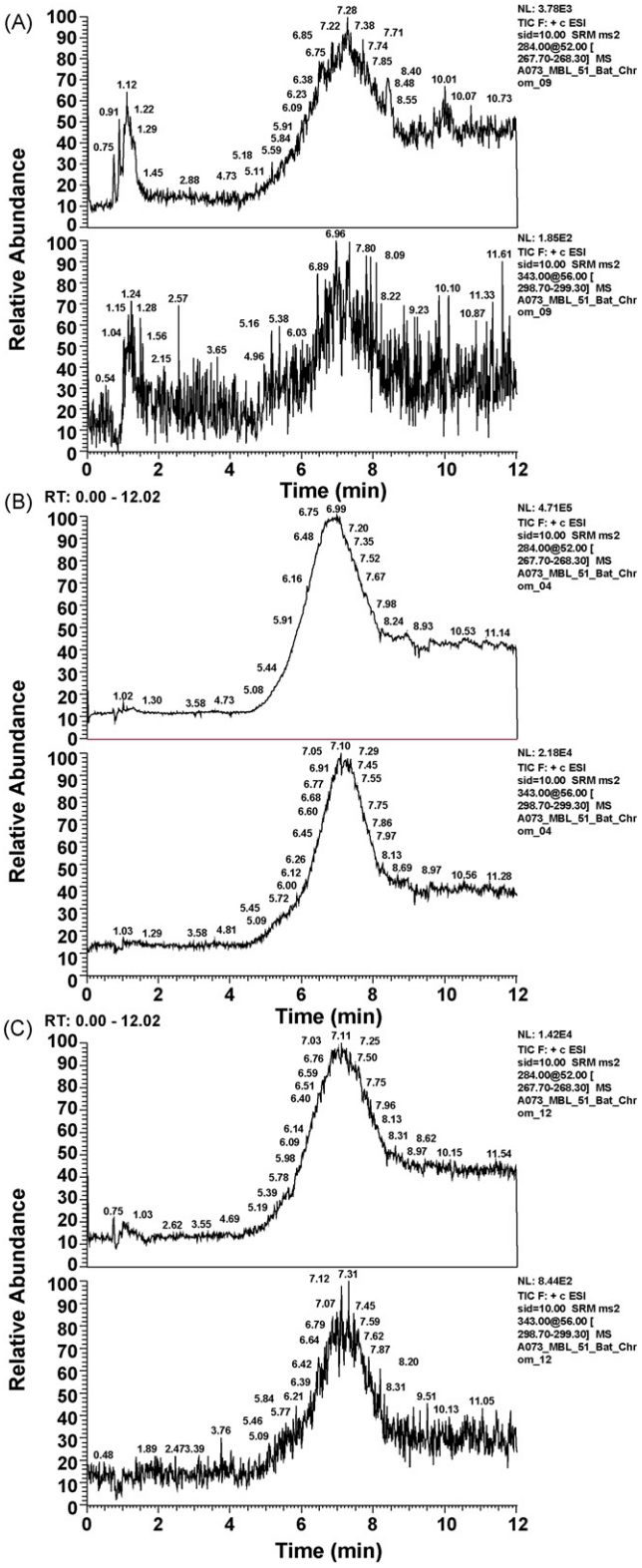

Fig. 6. LC/MS/MS chromatograms of processed blank whole blood (A), plasma (B), and paper spot (C) during post column infusion of methylene blue and methylene violet 3RAX for ion suppression determination. Upper traces: methylene blue 284.0 → 268.0, CID 42 V, Ar 2.0 mbar. Lower traces: methylene violet 3RAX 343.0 → 299.0, CID 46 V, Ar 2.0 mbar. Eluate was introduced without splitting from 0 to 12 min.

Table 1
Summary of validation results for methylene blue in different biological matrices

	Matrix								
	Whole blood			Plasma			Paper spot		
	QC A = 229.5[a]	QC B = 3214[a]	QC C = 6198[a]	QC A = 229.5	QC B = 3214	QC C = 6198	QC A = 229.5	QC B = 3214	QC C = 6198
Within-batch									
1									
Mean (ng/mL)	208.2	3467	6291	200.2	3282	5856	211.9	3655	6847
Accuracy (%)	−9.3	+7.9	+1.5	−12.8	+2.1	−5.5	−7.7	+13.7	+10.5
Precision (% CV)	5.3	4.0	3.7	9.3	5.5	6.0	16.3	3.7	3.8
2									
Mean (ng/mL)	233.8	3378	6109	229.5	3644	6463	218.3	3457	7009
Accuracy (%)	+1.9	+5.1	−1.4	0.0	+13.4	+4.3	−4.9	+7.6	+13.1
Precision (% CV)	5.9	3.9	5.7	8.4	3.5	4.2	9.3	6.3	4.1
3									
Mean (ng/mL)	215.4	3437	6620	233.1	3442	6060	216.4	3561	6802
Accuracy (%)	−6.1	6.9	+6.8	+1.6	+7.1	−2.2	−5.7	+10.8	+9.7
Precision (% CV)	5.9	3.7	1.4	11.0	6.4	2.6	4.3	2.8	5.1
Batch-to-batch									
Mean (ng/mL)	219.1	3427	6340	220.9	3456	6130	216.3	3558	6886
Accuracy (%)	−4.5	+6.6	+2.3	−3.7	+7.5	−1.1	−5.8	+10.7	+11.1
Precision (% CV)	7.4	3.8	5.0	11.8	6.6	6.1	8.5	4.8	4.3

[a] Nominal analyte concentrations (ng/mL).

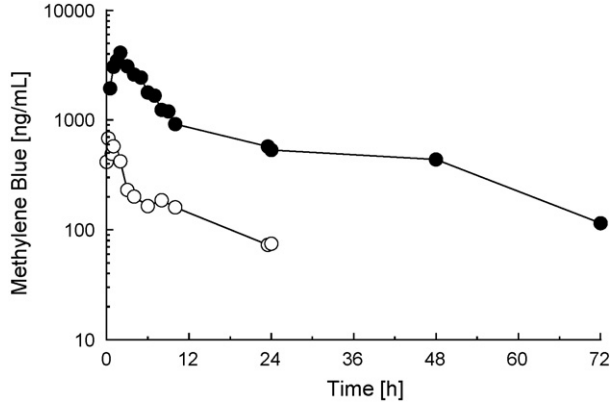

Fig. 7. Methylene blue plasma pharmacokinetics after oral (500 mg, black circles) and intravenous administration (50 mg, white circles).

from the respective study participant and a patient. The clinical data will be published elsewhere.

4. Conclusion

We established and validated an analytical assay for the monitoring of the anti-malarial MB in different biological matrices. A newly developed acidic protein precipitation procedures enabled efficient liberation of MB from its adsorption sites and to displace the drug from protein binding. MB as a cationic compound could be chromatographed on a mixed mode ion exchange column resulting in full separation of methylene blue from the internal standard and matrix compounds. The eluent contained acetic and formic acid and high amounts of the volatile buffer ammonium acetate and did not affect ionisation on the ESI tandem mass spectrometer. The combination of acidic protein precipitation, ion exchange chromatography, and tandem mass spectrometry enabled MB monitoring in whole blood, plasma, and paper spots up to 72 h after administration. The application of this method will be clinical studies with adult and paediatric patients and healthy individuals.

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References

[1] P. Guttmann, P. Ehrlich, Berlin. Klin. Wochenschr. 28 (1891) 953.
[2] R.H. Schirmer, B. Coulibaly, A. Stich, M. Scheiwein, H. Merkle, J. Eubel, K. Becker, H. Becher, O. Müller, T. Zich, W. Schiek, B. Kouyaté, Redox Rep. 8 (2003) 272.
[3] P.M. Färber, L.D. Arscott, C.H. Williams, K. Becker, R.H. Schirmer, FEBS Lett. 422 (1998) 311.
[4] S. Meierjohann, R.D. Walter, S. Müller, Biochem. J. 368 (2002) 761.
[5] H. Ginsburg, O. Famin, J. Zhang, M. Krugliak, Biochem. Pharmacol. 56 (1998) 1305.
[6] M. Akoachere, K. Buchholz, E. Fischer, J. Burhenne, W.E. Haefeli, H. Schirmer, K. Becker, Antimicrob. Agents Chemother. 49 (2005) 4592.
[7] J.L. Vennerstrom, M.T. Makler, C.K. Angerhofer, J.A. Williams, Antimicrob. Agents Chemother. 39 (1995) 2671.
[8] C. Peter, D. Hongwan, A. Küpfer, B.H. Lauterburg, Eur. J. Clin. Pharmacol. 56 (2000) 247.
[9] D.S. Chongtham, J. Phurailatpam, M.M. Singh, T.R. Singh, J. Postgrad. Med. 43 (1997) 73.
[10] J. Rengelshausen, J. Burhenne, M. Fröhlich, Y. Tayrouz, S.K. Singh, K.D. Riedel, O. Müller, T. Hoppe-Tichy, W.E. Haefeli, G. Mikus, I. Walter-Sack, Eur. J. Clin. Pharmacol. 60 (2004) 709.
[11] P.E. Meissner, G. Mandi, B. Coulibaly, S. Witte, T. Tapsoba, U. Mansmann, J. Rengelshausen, W. Schiek, A. Jahn, I. Walter-Sack, G. Mikus, J.

Burhenne, K.D. Riedel, R.H. Schirmer, B. Kouyaté, O. Müller, Malaria J. 5 (2006) 84.
[12] G. Mandi, S. Witte, P. Meissner, B. Coulibaly, U. Mansmann, J. Rengelshausen, W. Schiek, A. Jahn, M. Sanon, K. Wüst, I. Walter-Sack, G. Mikus, J. Burhenne, K.D. Riedel, H. Schirmer, B. Kouyaté, O. Müller, Trop. Med. Int. Health 10 (2005) 32.
[13] P.E. Meissner, G. Mandi, S. Witte, B. Coulibaly, U. Mansmann, J. Rengelshausen, W. Schiek, A. Jahn, M. Sanon, T. Tapsoba, I. Walter-Sack, G. Mikus, J. Burhenne, K.D. Riedel, H. Schirmer, B. Kouyaté, O. Müller, Malaria J. 4 (2005) 45.
[14] O.M. Minzi, A.Y. Massele, L.L. Gustafsson, O. Ericsson, J. Chromatogr. B 814 (2005) 179.
[15] A.M. Ronn, M.M. Lemnge, H.R. Angelo, I.C. Bygbjerg, Ther. Drug Monit. 17 (1995) 79.
[16] T. Koal, H. Burhenne, R. Römling, M. Svoboda, K. Resch, V. Kaever, Rapid Commun. Mass Spectrom. 19 (2005) 2995.
[17] R.K. Munns, D.C. Holland, J.E. Roybal, J.G. Meyer, J.A. Hurlbut, A.R. Long, J. AOAC Int. 75 (1992)796.
[18] H. Borwitzky, W.E. Haefeli, J. Burhenne, J. Chromatogr. B 826 (2005) 244.
[19] V.P. Shah, K.K. Midha, J.W. Findlay, H.M. Hill, J.D. Hulse, I.J. McGilveray, G. McKay, K.J. Miller, R.N. Patnaik, M.L. Powell, A. Tonelli, C.T. Viswanathan, A. Yacobi, Pharm. Res. 17 (2000) 1551.
[20] Guidance for Industry, Bioanalytical Method Validation, U.S. Department of Health and Human Services, Food and Drug Administration, May 2001, available at http://www.fda.gov/cder/guidance/4252fnl.htm (accessed December 13, 2007).
[21] T.M. Annesley, Clin. Chem. 49 (2003) 1041.
[22] R. Bonfiglio, R.C. King, T.V. Olah, K. Merkle, Rapid Commun. Mass Spectrom. 13 (1999) 1175.
[23] N. Belaz-David, L.A. Decosterd, M. Appenzeller, Y.A. Ruetsch, R. Chiolero, T. Buclin, J. Biollaz, Eur. J. Pharm. Sci. 5 (1999) 335.