Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 820 (2005) 83–93
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Liquid chromatography–electrospray mass spectrometry determination of a bis-thiazolium compound with potent antimalarial activity and its neutral bioprecursor in human plasma, whole blood and red blood cells

Olivier Nicolas[a,1], Delphine Margout[a], Nicolas Taudon[a,1], Michele Calas[b], Henri J. Vial[c], Françoise Bressolle[a,*]

[a] *Laboratoire de Pharmacocinétique Clinique, Faculté de Pharmacie, Université Montpellier I, 15 Avenue Charles Flahault, B.P. 14491, 34093 Montpellier Cedex 5, France*
[b] *UMR 5810 CNRS, Montpellier I and II University, France*
[c] *UMR 5539 CNRS, Montpellier II University, 34095 Montpellier Cedex 5, France*

Received 24 September 2004; accepted 14 March 2005

Abstract

Liquid chromatography–electrospray ionization mass spectrometry methods are described for the simultaneous quantification of a bis-thiazolium compound (T3), its related prodrug (TE3) and an intermediate compound (mTE3) that appeared during the prodrug/drug conversion process, in human plasma, whole blood and red blood cells (RBCs). The methods involve solid phase extraction (SPE) of the compounds and the internal standard (verapamil) from the three different matrices using Oasis®HLB columns with an elution solvent of 2 × 1 ml of acetonitrile containing 1 ml/l trifluoroacetic acid (TFA). HPLC separation was performed on a C_{18} encapped Xterra® column packed with 3.5 μm particles. The mobile phase used a 8 min gradient, from water containing 1 ml/l TFA to acetonitrile containing 1 ml/l TFA, at a flow rate of 400 μl/min. Verapamil and the TE3 compound were characterized by the protonated molecules at *m/z* 455 and *m/z* 541, respectively. The mTE3 species was detected through the $(M)^+$ ion at *m/z* 497. The T3 compound was detected by use of two ions, the quaternary ammonium salt ($M^{2+}/2$) at *m/z* 227.3 and by the adduct with TFA $(M + TFA)^+$ at *m/z* 567.3. The drug/internal standard peak area ratios were linked via a quadratic relationship to plasma (or whole blood) concentrations in the tested range of 6.4–1282 μg/l (12.8–2564 μg/kg) for T3, 20–2000 μg/l (40–4000 μg/kg) for mTE3 and 10–2000 μg/l (40–4000 μg/kg) for TE3, and to T3 concentrations in RBCs ranging from 12.8 to 2564 μg/kg. Inter-assay precision (in terms of R.S.D.) was below 13.5% and accuracy ranged from 95.4 to 107%. The dilution of the samples (plasma or whole blood) has no influence on the performance of the methods. The extraction recoveries averaged 87% for T3, 53% for mTE3 and 79% for TE3 in plasma; 79% for T3, 57% for mTE3 and 65% for TE3 in blood; and 93% for T3 in RBCs, and was constant across the calibration range. The lower limits of quantitation were 6.4 μg/l for T3, 20 μg/l for mTE3 and 10 μg/l for TE3 in plasma; 12.8 μg/kg for T3 and 40 μg/kg for mTE3 and TE3 in blood; and 12.8 μg/kg for T3 in RBCs. Stability tests under various conditions were also investigated. The three-step SPE procedure (loading, clean-up, and elution) described in this paper to quantify these new anti-malarial compounds in plasma, whole blood and RBCs, can easily be automated by using either robotisation or an automated sample preparation system.
© 2005 Elsevier B.V. All rights reserved.

Keywords: Bis-thiazolium compound; Neutral bioprecursor; Antimalarial activity; Human plasma; Whole blood; Red blood cells; Liquid chromatography–electrospray mass spectrometry; Validation

1. Introduction

Malaria is an infectious disease caused by unicellular, protozoan parasites of the genus *Plasmodium*. Four species of plasmodia are infectious in humans: *P. falciparum*, *P. vivax*,

* Corresponding author. Tel.: +33 4 67 54 80 75; fax: +33 4 67 54 80 75.
E-mail address: fbressolle@aol.com (F. Bressolle).
[1] Olivier Nicolas and Nicolas Taudon contributed equally to this work.

1570-0232/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2005.03.022

P. malariae and *P. ovale*. *P. falciparum* is responsible for the vast majority of deaths from malaria and has demonstrated an ability to develop resistance to most, if not all, of the antimalarial agents presently available [1]. It is becoming an increasingly more widespread problem because of drug and insecticide resistance, as well as social and environmental changes [2]. It is quite possible that within the next decade there will be parts of the world in which malaria is an untreatable disease. Consequently, unless new treatments are developed, the already significant number of fatalities arising from this disease will increase dramatically. In addition the development of new anti-malarial strategies, there is an urgent need for the identification of new chemotherapeutic agents that work through new, independent mechanisms of action and which are structurally unrelated to existing anti-malarial agents.

The growth and asexual reproduction of the intracellular malaria parasite within the red blood cells of its vertebrate host entails the synthesis of new membranes. The phospholipid content (phosphatidylcholine and phosphatidylethanolamine representing about 85%) of the malaria-infected erythrocyte increases by up to six-fold during the course of parasite development [3]. The de novo phosphatidylcholine biosynthesis within the intracellular parasite is dependant upon the uptake of choline from the external medium. Thus, any compounds inhibiting this de novo phosphatidylcholine biosynthesis may potentially be effective as anti-malarial agents [4,5].

During the last 10 years, compounds mimicking the choline structure have been synthesized and have been shown to be highly effective against multiresistant *P. falciparum* malaria [6–13]. Three generations of compounds have been synthesized, the first two of which consisted of quaternary ammonium salts [9,10], and amidine and guanidine compounds (manuscript in preparation). These compounds were found to be highly potent both in vitro and in vivo against *P. falciparum* and *P. cynomolgi*, a *P. vivax*-related parasite with a high therapeutic index [6,8,12]. These drugs possess a permanently charged cationic group [9,10] that is essential for activity but poorly absorbed orally. The third generation consisted of neutral prodrugs that deliver bis-thiazolium compounds with antimalarial activity in the nanomolar range [13]. These prodrugs are converted into an active ionized form by enzymes present in plasma [13]. From in vitro experiments performed in 80% plasma, we have shown that human plasma promoted rapid conversion of TE3 into T3 [13]. Moreover, the bis-thiazolium compounds were shown to be specifically and highly accumulated in infected erythrocytes. This chemical series is currently under evaluation for further development.

In this paper, liquid chromatography–electrospray ionization mass spectrometry methods (LC–ESI-MS) were developed in human plasma and whole blood to simultaneously quantify the TE3 neutral prodrug, its enzyme-converted active bis-cationic metabolite, T3, and an intermediate mono-cationic compound, mTE3, formed during prodrug/drug conversion. In a previous published paper, we have shown that the bis-cationic compounds accumulate within infected red blood cells (RBCs) and that 60% of total uptake was within the parasite [13]. Thus, a method to quantify T3 in RBCs was also developed and validated to further quantify this drug in infected RBCs during pharmacokinetic studies carried out in infected mice or monkey. The structures of these compounds are presented in Fig. 1. Validated methods were required in order to investigate the pharmacokinetic properties of this new class of drugs. These methods described here are based on solid-phase extraction and verapamil was selected as internal standard. Complete validation of these assays was carried out according to validation procedures, parameters and acceptance criteria [14–17] based on the recommendations of Shah et al. [16] and USP XXIII guidelines [15]. These methods have enhanced precision due to the high selectivity of liquid chromatography coupled with mass spectrometry.

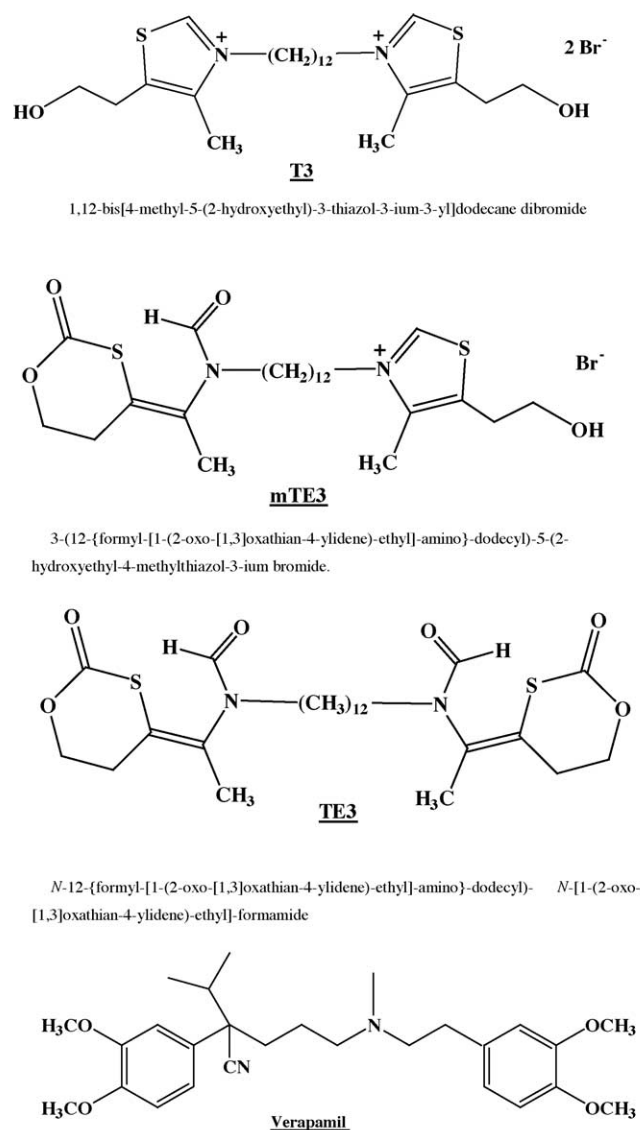

Fig. 1. Chemical structures of T3, mTE3, TE3 and verapamil.

2. Experimental

2.1. Chemicals and reagents

T3 (1,12-bis[4-methyl-5-(2-hydroxyethyl)-3-thiazol-3-ium-3-yl]dodecane dibromide, MW 614.7), TE3 (*N*-12-{formyl-[1-(2-oxo-[1,3]oxathian-4-ylidene)-ethyl]-amino}-dodecyl)- *N*-[1-(2-oxo-[1,3]oxathian-4-ylidene)-ethyl]-formamide, MW = 540.7) and mTE3 (3-(12-{formyl-[1-(2-oxo-[1,3]oxathian-4-ylidene)-ethyl]-amino}-dodecyl)-5-(2-hydroxyethyl-4-methylthiazol-3-ium bromide, MW = 577) are characterized products of Laboratoire des Aminoacides, Peptides et Proteines, *UMR* 5810 (Montpellier I and II Universities, France) (Fig. 1) [18]. The purity of these standards was evaluated by elemental analysis; results were within ±0.4 of calculated values. T3, TE3 and mTE3 were stored at ambient temperature and protected from light. Trifluoroacetic acid (TFA) and the internal standard (verapamil) were from Sigma (St. Louis, MO, USA). Verapamil was stored at room temperature (20 °C) and protected from light. HPLC-grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). In-house deionised water was further purified with a Milli-Q water-purifying system (Millipore, Bedford, MA, USA).

For method validation, whole blood, human plasma and RBCs were obtained from pooled blood samples collected from healthy volunteers not undergoing drug therapy. Coagulation was prevented by adding EDTA-sodium salt. The blood was centrifuged at 2000 × *g* for 10 min to obtain plasma. RBCs were washed twice with an equal volume of 0.9% sodium chloride before storage. The drug-free whole blood, plasma, and RBCs were stored at −80 °C before use.

A stock standard solution of T3 was prepared by dissolving an accurately weighted amount of the drug in deionised water to give a stock solution containing 64 mg/l of T3 expressed in the form of the doubly charged species. A stock solution of mTE3 (100 mg/l) was prepared in deionised water containing 10 ml/l TFA for enhanced stability. Stock solutions of TE3 (100 mg/l) and verapamil (internal standard, 250 mg/l) were prepared in acetonitrile–deionised water (50:50, v/v) and methanol–deionised water (1:100, v/v), respectively due to their poor solubility in pure water. For each compound, two separate stock standard solutions were prepared; one which was used for the preparation of the calibration curve standards and the second which was used for the preparation of quality control (QC) samples. Stock solutions were stored at +4 °C and protected from light.

2.2. Equipment

The LC–MS analysis was performed using a Hewlett-Packard (Les Ulis, France) Agilent 1100 mass spectrometer equipped with an electrospray interface and a data acquisition station. The mass spectrometer was coupled to a Hewlett-Packard LC system equipped with a ternary pumping unit (G1312A) and an autosampler set at 4 °C (G1329A). The LC column, C_{18} encapped Xterra, 3.5 μm, 30 mm × 2.1 mm, i.d., was obtained from Waters (Saint Quentin, France). Solid-phase extraction (SPE) columns (Oasis HLB, 1 cm^3) were purchased from Waters.

2.3. Liquid chromatography–mass spectrometry conditions

The mobile phase used was a linear gradient over 8 min from 1 ml/l TFA in water (solvent A) to 1 ml/l TFA in acetonitrile as organic modifier (solvent B). The column was then washed for 1 min with the final gradient solution, brought back to the initial conditions over 1 min and re-equilibrated for 4 min. The total run cycle was therefore 14 min. The column was thermostatted at 20 °C and the injection volume was 10 μl. The flow rate through the LC column was 400 μl/min and the effluent was split so that the flow rate entering the ion source was 35 μl/min.

The mass spectrometer was calibrated in the positive ion mode using a mixture of NaI and CsI. Voltages were set at +3.0 kV for the capillary and +0.5 kV for the skimmer lens. The heated nebulizer temperature was 350 °C; nebulizer pressure was 35 psi and drying gas flow was 720 l/h. The sampling cone voltage was set at 30 V.

Mass spectrometric data were acquired in single ion recording (SIR) mode. Verapamil and the TE3 prodrug were characterized by the protonated molecules $(M+H)^+$ at *m/z* 455.3 and *m/z* 541, respectively. mTE3 was detected through the $(M)^+$ ion at *m/z* 497. The T3 compound was detected by use of two ions, the quaternary ammonium salt ($M^{2+}/2$) at *m/z* 227.3 and by the adduct with TFA $(M+TFA)^+$ at *m/z* 567.3. The mass spectra (scan mode) of T3, mTE3, TE3 and verapamil are shown in Fig. 2.

2.4. Working standards

Stock solutions were extemporaneously diluted to obtain 11 working standards (T3: 0.16–32 mg/l in purified water; TE3: 0.25–50 mg/l in acetonitrile–purified water (50:50, v/v); mTE3: 0.50–50 mg/l in purified water containing 10 ml/l TFA). The stock solution of verapamil was extemporaneously diluted 100-fold in distilled water before use.

A reference standard solution containing T3 (1.3 mg/l), TE3 and mTE3 (2 mg/l) and the internal standard (0.5 mg/l) was prepared daily in acetonitrile containing 1 ml/l TFA and injected before each run to verify the performance of the LC–ESI-MS system.

2.5. Preparation of standards and quality-control (QC) samples

Calibration standards were prepared in acidified plasma or whole blood (0.5 ml drug-free plasma or 0.25 g drug-free whole blood diluted with 0.25 ml of deionised water +0.5 ml deionised water containing 10 ml/l TFA) to avoid TE3/T3 and mTE3/T3 conversions. In blood, the acidic solution was dripped while the mixture was vortex mixed in order to obtain smaller particles of precipitate which

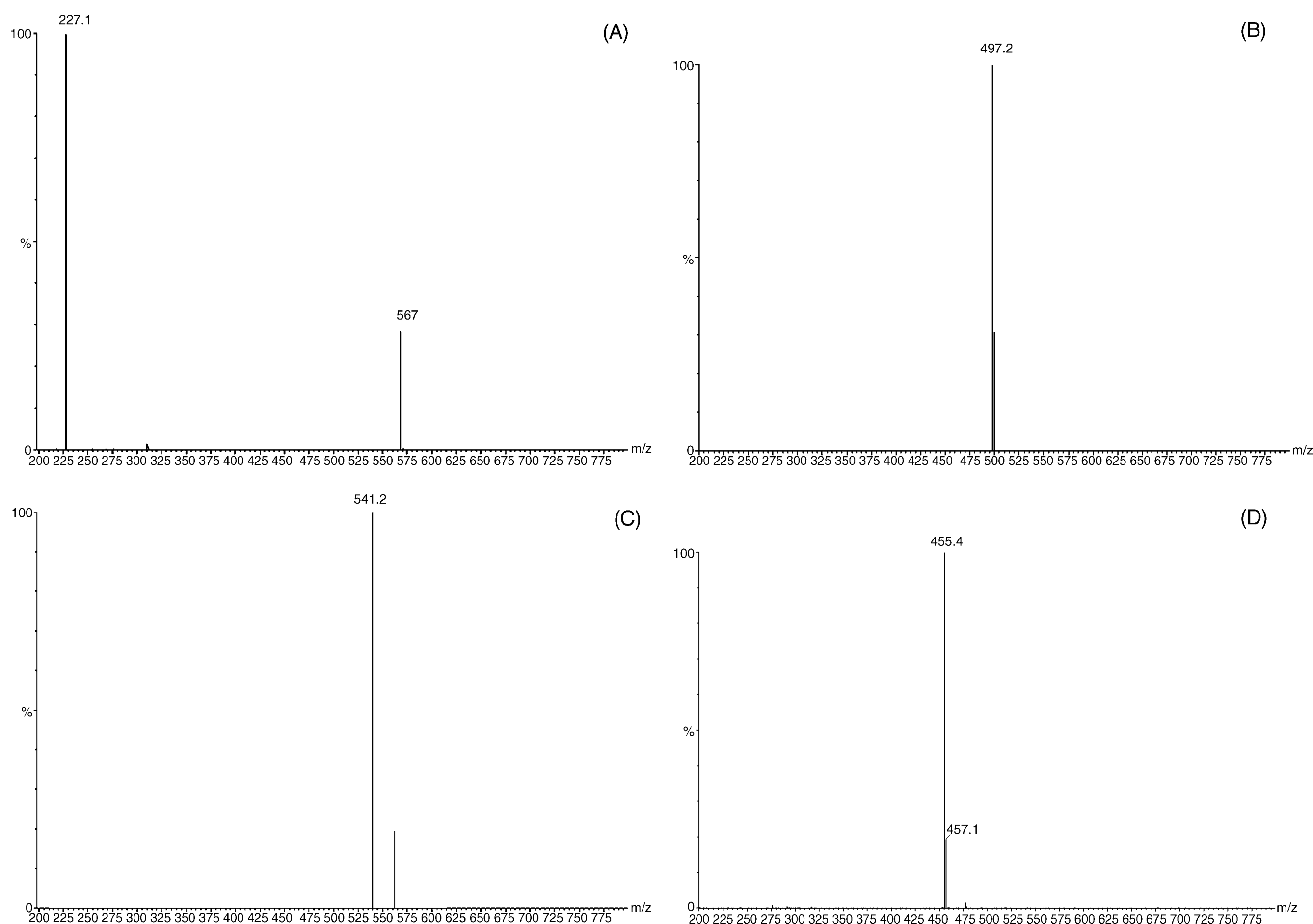

Fig. 2. Mass spectra for T3 (A), mTE3 (B), TE3 (C) and verapamil (D).

avoided important losses of analytes. The calibration set consisted of seven to eight concentrations, prepared by spiking drug-free acidified matrices with 20 μl of the appropriate working solutions (TE3, mTE3 and T3). Concentration ranges were 6.4–1282 μg/l for T3 (expressed in the form of doubly charged species), 20–2000 μg/l for mTE3 and 10–2000 μg/l for TE3 in plasma. They were 12.8–2564 μg/kg for T3 (expressed in the form of doubly charged species) and 40–4000 μg/kg for mTE3 and TE3 in blood. Calibration curves in drug-free RBCs were prepared by adding 20 μl of the appropriate working solution of the T3 compound to 0.25 g of RBCs diluted with 0.25 ml of deionised water to obtain concentrations ranging from 12.8 to 2564 μg/kg. The volume added was always less than or equal to 4% of total volume of the samples, so that the integrity of the matrix was maintained.

QC samples used in the validation study were prepared in the same way as the calibration standards, by mixing drug-free plasma, whole blood or RBCs with appropriate volumes of working solutions to obtain three different concentrations, low, medium and high (T3: 16, 160.3 and 961.9 μg/l in plasma and 32, 320.6 and 1923.8 μg/kg in whole blood and RBCs; TE3 and mTE3: 25, 250 and 1500 μg/l in plasma and 50, 500 and 3000 μg/kg in whole blood).

Before the SPE procedure, standards and QC samples previously vortex-mixed were incubated at 4 °C for 30 min, to allow a steady-state with the matrix components.

2.6. *Sample preparation procedure*

The SPE column was pre-washed with 1 ml of methanol followed by 1 ml of purified water. A volume of 20 μl of internal standard (2.5 mg/l) was added to 1 ml of acidified (pH, 3.6) plasma samples or standards. The mixture was shaken and centrifuged at 4 °C for 15 min at 1500 × *g*; the supernatant was then loaded onto the cartridge under a light vacuum (approximately 86 kPa) using a Vac Elut 20® (Varian, Les Ulis, France). The column was rinsed with 1 ml of purified water and was then dried for 2 min by vacuum aspiration (approximately 27 kPa). The compounds of interest were eluted from the column with 2 × 1 ml of acetonitrile containing 1 ml/l TFA under a light vacuum (approximately 86 kPa). The eluate was dried at 40 °C under a stream of nitrogen for 30 min. The dried residue was reconstituted in 100 μl of acetonitrile containing 1 ml/l TFA and ultrasonicated for 1 min. A 10 μl volume was injected into the HPLC system for analysis.

Twenty microlitres of internal standard (2.5 mg/l) was added to 0.5 ml of diluted RBC samples or standards, mixed with 0.5 ml of water containing 10 ml/l TFA (added drop by drop as described above), or to 1 ml of acidified whole blood samples or standards (pH, 3.3). The resulting mixture was briefly vortex-mixed then centrifuged for 20 min at 17,562 × *g*. The assay procedure was as described above for the plasma samples, except that interfering material was removed by washing with 2 × 0.75 ml of purified water.

2.7. Data analysis

Analyte-to-internal standard peak area ratios were recorded and calibration curves were constructed from the calibration standard data using a quadratic equation, $Y = aX^2 + bX + c$, in which Y is the peak area ratio and X is the concentration of the analyte. The regression curve was not forced through zero. The resulting a, b and c parameters were used to determine back-calculated concentrations, which were then statistically evaluated. The normal distribution of the residuals (the difference between nominal and back-calculated concentrations) was verified. Moreover, the mean residual values (or mean predictor error) was computed and compared to zero (Student *t*-test); the 95% confidence interval was also determined.

2.8. Ion suppression and specificity studies

The absence of ion suppression attributable to the matrices was demonstrated by the method of Matuszewski et al. [19]. A total of 10 human EDTA-anticoagulant blood samples from 10 different donors were used. Plasma, whole blood and RBCs were extracted in duplicate ($n = 20$ per matrix) by the method described above. The final dried extracts obtained from plasma and blood samples were reconstituted with two reference solutions containing the three drugs and the internal standard in 100 μl acetonitrile–1 ml/l TFA at final nominal concentrations of 800 and 4800 μg/l (T3), 1250 and 5000 μg/l (TE3 and m TE3) and 500 μg/l (internal standard). The dried residues obtained from RBCs were reconstituted with two different solutions containing T3 (800 and 4800 μg/l) and the internal standard (500 μg/l) in 100 μl acetonitrile-1 ml/l TFA. A reference solution comprising 100 μL of acetonitrile containing 1 ml/l TFA was also enriched with the four drugs to the same nominal concentrations. The reconstituted extracts and reference solutions were injected onto the analytical column. The peak areas for T3, TE3 and mTE3, and the internal standard for the extracted samples were compared with the mean of those produced by the reference solutions ($n = 6$ injections). The peak area ratios (extracted samples/references) in the three matrices were as follows: T3, 1.01–1.03; TE3, 1.02–1.05; mTE3, 1.00–1.03 and verapamil 1.03–1.06. These findings confirmed that there was no influence of the matrix on the detection of either T3, TE3, mTE3 or the internal standard.

Selectivity was studied by analysing control human plasma, blood and RBCs from 10 different healthy subjects by the described procedures and the chromatograms examined for visible evidence of interfering endogenous compounds.

The possible interference by other commonly-used antimalarial drugs was also verified. The following drugs were checked: chloroquine, quinine, amodiaquine, mefloquine, sulfadoxine and pyrimethamine.

2.9. Validation

Validation of the assay procedure was carried out to establish inter-assay variability, selectivity and extraction efficiency over the calibration range. The inter-assay variability was determined by calculating the accuracy and precision of the assay. The accuracy is expressed as the percent recovery in terms of the nominal concentration (i.e., [mean found concentration/theoretical concentration] × 100), and precision is expressed by the relative standard deviation (R.S.D.).

Inter-assay variability was determined from the analysis of QC samples (T3: 16, 160.3 and 961.9 μg/l in plasma, 32, 320.6 and 1923.8 μg/kg in RBCs and whole blood; TE3 and mTE3: 25, 250 and 1500 μg/l in plasma and 50, 500 and 3000 μg/kg in whole blood) assayed against independent calibration curves on 7–33 separate occasions.

In order to establish that dilution of plasma or whole blood samples with T3 concentrations above the upper limit of the standard curve does not interfere with method performance, QC samples containing this compound at concentrations of 1300, 3250 and 6500 μg/l in plasma and 2600, 6500 and 13,000 μg/kg in whole blood (expressed in the form of bis-charged compound) were prepared. The three QC samples were diluted 2-, 5- and 10-fold, respectively, with drug-free human matrix in order to bring concentrations within the range of standard curves. Each analysis was performed six times at each concentration, using calibration curves and QC samples. The found concentrations were compared to the nominal concentrations.

The absolute extraction recoveries were determined based on the comparison of the areas under the peaks of the extracted QC samples as described above with those of unextracted reference standard solutions containing the corresponding concentrations. Three replicate analyses were performed at each concentration point. The extraction efficiency was also determined for the internal standard.

The lower limit of quantitation (LLOQ) was determined to be that concentration where the inter-assay precision and accuracy were acceptable (accuracy 80–120% and precision <20%) [14–17] based on six replicate analyses of fortified plasma, whole blood or RBCs.

2.10. Stability studies

The stability of stock solutions was determined at 4 °C. Appropriately diluted stock solutions were injected in triplicate into the LC–MS system immediately after preparation

(time 0) and at periodic intervals after storage at 4 °C over a span of 1 month.

Stability assays in plasma and whole blood were assessed by use of QC samples at the following concentrations: T3: 16, 160.3 and 961.9 μg/l, mTE3 and TE3: 25, 50, 250 and 1500 μg/l in plasma; T3: 32, 320.6 and 1923.8 μg/kg, mTE3 and TE3: 50, 500 and 3000 μg/kg in whole blood. The stability assays were carried out as follows:

(a) By storing plasma and whole blood QC samples at ordinary laboratory conditions (20 °C and daylight exposure) and in a refrigerator at 4 °C for 6 h.
(b) By storing QC samples in plasma and whole blood at −30 °C for 4 and 1 months, respectively.

Special attention was paid to the preparation of QC samples. Before to supplement plasma or blood samples with TE3 and mTE3, polypropylene tubes containing 0.5 ml of drug-free plasma or 0.25 g of drug-free blood diluted with 0.25 ml of purified water were stored in the refrigerator for 1 h then immediately chilled in an ice water bath. Thereafter, each sample was enriched with TE3 and mTE3, stirred and immediately placed for 10 s in solid carbon dioxide pellets for immediate freezing. Samples were then stored at −30 °C. Spiked samples were analysed immediately after preparation (reference values) and at selected time intervals after storage over the study period. Prior to their analysis, before thawing, samples were acidified by addition of 10 ml/l TFA in water, brought to room temperature and thoroughly vortex-mixed. Concentrations were calculated at each time point using a calibration curve freshly prepared and QC samples. Each determination was performed in triplicate.

(c) By subjecting plasma and whole blood QC samples fortified with the T3 compound to three freeze–thaw cycles; frozen triplicate samples were allowed to thaw at room temperature for 1 h and were subsequently refrozen. Their T3 concentrations were compared with aliquots that had not been subjected to the freeze–thaw cycles. The freeze–thaw evaluation was also performed for mTE3 and TE3 in acidified plasma and blood QC samples.
(d) By storing the acidic extracts (acetonitrile containing 1 ml/l TFA) in the autosampler at 20 and 4 °C for 48 h.
(e) The potential influence of the evaporating step (40 °C) was also tested: samples were maintained for 1 h at 40 °C. Aliquots were injected into the LC column at selected time intervals.

The possible conversion of mTE3 and TE3 to T3 during the course of evaporation, storage and analysis was also assessed.

Stability tests of the T3 compound in RBCs were also carried out by storing fortified samples (32, 320.6 and 1923.8 μg/kg) at room temperature and at 4 °C for 2 h, and at −30 °C for 4 months. The freeze–thaw stability (three cycles) was also determined.

Compounds were considered stable when losses were less than 15%.

3. Results and discussion

3.1. Retention times and specificity

The high polarity and the ionic nature of T3 complicated the selective clean-up of the sample, which probably was the principal handicap for its determination in biological samples. The Oasis HLB sorbent (*N*-vinylpyrrolidone–divinybenzene copolymer) used in this study during sample pre-treatment retained ionised analytes more strongly than silica-based reversed-phase sorbents [20]. Moreover, this water-wettable sorbent retains both ionised and non-ionised compounds. The clean-up procedure by SPE was found to be a suitable method for eliminating interfering material from both plasma, blood and RBCs. As presented in Fig. 3, no discernible peaks due to the matrices were observed within the time frame in which the different analytes were detected. T3, mTE3 and TE3 were well resolved from endogenous matrix components as shown in the typical LC profiles (Figs. 4 and 5). Over the 4 months of the validation study, retention times ($n = 10$) were 4.7 min (R.S.D. = 1.6%) for T3, 6.1 min (R.S.D. = 0.4%) for mTE3, 7.3 min (R.S.D. = 1.0%) for TE3 and 5.6 min (R.S.D. = 1.1%) for the internal standard. Under the chromatographic conditions used, the number of theoretical plates (calculated from the internal standard peak) was approximately 13,500. The column was replaced when the number of theoretical plates decreased below 7000 (i.e., after 940 analyses; this value is the mean obtained from four columns).

No interference was found with all tested drugs.

3.2. Drug/response relationship

The drug/internal standard peak area ratios were linked to concentrations according to a quadratic equation. The quadratic regressions gave mean coefficients of determination greater than 0.997 for the three analytes. Mean parameters of the quadratic equations are given in Table 1. Tables 2 and 3 show precision and percent recovery calculated from the mean back-calculated concentrations. For each analyte, the goodness of fit between back-calculated concentrations and nominal concentrations was statistically evaluated (i) by comparing the regression line of back-calculated versus nominal concentrations to the reference line of slope = 1 and intercept = 0; no significant difference was observed; (ii) by studying the frequency distribution histogram of the residuals, which were normally distributed and centred around zero, the number of positive and negative values being approximately equal; and (iii) by comparing the bias to zero; a *t*-test showed that this parameter was not statistically different from zero; moreover, the 95% confidence interval included the zero value.

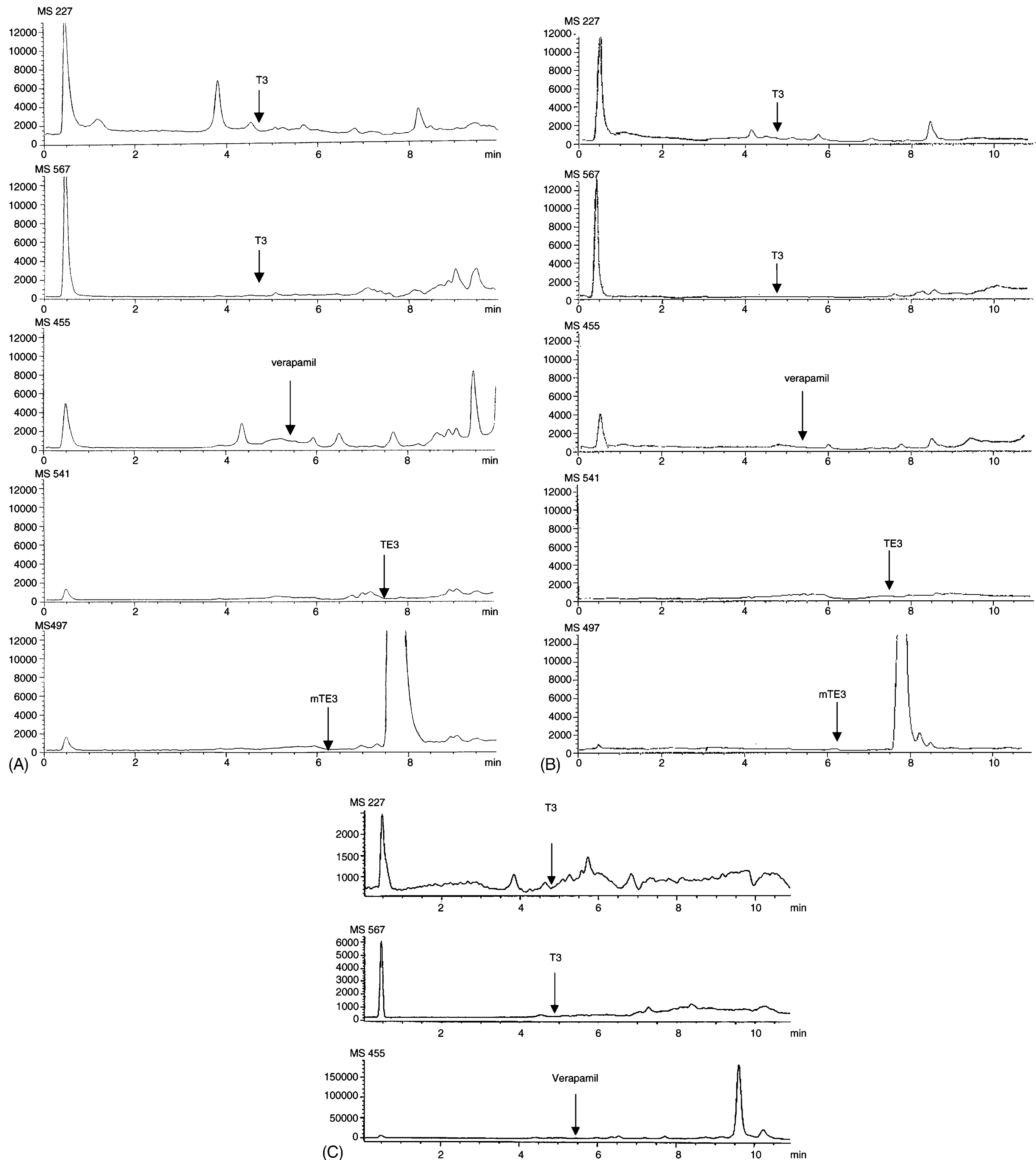

Fig. 3. Mass chromatograms obtained from 0.5 ml of blank human plasma (A), 0.25 g of blank human whole blood (B) and 0.25 g of blank human RBCs (C).

3.3. Precision, accuracy, extraction efficiency and LLOQ

The accuracy and precision determined during the validation procedure are presented in Table 4. At low, medium and high concentrations, the inter-assay precision was less than 13.5%, and accuracy ranged from 95.4 to 107%. Dilution has no influence on the performance of the method; following 2-, 5- and 10-fold dilutions of plasma and blood QC samples containing the T3 compound, precision ranged from 8.8 to 10.6% and accuracy was 94–103%.

In plasma, the mean extraction recoveries ($n = 9$) averaged 87% (R.S.D., 6.1%) for T3, 53% (R.S.D., 10%) for mTE3

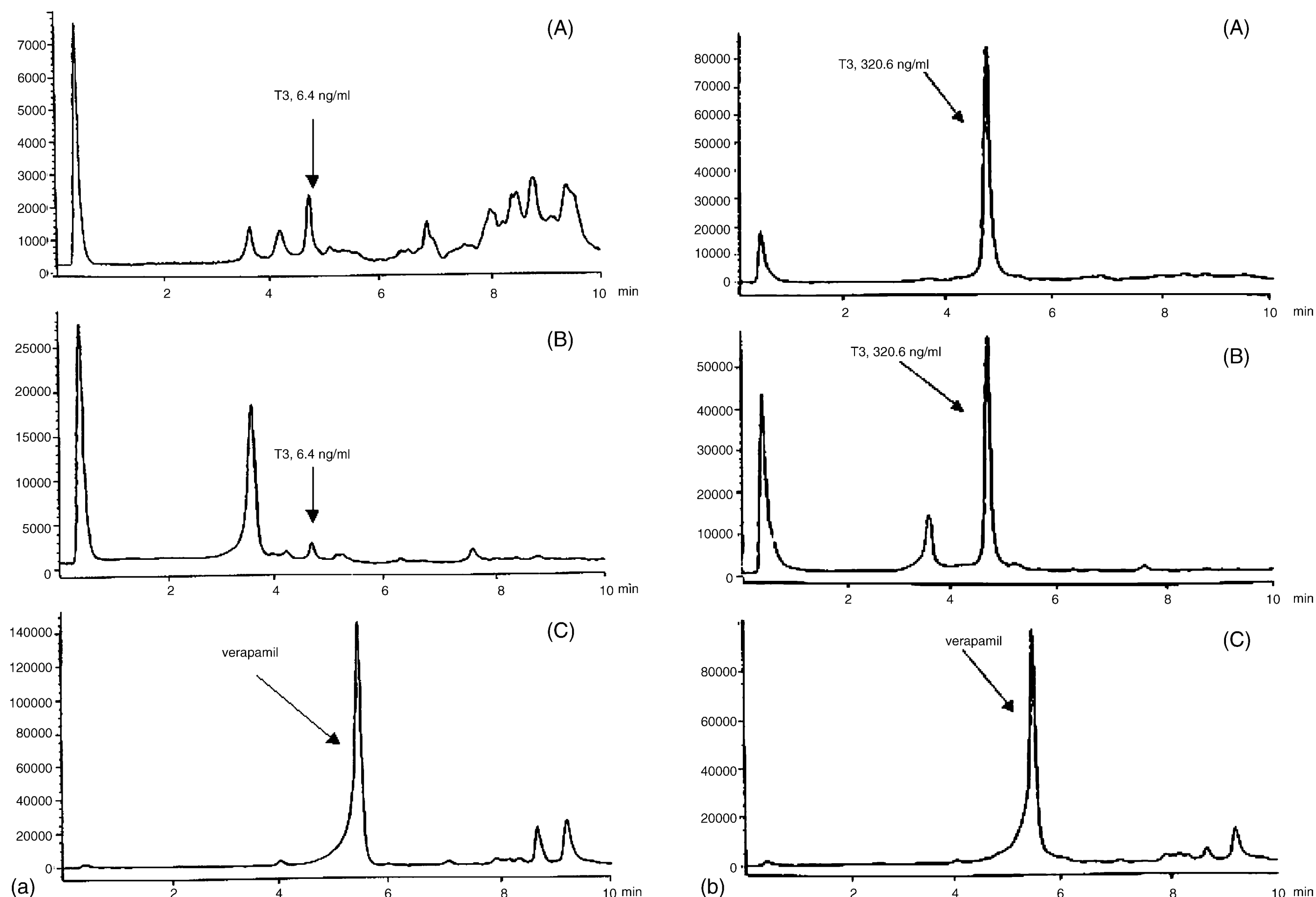

Fig. 4. Typical mass chromatograms of a blank plasma spiked with T3 at (a) 6.4 ng/ml (LLOQ) and (b) 320.6 ng/ml: (A) *m/z* 567; (B) *m/z* 227; and (C) *m/z* 455. For LC–MS conditions see Section 2.3. Concentrations are expressed in the form of bis-charged compound.

and 79% (R.S.D., 3.8%) for TE3. In the whole blood ($n = 9$), they were 79% (R.S.D., 10%) for T3, 57% (R.S.D., 8.2%) for mTE3 and 65% (R.S.D., 6.5%) for TE3. Recovery of T3 from RBCs was 93% (R.S.D., 9.2%, $n = 9$). These recoveries were not statistically different over the range of concentrations studied. The extraction recovery of the internal standard was determined to be 83% (R.S.D., 7.2%) ($n = 6$) from plasma, 70% (R.S.D., 11%) ($n = 9$) from whole blood and 72.5% (R.S.D., 12.6%) ($n = 7$) from RBCs.

Using 0.5 ml of plasma, the LLOQ was 6.4 μg/l for the bis-thiazolium compound, T3; 20 μg/l for the mTE3 compound and 10 μg/l for the lipophilic prodrug TE3. At these concentrations, accuracy and precision values were 84–110% and 18–20%, respectively. In blood and RBCs, the LLOQ was 12.8 μg/kg for the bis-thiazolium compound (accuracy, 82–120%; precision, 17%). It was 40 μg/kg for the mTE3 and TE3 compounds (accuracy, 90–115%; precision, 15%) in blood.

Table 1
Results of calibration curves[a]

Analyte	*a* (mean)	*b* (mean)	*c* (mean)
Plasma			
T3 ($n = 39$)	4.25×10^{-5}	1.93×10^{-3} (R.S.D. = 20%)	−0.058
mTE3 ($n = 6$)	-1.5×10^{-7}	8.6×10^{-3} (R.S.D. = 16.0%)	−0.22
TE3 ($n = 9$)	3.65×10^{-8}	3.72×10^{-4} (R.S.D. = 16.9%)	0.0153
Whole blood			
T3 ($n = 10$)	-3.19×10^{-7}	1.62×10^{-3} (R.S.D. = 13.5%)	0.018
mTE3 ($n = 5$)	9.07×10^{-8}	7.2×10^{-4} (R.S.D. = 8.87%)	−0.037
TE3 ($n = 5$)	1.74×10^{-8}	1.88×10^{-4} (R.S.D. = 14.6%)	0.0090
RBCs			
T3 ($n = 10$)	-1.86×10^{-6}	7.3×10^{-3} (R.S.D. = 16.7%)	−0.0605

n, number of replicates.
[a] $Y = ax^2 + bX + c$.

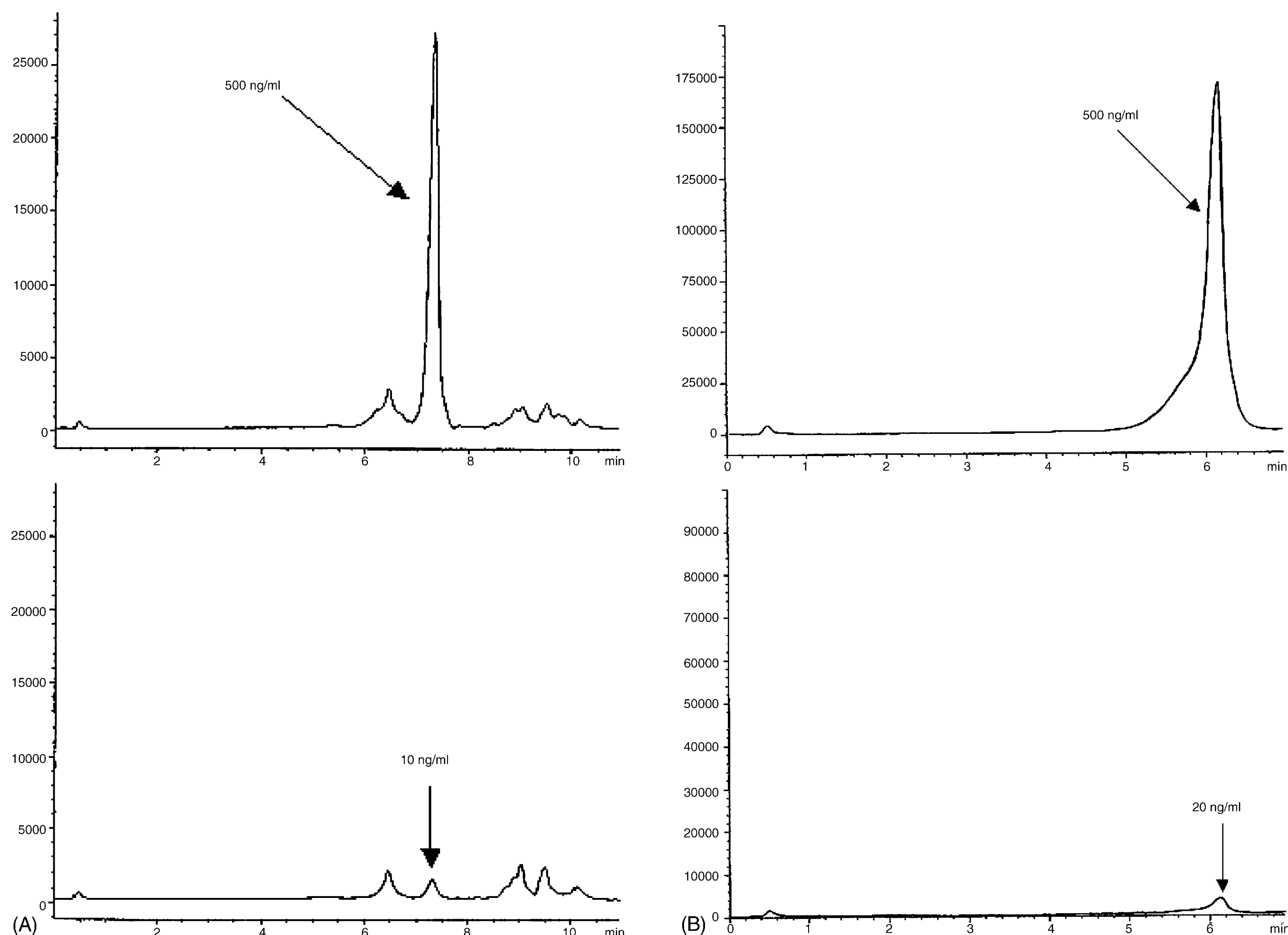

Fig. 5. Typical mass chromatograms of a blank plasma spiked with TE3 at 10 (LLOQ) and 500 ng/ml (*m/z* 541) (A) and mTE3 at 20 (LLOQ) and 500 ng/ml (*m/z* 497) (B). For LC–MS conditions see Section 2.3.

3.4. Stability

Stock solutions containing T3, mTE3 and TE3 were stable after storage for 1 month in a refrigerator (4 °C).

In plasma and blood, after bench-top storage at both room temperature and at 4 °C, T3 (whatever the concentration studied) was stable for 6 h; at each time point studied, no statistical difference was shown to exist by comparison with the reference values. In these two matrices, rapid mTE3/T3 and TE3 prodrug/T3 conversions occurred, at both 20 and 4 °C whatever the concentration studied. The lowest conversion rate was observed at 4 °C (conversion half-life ∼3 h); after 15 min mean percent recovery was 92–97%. The conversion rate increased with the tempera-

Table 2
Calibration curves in plasma: precision and recovery computed from mean back-calculated concentrations

T3 ($n = 39$)			mTE3 ($n = 6$)			TE3 ($n = 9$)	
Theoretical concentration[a] (μg/l)	R.S.D. (%)	Recovery (%)	Theoretical concentration (μg/l)	R.S.D. (%)	Recovery (%)	R.S.D. (%)	Recovery (%)
6.4	10.3	112	10	–	–	19.7	110
12.8	13.6	95.5	20	6.4	105.1	2.3	107
32.1	13.7	105	50	7.9	96.9	11.3	102
64.2	8.9	98.6	100	10.6	104.7	6.7	97.1
128.2	7.1	99.7	200	1.8	96.0	8.0	98.1
320.6	6.2	98.7	500	3.0	101.7	6.5	97.9
641.2	1.9	101	1000	1.6	99.5	1.1	100
1282	3.7	99.3	2000	0.1	100.1	5.3	98.0

[a] Expressed in the form of bis-charged compound.

Table 3
Calibration curves in RBCs and whole blood: precision and recovery computed from mean back-calculated concentrations

T3 theoretical concentration[a] (μg/kg)	RBCs (whole blood) ($n = 10$)		Whole blood ($n = 5$)				
	R.S.D. (%)	Recovery (%)	mTE3 and TE3 theoretical concentration (μg/kg)	mTE3		TE3	
				R.S.D. (%)	Recovery (%)	R.S.D. (%)	Recovery (%)
12.8	15.7 (14.9)	102 (96.8)	–	–	–	–	–
25.6	12.2 (13.1)	98.5 (95.4)	40	10.5	111.9	9.02	110.0
64.2	7.9 (5.0)	99.8 (102)	100	11.8	93.6	8.22	89.3
128.4	5.5 (6.0)	99.4 (101)	200	9.31	108.2	3.64	106.2
256.8	4.8 (3.9)	100 (97.0)	400	1.76	96.3	3.00	99.2
641.2	4.0 (3.6)	99.4 (102)	1000	0.64	99.9	0.30	100.0
1282.4	3.5 (3.8)	99.6 (97.3)	2000	2.95	107.7	0.10	100.0
2564	4.0 (4.4)	97.4 (103)	4000	1.20	99.5	6.74	96.7

RBCs: red blood cells.
[a] Expressed in the form of bis-charged compound.

ture. We have previously shown that, at 37 °C, the initial conversion half-life was about 5 min and this bioconversion is almost complete after 8 h (13). The decrease in the concentration of TE3 occurs concomitantly with the appearance of T3. Furthermore, this transformation involves plasma enzymatic activity such as esterases or thioesterases (data not shown). Acidification of plasma with TFA and operating at reduced temperatures considerably lower TE3/T3 conversion, enabling preparation of calibration curves and QC samples. Thus, for pharmacokinetic studies, special attention must be paid during the handling of samples containing TE3 and mTE3. Blood must be collected in tubes placed in an ice water bath then immediately centrifuged at 4 °C. The storage of plasma samples in acidified medium (TFA) avoided TE3/T3 and mTE3/T3 conversions during the freezing and thawing steps. Working in whole blood instead of plasma should be more convenient as it was possible to immediately stabilize blood with TFA after sampling.

When stored at −30 °C for 4 months in plasma and 1 month in whole blood, T3, TE3 and mTE3 were stable, all samples retaining more than 90% of their original concentration values.

All analytes were stable during the evaporation process. In extracts after sample pre-treatment (i.e., in acetonitrile containing 1 ml/l TFA), T3, mTE3 and TE3 were stable whatever the concentration studied for at least 24 h at both room temperature and +4 °C. Extracts must not be stored frozen to avoid precipitation of the T3 compound.

Finally, it was determined that three freeze–thaw cycles of plasma or whole blood QC samples fortified with T3 or acidified plasma and whole blood QC samples fortified with TE3 and mTE3 were well tolerated with no significant losses of the compounds (<10%).

RBC samples spiked with T3 and allowed to stand at room temperature and at 4 °C for 2 h did not demonstrate a significant reduction in the nominal starting concentrations. When stored at −30 °C for 4 months, T3 was stable.

Table 4
Between-day accuracy and precision of the assay in plasma, whole blood and RBCs

T3[a] (plasma, $n = 33$; RBCs, $n = 17$)						mTE3 and TE3 plasma	mTE3 ($n = 15$)		TE3 ($n = 13$)	
Theoretical concentration		Precision (%)		Accuracy (%)		Theoretical concentration (μg/l)	Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)
Plasma (μg/l)	RBCs (μg/kg)	Plasma	RBCs	Plasma	RBCs					
16.0	32.0	11.7	12.9	104	98.7	25	6.2	105	5.1	105
160.3	320.6	12.4	7.7	99.5	102	250	6.2	102	10.3	103
961.9	1923.7	10.6	9.2	102	101	1500	5.3	98.3	4.0	99.7
T3 Whole blood ($n = 7$)						**mTE3 and TE3 plasma**	**mTE3 ($n = 10$)**		**TE3 ($n = 10$)**	
Theoretical concentration (μg/kg)		Precision (%)		Accuracy (%)		Theoretical concentration (μg/kg)	Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)
32.0		13.2		100		50	10.2	101	8.3	98.3
320.6		3.79		107		500	9.6	95.4	10.9	99.3
1923.7		11.2		96.3		3000	9.9	96.1	11.1	100

n, number of replicates; RBCs: red blood cells.
[a] Expressed in the form of bis-charged compound.

4. Conclusion

LC–MS methods, with good precision and accuracy have been developed for the simultaneous determination of a bis-thiazolium cationic compound, T3, its related neutral prodrug, TE3, and an intermediate mono-cationic compound, mTE3, in plasma and whole blood. This last compound appears in blood during the prodrug/drug conversion. A method has been also validated to quantify T3 in RBCs. Distinct advantages include simplicity and rapidity of sample preparation and chromatographic operating conditions, and good resolution between the analytes. The SPE procedure can be easily automated either by robotisation or with an automated sample preparation system giving high-quality, high-throughput analyses in support of pharmacokinetic studies of this new class of antimalarial drugs.

Acknowledgments

This study was supported by the European Community (QLK2-CT-2000-01166), Ministère de l'Education Nationale et Recherche Scientifique (PAL^{+}).

References

[1] C. Wongsrichanalai, A.L. Pickard, W.H. Wernsdorfer, S.R. Meshnick, Lancet Infect. Dis. 2 (2002) 209.
[2] B. Greenwood, T. Mutabingwa, Nature 670 (2002) 670.
[3] G.G. Holz Jr., Bull. Wld. Hlth. Organiz. (WHO) 55 (1977) 237.
[4] H.J. Vial, M.L. Ancelin, J.R. Philippot, M.J. Thuet, Blood Cells 16 (1990) 531.
[5] H.J. Vial, M. Calas, Inhibitors of phospholipid metabolism, in: P.J. Rosenthal (Ed.), Antimalarial Chemotherapy Mechanism of Action, Resistance, and New Directions in Drug Discovery, Humana Press Inc., Totowa, NJ, 2000, pp. 347–365.
[6] M.L. Ancelin, M. Calas, J. Bompart, G. Cordina, D. Martin, M. Ben Bari, H. Vial, Blood 91 (1998) 1426.
[7] M.L. Ancelin, M. Calas, V. Vidal-Sailhan, S. Herbute, P. Ringwald, H.J. Vial, Antimicrob. Agents Chemother. 47 (2003) 2590.
[8] M.L. Ancelin, M. Calas, A. Bonhoure, S. Herbute, H.J. Vial, Antimicrob. Agents Chemother. 47 (2003) 2598.
[9] M. Calas, G. Cordina, J. Bompart, M. Ben Bari, T. Jei, M.L. Ancelin, H. Vial, J. Med. Chem. 40 (1997) 3557.
[10] M. Calas, M.L. Ancelin, G. Cordina, P. Portefaix, G. Piquet, V. Vidal-Sailhan, H. Vial, J. Med. Chem. 43 (2000) 505.
[11] H.J. Vial, M.L. Ancelin, J.R. Philippot, M.J. Thuet, Blood Cells 16 (1990) 531.
[12] K. Wengelnik, V. Vidal, M.L. Ancelin, A.M. Cathiard, J.L. Morgat, C.H. Kocken, M. Calas, S. Herrera, A.W. Thomas, H.J. Vial, Science 295 (2002) 1311.
[13] H. Vial, S. Wein, C. Farenc, C. Kocken, O. Nicolas, M.L. Ancelin, F. Bressolle, A. Thomas, M. Calas, Proc. Natl. Acad. Sci. U.S.A. 101 (2004) 15458.
[14] US Food and Drug Administration, Guidance for Industry, Bioanalytical Method Validation, May 2001, http://www.fda.gov/cder/guidance/4252fnl.htm (accessed May 2004).
[15] United States Pharmacopoeia XXXIII, The United States Pharmacopoeia Convention, Rockville, MD, 2003, p. 2439.
[16] V.P. Shah, K.K. Midha, S. Dighe, I.J. McGilveray, J.P. Skelly, A. Yacobi, T. Layloff, C.T. Viswanathan, C.E. Cook, R.D. McDowall, K.A. Pittman, S. Spector, J. Pharm. Sci. 81 (1992) 309.
[17] F. Bressolle, M. Bromet-Petit, M. Audran, J. Chromatogr. B 686 (1996) 3.
[18] H. Vial, M. Calas, J.J. Bourguignon, M.L. Ancelin, V. Vidal, E. Rubi, Précurseurs de sels de bis-ammonium quaternaire et leurs applications comme prodrogues ayant une activité antiparasitaire, Patent 99 09471, applied by CNRS, 21 July 1999, WO 0105742 A1, published 25 January 2001.
[19] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 70 (1998) 882.
[20] F. Bressolle, O. Nicolas, M.T. Kelly, Clinical analysis: sample handling, in: Encyclopedia of Analytical Sciences, Elsevier Ltd., Amsterdam, 2005, pp. 133–141.