Journal of Pharmaceutical and Biomedical Analysis 48 (2008) 1001–1005

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

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

Short communication

A liquid chromatography–mass spectrometry assay for simultaneous determination of two antimalarial thiazolium compounds in human and rat matrices

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ARTICLE INFO

Article history:
Received 2 May 2008
Received in revised form 23 June 2008
Accepted 25 June 2008
Available online 5 July 2008

Keywords:
Antimalarial mono-ammonium salts
Bioanalysis
Validation
Human and rat matrices
LC–ESI-MS

ABSTRACT

A new class of antimalarial drugs targeting phospholipid metabolism of the malarial parasite is now in development. In the strategy of this development, two mono-thiazolium salts, T1 and T2, need to be monitored. A liquid chromatography–mass spectrometry (LC–MS) method has been developed and validated according to FDA guidelines for simultaneous determination of T1 and T2 in plasma, whole blood and red blood cells (RBCs) from human and rat. The sample-pre-treatment procedure involved solid phase extraction after protein precipitation. Chromatography was carried out on a Zorbax eclipse XDB C8 column and mass spectrometric analysis was performed using an Agilent 1100 quadrupole mass spectrometer working with an electrospray ionization source. LC–MS data were acquired in single ion monitoring mode at *m/z* 312, 326 and 227 for T1, T2 and the internal standard (T3), respectively. The drug/internal standard peak area ratios were linked via a quadratic relationship to concentrations (human and rat plasma: 2.25–900 μg/l; human blood and rat RBCs: 4.5–900 μg/kg). Precision was below 14.5% for T1 and below 13% for T2. Accuracy was 92.6–111% for T1 and 95.6–108% for T2. Extraction recoveries were ≥85% in plasma and ≥53% in blood and RBCs. For T1 and T2, the lower limits of quantitation were 2.25 μg/l in plasma, and 4.5 μg/kg in whole blood and RBCs. Stability tests under various conditions were also investigated. This highly specific and sensitive method was useful to analyse samples from pharmacokinetic studies carried out in rat and would also be useful in clinical trials at a later stage.
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1. Introduction

The emergence of *Plasmodium falciparum* resistance to many antimalarial drugs is becoming a severe problem all over the world, especially on the African continent [1]. Thus, the development of new chemotherapies is urgently needed, especially compounds that work through new independent mechanisms of action. During its intraerythrocytic development, the parasite synthesizes considerable amounts of membranes, with a quasi-absence of cholesterol [2]. As a consequence, its development depends on an active and temporally controlled synthesis of new membranes, which is fuelled by phospholipid (PL) precursors including serine, ethanolamine, choline and fatty acids, scavenged from the human host. The erythrocyte PL content increases by as much as 500% after infection [2]. Phosphatidylcholine is the major PL of infected erythrocytes (~45% of the total PL). Thus, we have developed a new strategy of antimalarial treatment targeting phospholipid metabolism of the malarial parasite through compounds that mimic choline, the polar head of phosphatidylcholine [3–11].

Both mono- and bis-quaternary ammonium salts have been synthesized [5,6,8]. These drugs have inhibitory concentrations ranging from 10^{-6} to 10^{-12} mol/l. Among them, the T3 and T4 bis-quaternary ammonium salts exhibited the highest in vitro and in vivo activities against *P. falciparum* [10]. We have recently published analytical methods to quantify these compounds and their bioprecursors (prodrugs) in different matrices [12–14]. The pharmacokinetic properties of T3 and its prodrug (TE3) have also been studied in rat [15]. In the strategy of the development of this new class of antimalarial drugs, it appeared interesting to study the mono-quaternary ammonium salts, especially T1 and T2. T1 and T2 are the mono-cationic analogues of T3 and T4, respectively; they structures are presented in Fig. 1. These two compounds might be

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doi:10.1016/j.jpba.2008.06.018

of interest for other antiparasitic activities against plasmodium-unrelated parasites (unpublished data).

The aim of this paper was to describe analytical methods to simultaneously quantify T1 and T2 in plasma, whole blood and red blood cells (RBCs) from human and rat. T2 is a T1-like monothiazolium compound in which the hydroxyl function has been replaced by a methoxy group. The developed method was used to assay plasma and RBC samples from a preclinical study carried out in rats after intravenous administrations of T1 and T2.

2. Materials and methods

2.1. Chemicals and reagents

T1 (MW 392), T2 (MW 406) and T3 (internal standard, MW 614.7) were synthesized by the Laboratoire des Aminoacides, Peptides et Proteines (UMR 5247, Montpellier I and II Universities) with purity, evaluated by elemental analysis; greater than 99.6% (Fig. 1). They were stored at 4 °C in the refrigerator. All solvents and chemicals were of analytical grade. Trifluoroacetic acid (TFA) was obtained from Sigma (St. Louis, MO, USA). Acetonitrile, methanol, ammonium formate, formic acid and trimethylamine were purchased from Merck (Darmstadt, Germany). The formate buffer

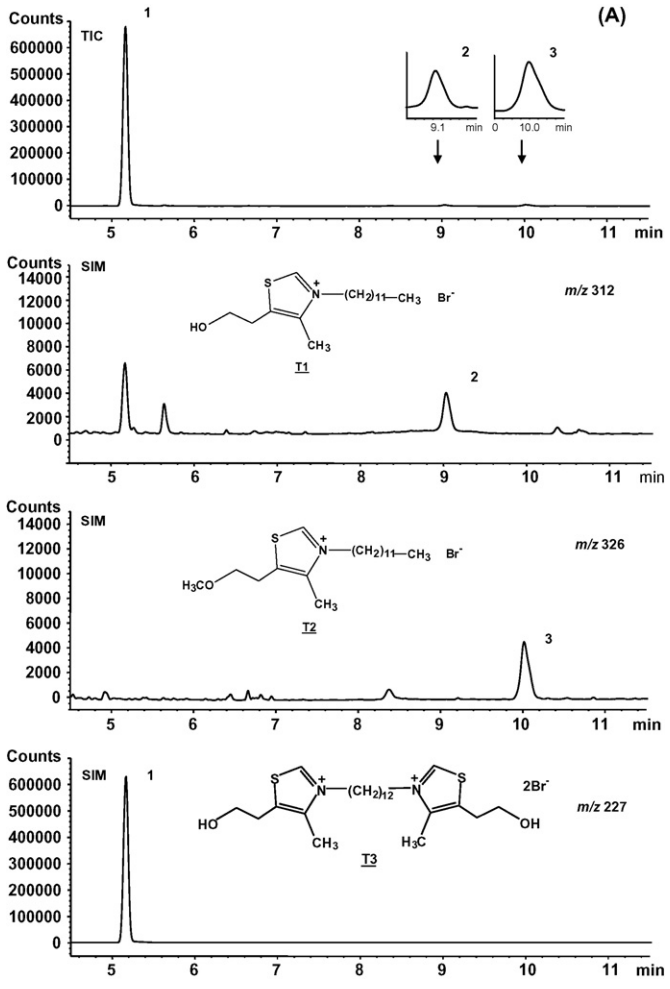

Fig. 1. Typical chromatograms of blank human whole blood spiked with T1 and T2 at the following concentrations: 4.5 μg/kg. For LC–MS conditions see Section 2.2. Peak 1 = T3 (internal standard); peak 2 = T1 and peak 3 = T2 (TIC, total ionic current; SIM, single ion monitoring). MS parameters: capillary voltage, 4.0 kV; cone voltage, 80 V; heated N_2 gas (350 °C, 10 l/min); and compressed N_2 gas (13 psi).

Table 1
Gradient schedule

Time (min)	% solvent	
	A	B
0	15	85
10	100	0
12	100	0
14	15	85
19	15	85

(126 mg/l ammonium formate) was prepared in purified water and adjusted to pH 3 with formic acid. In-house deionised water was further purified with a Milli-Q water-purifying system (Millipore, Bedford, MA, USA). Oasis HLB cartridges (30 mg of sorbent, average particle diameter 30 μm) were supplied by Waters (Saint Quentin, France). For method validation, human whole blood and plasma were obtained from pooled blood samples collected from healthy volunteers not undergoing drug therapy (Etablissement Français du sang, Montpellier, France). Coagulation was prevented by adding EDTA–sodium salt. Blood samples from Sprague–Dawley rats (Charles River Laboratories, L'Arbresle Cedex, France) were collected with sodium heparin. 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, RBCs and plasma were aliquoted, stored at −80 °C and then used during the study in the preparation of standards and quality control (QC) samples.

Individual stock solutions of T1 (45 mg/l), T2 (45 mg/l) and T3 (65 mg/l), expressed in the form of charged compounds, were prepared in purified water then stored at 4 °C in the refrigerator. For each compound, two separate stock solutions were prepared: one was used for the preparation of calibrators and the second was used for the preparation of quality control (QC) samples.

2.2. Instrumentation and chromatographic conditions

The liquid chromatography–mass spectrometry (LC–MS) analysis was performed using an Agilent 1100 quadrupole mass spectrometer (Agilent Technologies, Les Ulis, France) working with an electrospray ionization (ESI) source in the positive mode. The mass spectrometer was coupled to a Hewlett Packard LC system and an HP Chemstation. The autosampler was set at 4 °C. A Zorbax Eclipse XDB C8 column (150 mm × 4.6 mm, 5 μm, Agilent Technologies) with a C_8 symmetry guard column (20 mm × 3.9 mm, 5 μm, Waters, Milford, MA, USA) was used for LC separation. The column temperature was 20 °C. Chromatography was carried out via a gradient system (mobile phase A: 130 μl/l trimethylamine in acetonitrile, mobile phase B 2 mmol/l ammonium formate buffer pH 3.0, Table 1). The flow rate was set at 800 μl/min (injected volume, 10 μl). The optimum mass spectrometric parameters are given in Fig. 1. From the full-scan spectra, T1 and T2 were detected through the $(M)^+$ ions at *m*/*z* 312 and *m*/*z* 326, respectively. The T3 compound (internal standard) was detected by use of the quaternary ammonium salt ($M^{2+}/2$) at *m*/*z* 227.

2.3. Working standards, preparation of calibration curves and quality control samples

Stock solutions of T1 and T2 were appropriately diluted in purified water (1/2 to 1/1600) to obtain the 13 working solutions (0.028–22.5 mg/l) used to spike calibrators and QC samples. The stock solution of T3 was diluted eightfold (8.125 mg/l) in purified water before use. An unextracted solution prepared in a mixture of water–acetonitrile–TFA (50:50:0.1, v/v/v) containing T1 and

T2 (2.25 mg/l) and the internal standard (1.62 mg/l) was injected before each run to check the performance of the LC–ESI-MS system.

In plasma, the calibration set consisted of nine concentrations, prepared by adding 20 μl of the relevant working solutions to 0.5 ml of drug-free matrices. In whole blood or RBCs, calibration set consisted of eight concentrations, prepared by adding 20 μl of the relevant working solutions to 0.25 g of drug-free matrices diluted with 0.25 ml of purified water. Thus, concentrations across a range of 2.25–900 μg/l (expressed in the form of charged compound) for T1 and T2 in plasma and of 4.5–900 μg/kg for T1 and T2 in whole blood or RBCs were obtained. A calibration curve also included a blank matrix. QC samples at four different levels (3.75, 11.2, 112 and 675 μg/l in plasma; 7.5, 22.5, 225 and 600 μg/kg in whole blood or RBCs) were prepared in the same way as the calibrators by mixing drug-free matrices (0.5 ml plasma, or 0.25 g RBC or whole blood diluted with 0.25 ml of purified water) with 20 μl of the appropriate working solutions.

2.4. Sample preparation procedure

The same sample pre-treatment procedure described to analyse other compounds of the same chemical series (bis-thiazolium salts and their prodrugs) [12–14] has been used with success to quantify these two new compounds. Briefly, plasma, whole blood and RBC samples were subjected to a solid–liquid extraction using Oasis HLB cartridges after protein precipitation. Thus, to 0.5 ml of plasma or 0.25 g of whole blood (or RBCs) diluted with 0.25 ml of purified water, 20 μl of internal standard (T3 at 8.125 mg/l) and 0.5 ml of water containing 10 ml/l TFA were added before solid-phase extraction. Cartridges were washed with purified water then the analytes were desorbed by acetonitrile containing 1 ml/l TFA. After evaporation of the eluate fractions, the dry residue was dissolved in 100 μl of the mixture water–acetonitrile–TFA (50:50:0.1, v/v/v). A 10-μl volume was injected onto the LC system for analysis.

2.5. Data analysis

From recorded peak areas, the ratios of each analyte to internal standard were calculated. To link peak area ratios and theoretical concentrations of each analyte in the three matrices, different models were tested: (i) unweighted or weighted least-squares linear regression analysis ($Y = aX + b$) and (ii) quadratic relationship as $Y = aX^2 + bX + c$. The regression curve was not forced through zero. The resulting equation parameters were used to calculate "back-calculated" concentrations for the calibrators, which were then statistically evaluated [16].

2.6. Matrix effects

The suppression or enhancement of ionization of analytes by the presence of matrix components in the extracts was investigated by calculating the matrix factor [17]. For this procedure, six different batches of each drug-free matrix (human and rat plasma, human whole blood and rat RBCs) were treated as described above in duplicate ($n = 12$ per matrix and per studied concentration ($n = 4$)). The dried extracts obtained from plasma, whole blood or RBCs were then reconstituted with 100 μl of the TFA–acetonitrile–water mixture containing T1 and T2, both at concentrations of 18.75, 56, 560 and 3375 μg/l, and the internal standard at concentrations of 1620 μg/l. Reference solutions comprising 100 μl of the TFA–acetonitrile–water mixture were also enriched with the three drugs at the same four nominal concentrations. The reconstituted extracts and reference solutions were injected onto the analytical column. The matrix factors were calculated from the peak areas of T1, T2 and internal standard obtained from the reconstituted extracts (in presence of matrix ions) divided by the corresponding peak areas produced by the reference solutions ($n = 6$ injections per concentration, absence of matrix ions). Matrix factor values between 0.85 and 1.15 were jugged acceptable.

2.7. Validation procedure [16,18,19]

The specificity of the analytical methods was determined by the analysis of ten different independent sources of the same biological matrix. The retention times of endogenous compounds in the matrices were compared with those of the compounds of interest. The possible interference by other commonly used antimalarial drugs was also verified. The following drugs were checked: chloroquine, quinine, amodiaquine, mefloquine, sulfadoxine and pyrimethamine.

We assessed both intra- and inter-assay precision and accuracy by analysing QC samples at the above mentioned four concentrations against calibration curves. For intraassay precision and accuracy, replicate analyses ($n = 6$) of each QC sample were performed the same day. For interassay precision and accuracy, each of the four QC samples were analysed once a day, on 6–10 different days. The accuracy was evaluated as [mean found concentration/nominal concentration] × 100. Precision was given by the percent relative standard deviation (R.S.D.).

Extraction recoveries of T1 and T2 from human and rat plasma, human blood and rat RBCs were measured six times at each concentration of QC samples. The areas under the peaks of extracted QC samples were compared with those of the authentic (unextracted) calibrators in the relevant concentration range prepared in the TFA–acetonitrile–water mixture. The extraction recovery was also determined for the internal standard.

The LLOQ was defined as the lowest concentration that could be determined with accuracy within 80–120% and a precision ≤20% on a day-to-day basis. To determine the analytical error in the LLOQ, spiked QC samples were used.

2.8. Stability study

The stability of stock solutions was determined at 4 °C over a span of 1 month.

The stability studies of T1 and T2 in the four matrices (human and rat plasma, human whole blood and rat RBCs) were performed by analysing QC samples at the above mentioned concentrations ($n = 3$ per concentration) against a calibration curve immediately following preparation then after different storage conditions: (i) 20 and 4 °C over 6 h, (ii) in the freezer (−20 and −80 °C) over 8 months and (iii) during up to three freeze–thaw cycles. At specific time intervals, samples were analysed alongside replicates of identical concentrations of freshly prepared QC samples. Run-time stability at 20 and 4 °C over 24 h for processed samples after extraction was also determined for each calibration point.

Compounds were considered stable when losses were <15%.

2.9. In vitro and in vivo studies

The LC–MS method was used to quantify T1 and T2 in plasma and RBCs from Sprague–Dawley rats (Charles River, L'Arbresle Cedex, France), age 10 weeks weighing 250–300 g, receiving intravenous administration of T1 (2.10 mg/kg, expressed in the form of charged compound) or T2 (2.15 mg/kg, expressed in the form of charged compound). This research adhered to the "Principles of Laboratory Animal Care" (NIH publication #85-23, revised 1985). The animal study was approved by the local Animal Use Committee. Blood samples (one sample *per* rat) were drawn in heparinized polypropylene tubes at the following time-points (3 animals *per* time-point),

before administration, 5, 10 and 30 min, and 1, 2, 4, 8, 11, 24 and 35 h after drug administration. Blood samples were collected after sacrifice of the animal by section of the carotid artery then centrifuged at 4 °C (2000 × *g* for 20 min). Plasma samples were transferred into polypropylene tubes and stored at −80 °C until assay. RBCs were washed twice with an equal volume of 0.9% sodium chloride to limit plasma contamination before storage at −80 °C.

Pharmacokinetic parameters were computed using a compartmental approach from the average concentration values at each time point [20,21].

3. Results

3.1. Ion suppression study

In human matrices, the peak area ratios (reconstituted extracts/reference solutions) were 1.05 and 0.97 in plasma; 1.03 and 0.97 in whole blood for T1 and T2, respectively (R.S.D., 0.8–5.9%). They were 0.93 in plasma and 1.13 in blood (R.S.D., 5.0–6.0%) for the internal standard (T3). In rat matrices, the peak area ratios ranged from 0.93 to 1.10 (R.S.D., 2.1–6.5%). These findings confirmed that the matrix had no influence on the detection of T1, T2 or the internal standard.

3.2. Retention times and specificity

Fig. 1 shows typical chromatograms obtained from extracts of drug-free whole blood spiked with the three analytes. Observed retention times ($n = 30$) were 9.03 min for T1, 9.98 min for T2 and 5.11 min for the internal standard (R.S.D.s, 0.09–0.14%). The k'-values were 3.1, 3.5 and 1.3, respectively. The asymmetry coefficient was 0.96 for T1, 0.87 for T2 and 0.90 for T3. The specificity of this method was demonstrated by representative chromatograms of blank matrices which indicated that each analyte was well resolved from the matrix endogenous peaks. No interference was found with any tested drugs.

3.3. Drug/response relationship

For both T1 and T2, quadratic calibration curves gave the best fit based on the statistical analysis results and accuracy and precision of QC samples. Moreover, quadratic calibration curves clearly provide an extended measurement interval. R.S.D. values on the slope, *b*, were ≤15% (6.1–9.3% in human plasma; 14–15% in rat plasma; 7.6–12.5% in human blood and 7.5% in rat RBCs). R.S.D. and recovery values around the mean back-calculated concentrations were 0.7–19.3% and 89.9–108%, respectively. For each analyte, the goodness of fit between back-calculated concentrations and nominal concentrations was statistically verified [14].

3.4. Precision, accuracy, extraction efficiency and LLOQ

Results for intra-day and inter-day precision and accuracy are presented in Table 2.

For T1, the mean extraction recoveries were 87.0, 86.5, 53.0 and 53.5% (R.S.D., 6.1–10%) from human plasma, rat plasma; human whole blood and rat RBCs, respectively. For T2, they were 85.0, 85.3, 56.0 and 55.3% (R.S.D., 8.6–11%), respectively. In the four matrices,

Table 2
Accuracy and precision of the method

Matrices	Theoretical concentration (μg/l or μg/kg)[a]	T1		T2	
		Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)
Human matrices					
Plasma	Within-day ($n = 6$)				
	3.75	8.0	101	8.6	103
	11.2	4.9	111	10.4	108
	112	5.8	102	9.6	99.2
	675	6.3	101	9.9	96.0
	Between-day ($n = 6$)				
	3.75	8.5	98.9	12.7	97.7
	11.2	5.3	102	4.3	97.1
	112	4.9	99.5	2.9	102
	675	6.1	102	2.8	99.9
Whole blood	Within-day ($n = 6$)				
	7.5	9.0	99.3	7.2	101
	22.5	12.3	94.8	9.8	95.6
	225	7.6	95.5	11.0	95.6
	600	9.0	96.1	9.2	98.6
	Between-day ($n = 6$)				
	7.5	14.3	98.8	9.8	101
	22.5	11.6	100	9.6	99.3
	225	12.4	94.7	10.0	100
	600	10.0	92.6	9.0	97.2
Rat matrices					
Plasma	Between-day ($n = 10$)				
	3.75	10.1	97.0	8.6	102
	11.2	13.5	99.0	9.8	103
	112	7.5	102	9.4	101
	675	10.7	102	9.0	100
RBCs	Between-day ($n = 7$)				
	7.5	10.4	101	10.0	102
	22.5	10.0	97.8	9.1	98.7
	225	6.8	103	8.5	101
	600	9.0	98.8	9.4	101

[a] Expressed in the form of charged compound, μg/kg applies for whole blood and RBCs; *n*, number of replicates; RBCs: red blood cells.

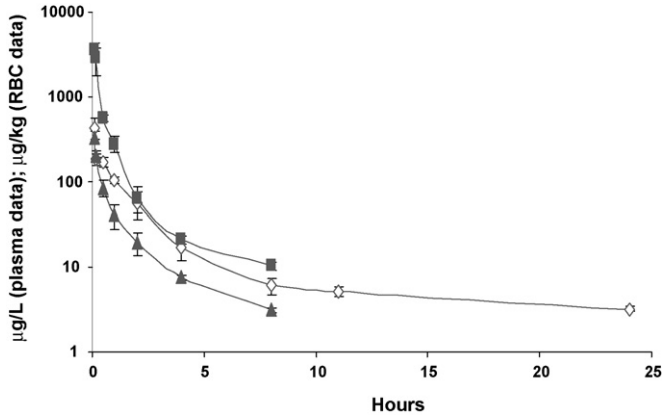

Fig. 2. Mean (±S.D.) RBC (◊) concentration versus time curve after single intravenous (2.10 mg/kg) administration of T1 in rat. Mean (±S.D.) plasma (▲) and RBC (■) concentration versus time curves after single intravenous (2.15 mg/kg) administration of T2 in rat.

the extraction recoveries of the internal standard were 89.0, 91.2, 51.0 and 51.8% (R.S.D., 4.5–8.7%), respectively.

Based upon the analysis of low concentration replicate QC samples in each validation run, the LLOQ value was 2.25 μg/l for both T1 and T2 in plasma. In whole blood and RBCs, the corresponding values were 4.5 μg/kg. At these levels, the precision was <19.5% R.S.D. and accuracy was 89.8–108%.

3.5. Stability

When stored at 4 °C in the refrigerator for a period of 1 month, stock solutions of T1 and T2 did not reveal any appreciable degradation.

Frozen at −20 and −80 °C, QC samples showed no sign of either degradation or losses. In all studied matrices, at least three freeze–thaw cycles can be tolerated without losses higher than 10%. Human or rat plasma, human whole blood and rat RBC samples spiked with T1 and T2 allowed standing at 20 or 4 °C for 6 h showed no sign of decrease in the nominal starting concentration. T1 and T2 were stable during the evaporation process. At 4 °C in the autosampler, reconstituted extracts were stable for 24 h.

3.6. In vivo studies

Semilogarithmic plots of the mean (±S.D.) T1 RBC, and T2 plasma and RBC concentration–time profiles are shown in Fig. 2. Five and 10 min after administration, plasma T1 concentrations were 108 ± 48.1 μg/l and 22.4 ± 4.82 μg/l, respectively. They were below the LLOQ of the analytical method at the third sample and not detected at 1 h. From RBC data, the half-life of the terminal part of the curve was 18 h. After T2 administration, pharmacokinetic parameters were: (i) from plasma data: total clearance, 10 l/(h kg); steady-state volume of distribution, 33.8 l/kg; and terminal half-life, 2.33 h; (ii) from RBC data: terminal half-life, 2.62 h. For T2, the RBC/plasma area under curve ratio was 7.9. The rate of transfer within RBCs is higher for T1 than for T2. These findings were in accordance with in vitro results (data not shown). Such an uptake was not observed for the bis-thiazolium compounds [9–11].

4. Discussion and conclusion

In the present paper, a LC–MS method, with good precision and accuracy has been developed for the simultaneous determination of two mono-ammonium compounds, T1 and T2, in human and rat matrices. We have shown that in healthy rat, T1 and T2 concentrations were higher in RBCs than in plasma. It has been shown that amphiphile compounds, as the alkylammoniums, are presumed to intercalate by their hydrophobic ends into the lipid bilayer of the erythrocyte membrane inducing swelling and shape alterations in erythrocytes [22–24]. Thus, T1 and T2 could be mainly retained within the lipid bilayer of the erythrocyte membrane instead of in the cytosol of the cell.

Acknowledgments

This study was supported by the European Community (PCRDT FP6, Integrated Projects Antimal “Development of New Drugs for the treatment of Malaria”) and Sanofi-Aventis Laboratories (Montpellier, France).

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