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Quantitative analysis of a bis-thiazolium antimalarial compound, SAR97276, in mouse plasma and red blood cell samples, using liquid chromatography mass spectrometry

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Abstract

A sensitive and selective liquid chromatography–mass spectrometry (LC–MS) method has been developed for the determination of a new antimalarial bisthiazolium salt, SAR97276, in mouse plasma and red blood cells (RBCs). A drug of the same chemical series as the test drug, T2, was used as internal standard. The method involved solid phase extraction of the compound and the internal standard from the two matrices using Oasis®HLB columns. LC separation was performed on a Zorbax eclipse XDB C8 column (5 μm) with a mobile phase of acetonitrile containing trimethylamine (130 μl/l, solvent A) and 2 mM ammonium formate buffer (solvent B). MS data were acquired in single ion monitoring mode at *m/z* 227 for SAR97276 and *m/z* 326 for T2. The matrix had no influence on the detection of either SAR97276 or T2. The drug/internal standard peak area ratios were linked via quadratic relationships to plasma (1.65–1322 ng/ml) and RBC concentrations (3.31–2644 ng/ml). Precision was below 14% and accuracy was 91.4–104%. Dilution of the samples had no influence on the performance of the method. Extraction recoveries of SAR97276 were ≥90% in plasma and ≥60% in RBCs. The lower limits of quantitation were 1.65 ng/ml in plasma and 3.31 ng/ml in RBCs. Stability tests under various conditions were also investigated. The method was successfully used to determine the pharmacokinetic profile of SAR97276 in healthy mouse.

Keywords: Antimalarial bis-thiazolium salt; SAR97276; Red blood cells; Plasma; Quantitation; LC-ESI-MS

1. Introduction

Malaria is one of the most common infectious diseases and an enormous public-health problem. A 300–500 million people worldwide are affected by malaria and between 1 and 1.5 million people die from it every year. Previously extremely widespread, the malaria is now mainly confined to Africa, Asia and Latin America. The problems of controlling malaria in these countries are aggravated by inadequate health structures and poor socioeconomic conditions [1,2]. The situation has become even more complex over the last few years with the increase in resistance to antimalarial drugs normally used to combat the parasite that causes the disease and the increase in insecticide resistance [3]. Unfortunately, no vaccine is currently available for malaria; moreover, prophylactic treatments are simply too expensive for most people living in endemic areas. Thus, the development of new chemotherapies is urgently needed; especially compounds that work through new independent mechanisms of action and that are structurally unrelated to existing antimalarial agents.

Lipid biogenesis in *Plasmodium* constitutes metabolic processes that are required for the synthesis of the malarial membranes that serve as permeability barriers between the parasite and its host, the membranes of the subcellular organelles, the membrane networks in the cytoplasm of infected erythrocytes, and also the lipid-derived signalling molecules that regulate

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parasite development and proliferation. Lipids are among the most critical components that need to be duplicated, and there is considerable evidence supporting the crucial role of lipids and the enzymes involved in their synthesis in parasite growth, differentiation and various other cellular events such as signaling and hemozoin formation. The intracellular malarial parasites obtain the necessary lipid species by active synthesis through *de novo* pathways using precursors that are actively transported from the host cytoplasm [4,5].

During the last 10 years, a new antimalarial pharmacological approach based on inhibition of the plasmodial phospholipid metabolism has been identified and developed. Both mono- [6,7] and bis-quaternary ammonium compounds [7–9], and amidine and guanidine compounds have been designed and evaluated for antimalarial activities. These compounds mimic the choline structure, and have been shown to be specifically accumulated inside infected erythrocytes [10,11], thus ensuring both potency and selectivity. They were shown to block the plasmodial phosphatidylcholine biosynthesis [10,11], but also to interact with heme/hemozoin (plasmodial haemoglobin metabolites) [12]. Their dual mechanism of action should limit the risk of emergence of resistance. Although much data indicate that the lethal effect is closely related to the blockage of phosphatidylcholine biosynthesis, the exact steps that mediate selective inhibition of this metabolic pathway have yet to be clarified. The bis-quaternary ammonium salts showed the highest activities and have an antimalarial activity in the nanomolar range [10]. These drugs exert a very rapid cytotoxic effect against malarial parasites and are active in vivo against *P. vinckei*-infected mice after intraperitoneal, intra-rectal or oral administration as well as against *P. cynomolgi* and *P. falciparum malaria* in the monkey [10,11].

SAR97276 has shown potent in vitro as well as in vivo antimalarial activities at low doses in the murine model (50% efficient dose (ED_{50}) of 0.2–0.25 mg/kg by the intraperitoneal route) [13] and in *P. cynomolgi*-infected *Rhesus* monkey [10]. Its pharmacokinetic profile has been also studied in the rat [13].

The present work involves the development of a new technique for the identification of this new series of antimalarial drugs. Recently, liquid chromatography–mass spectrometry (LC–MS) methods were developed in our laboratory for the quantitation of SAR97276 and of another bis-quaternary ammonium salt (T4), and their corresponding prodrugs in human and rat matrices [14,15]. Applying these methods to mouse matrices an interfering compound at the retention time of SAR97276 was detected. Thus, LC–MS conditions have been modified and optimized, and we developed a new method that is required to carry out pharmacokinetic/pharmacodynamic studies in healthy and infected animal.

We report in this paper a LC–MS method to quantify the SAR97276 compound in plasma and red blood cells from mouse. This method was validated according to validation procedures, parameters and acceptance criteria [16–19]. It was used to assay samples from a preclinical study performed in the mouse. Pharmacokinetic results in healthy animals are presented.

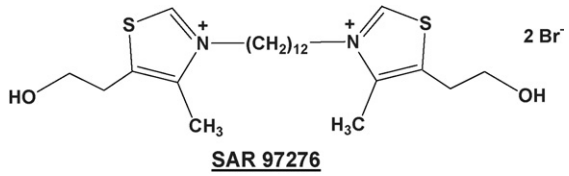
2. Experimental

2.1. Chemicals and reagents

SAR97276 (1,12-bis[4-methyl-5-(2-hydroxyethyl)-3-thiazol-3-ium-3-yl]dodecane dibromide, MW 614.7) and T2 (3-dodecyl-5-(2-methoxyethyl)-4-methyl-1,3-thiazol-3-ium bromide, MW 405.9) are characterized products of Laboratoire des Aminoacides, Peptides et Proteines, UMR 5247 (Montpellier I and II Universities, France) (Fig. 1) [20]. The purity of these standards was evaluated by elemental analysis; results were within ±0.4% for each element of calculated values. SAR97276 and T2 were stored at ambient temperature and protected from light.

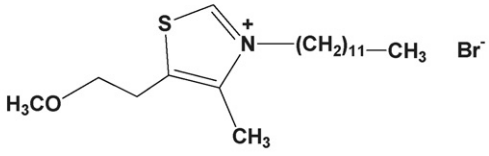
Acetonitrile and methanol (HPLC grades), ammonium formate, formic acid and trimethylamine were obtained from Merck (Darmstadt, Germany). Trifluoroacetic acid (TFA) was from Sigma (St. Louis, MO, USA). The formate buffer solution consisted of ammonium formate (2 mM, 126 mg/l) in purified water and adjusted to pH 3 ± 0.05 with formic acid. Ultra-high quality water was obtained from 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).

Blood samples used for the development and validation of the procedure were collected with sodium heparin and were obtained from female Swiss mice (body weight 29.8 ± 2.5 g; Charles River Laboratories, L'Arbresle Cedex, France). 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 RBCs and plasma were aliquoted, kept frozen at −80 °C and then used during the study in the preparation of standards and quality control (QC) samples.



SAR 97276

1,12-dodecamethylenebis[4-methyl-5-(2-hydroxyethyl)thiazol-3-ium] dibromide



T2

3-dodecyl-5-(2-methoxyethyl)-4-methyl-1,3-thiazol-3-ium bromide

Fig. 1. Structural formulae of SAR97276 and T2 (internal standard).

2.2. Instrumentation and chromatographic conditions

Optimization of various experimental parameters including the nature of the stationary phase, composition of the eluent, nature of the organic modifier, capillary voltage, nebulizer pressure and sampling cone voltage was carried out (data not shown).

The chromatographic analysis was carried out using a Hewlett Packard Agilent 1100 chromatograph (Agilent Technologies, Les Ulis, France) equipped with a ternary pumping unit, a degasser and an autosampler fitted with a rheodyne loading valve and set at 4 °C. Chromatographic separation was achieved using a Zorbax Eclipse XDB C8 column (150 mm × 4.6 mm, i.d., particle size 5 μm) purchased from Agilent technologies protected by a C_8 symmetry column (20 mm × 3.9 mm, i.d., 5 μm-size from Waters). The column temperature was 20 °C. Chromatography was carried out via a gradient system at a flow rate of 800 μl/min. The injected volume was 10 μl. The mobile phase involved a mixture of eluent A, 130 μl/l trimethylamine in acetonitrile and eluent B, ammonium formate buffer. The starting eluent was 15% A and 85% B after which the proportion of eluent A was increased linearly to 100% in 10 min then held for 2 min in order to wash the column, returned to its initial conditions within 2 min and then was re-equilibrated for 5 min.

The mass spectrometer (Agilent Technologies) equipped with an electrospray source (mass range: *m/z* 50–2000) was run in positive mode (ES+). Mass spectrometric data were acquired in single ion monitoring mode. A standard 0.50 mm capillary was used in the electrospray interface. Data acquisition and analysis were performed using the HPChem software (version 08.04) from Agilent Technologies. The mass spectrometer was calibrated using a mixture of NaI and CsI (peak width of the mass: 0.6–0.7 amu). The drying gas temperature and flow were maintained at 350 °C and 10 l/min, respectively, and the nebulizer pressure was set at 13 psi. The dwell time was set at 98 ms, the values of the capillary voltage and the cone voltage were 4.0 kV and 80 V, respectively. From the full-scan spectra, T2 was detected through the $(M)^+$ ion at *m/z* 326; SAR97276 was detected by use of the quaternary ammonium salt ($M^{2+}/2$) at *m/z* 227. The mass spectra (scan mode) of T2 and SAR97276 are shown in Fig. 2.

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

Stock solutions of T2 (45 mg/l) or SAR97276 (64 mg/l), expressed in the form of charged compounds, were prepared in purified water. For each compound, two separate stock standard solutions were prepared: one was used for the preparation of the calibration curve standards and the second was used for the preparation of QC samples. Stock solutions were stored at 4 °C and were brought to room temperature before use. In these storage conditions; they were stable for at least 1 month [14,15]. For the SAR97276 compound, 13 standard working solutions (from 0.064 to 32.0 mg/l) were obtained extemporaneously by making appropriate dilutions of the stock solutions with purified water. The stock solutions of the internal standard,

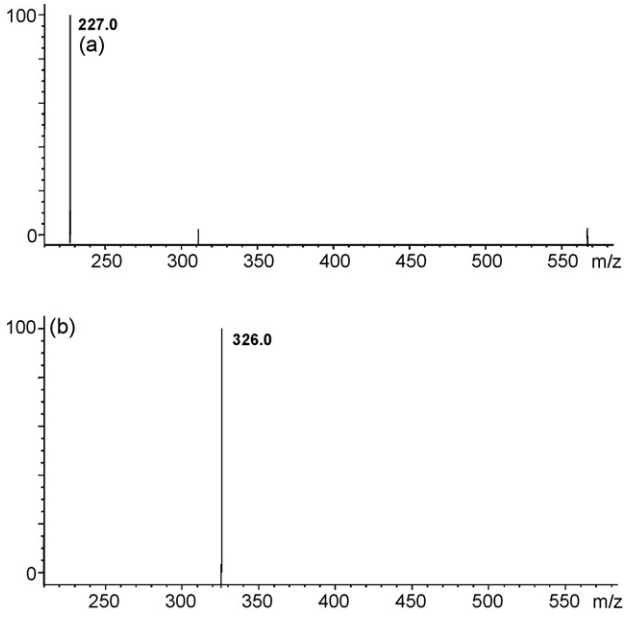


Fig. 2. Mass spectra (scan mode) for (a) SAR97276 and (b) T2.

T2, were diluted 40-fold (1.125 mg/l) in-purified water before use.

The nominal concentration of calibration standards were prepared by adding appropriate volumes of working solutions to drug-free matrix. Calibration curves consisted of 10 calibration points covering 1.65–1322 ng/ml for plasma and 3.31–2644 ng/ml for RBCs. A calibration curve also included a blank matrix. Four levels of QC samples were prepared at the concentrations of 3.31 (low QC samples), 16.5 (medium QC samples), 132 (medium QC samples) and 992 ng/ml (high QC samples) for plasma and 6.61, 33.1, 331 and 1983 ng/ml for RBCs. In order to test whether it is possible to apply the described method to samples whose concentrations are higher than the last calibration point, QC samples spiked at 1300 and 2600 ng/ml in plasma and 2594 and 5085 ng/ml in RBCs were prepared. They were diluted 2-, 5- and 10-fold with blank mouse matrix (RBCs or plasma) in order to bring concentration within the range of standard curve. Inter-day variability was determined by performing replicate analyses of QC samples against a calibration curve. The found concentrations were reported and compared to the nominal one.

After preparation, standards and QC samples previously vortex-mixed (10 s) were incubated at 4 °C protected from light, for 20 min, to allow a steady state with the matrix components.

Before each analytical run, a reference standard solution prepared in a mixture acetonitrile/water/TFA (50:50:0.1, v/v) containing SAR97276 (3.25 mg/l) and the internal standard (0.225 mg/l) was injected in the LC–MS system to verify its performance.

2.4. Sample preparation procedure

Sample clean-up was achieved by solid phase extraction (SPE) after protein precipitation using a method similar to that previously published [14,15]. The SPE column was pre-washed

with 1 ml of methanol followed by 1 ml of purified water before use.

After adding 20 μl of internal standard solution (1.125 mg/l) to a 500 μl plasma sample aliquot, 0.5 ml of purified water containing 10 ml/l TFA was added. The mixture was vortex-mixed and centrifuged at 4 °C (3000 × *g*) for 10 min. The supernatant was then loaded onto the conditioned cartridge under a light vacuum (approximately 86 kPa) using a Vac Elut 20® (Varian, Les Ulis, France). The extraction column was washed with 1 ml of purified water and was then dried for 2 min by vacuum aspiration (approximately 27 kPa). The analytes were eluted with 2× 1 ml of acetonitrile containing 1 ml/l TFA under a light vacuum (approximately 86 kPa). The eluate fractions were collected in a 5-ml polypropylene tube then evaporated to dryness under a stream of nitrogen for 30 min at 40 °C. The residue was reconstituted in 100 μl of a mixture water/acetonitrile/TFA (50:50:0.1, v/v) followed by vortex-mixed for 1 min. A 10-μl volume was injected into the LC–MS system for analysis.

To a 250 μl RBC sample aliquot diluted with 250 μl of purified water, 20 μl of internal standard (1.125 mg/l) were added. To precipitate proteins before SPE extraction, three types of precipitation reagents were investigated (TFA, methanol and acetonitrile) during the experiment. A mixture of 0.5 ml of water containing 10 ml/l TFA was proved to be the best among the three reagents in terms of peak shape and higher recovery. This precipitation reagent was added drop-wise while the mixture was vortex-mixed in order to obtain smaller precipitate particles which avoid significant analyte losses. After centrifugation at 4 °C for 10 min at 17,562 × *g*, the assay procedure was as described above for the plasma sample, except that interfering material was removed by washing with 2× 0.75 ml of purified water.

2.5. Data analysis

Analyte-to-internal standard peak area ratios were used as the assay parameter. To define the relationship between peak area ratios and nominal SAR97276 concentrations in the two matrices, two different models were tested: (i) unweighted or weighted linear regression model ($Y = aX + b$) and (ii) quadratic regression model ($Y = aX^2 + bX + c$) in which Y is the peak area ratio and X is the nominal concentration of the analyte. The regression curve was not forced through zero. The resulting equation parameters were used to calculate "back-calculated" concentrations for the calibrators. The good agreement between added and back-calculated concentrations was 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's *t*-test); the 95% confidence interval was also determined.

2.6. Matrix effect and specificity studies

Undetected matrix components, which co-eluting with analytes, may adversely affect the reproducibility of analyte ionisation in electrospray source of mass spectrometer [21]. To investigate potential ion suppression or ion amplification effects attributable to the matrix, we performed the following experiments. A total of six different batches of mouse matrix (plasma and RBCs) were extracted by the method described above in duplicate ($n = 12$ per matrix and per studied concentration). The dried extracts were reconstituted in 100 μl of a mixture of water/acetonitrile/TFA (50:50:0.1, v/v) containing SAR97276 at three reference concentrations (16.5, 660 and 4960 ng/ml) and the internal standard (225 ng/ml). A reference solution comprising 100 μl of water/acetonitrile/TFA (50:50:0.1, v/v) was also enriched with the two drugs to the same nominal concentrations. The 36 supernatants and 3 preparations from the reference solutions were injected into the analytical column, and the peak areas obtained from the supernatants were compared with the corresponding peak areas produced by the reference solutions (each of them being injected fivefold in the LC–MS system). The ratios for the mean peak areas in matrix to those in the reference solution were calculated. This ratio must be near to 1. An interval of 0.85–1.15 was jugged acceptable.

Selectivity was demonstrated by running drug-free mouse plasma and RBC samples from six different pools and by examining the chromatograms 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.

2.7. Accuracy, precision, extraction recovery and lower limit of quantitation (LLOQ)

Precision and accuracy were assessed by performing replicate analyses of QC samples in plasma and RBCs at the abovementioned four concentrations against a calibration curve. The procedure was repeated on different days ($n = 9$–10) to determine inter-day accuracy and precision validation data. The percent relative standard deviation (R.S.D.) served as the measure of precision. The accuracy was evaluated as [mean found concentration/nominal concentration] × 100.

The extraction recoveries of SAR97276 over the QC ranges were determined by comparing peak areas of SAR97276 obtained from each QC sample prepared in plasma (3.31–992 ng/ml) or RBCs (6.61–1983 ng/ml), extracted as described above and those obtained from direct injection of a solution containing SAR97276 at the same concentrations dissolved in a mixture water/acetonitrile/TFA (50:50:0.1, v/v). The extraction recovery was also determined for the internal standard.

The LLOQ was defined as the lowest concentration on the calibration curves for which an acceptable accuracy (within 80–120%) and precision (≤20%) were obtained [16–19].

2.8. Stability

The stability of SAR97276 in mouse plasma and RBCs was evaluated in reconstituted extracts (at 4 °C in the autosampler

for 40 h) and in the matrices (at 4 °C for 6 h, and at −80 °C for 18 months). Stability data are based on replicate determinations of QC samples at the following concentrations: 16.5, 165 and 992 ng/ml in plasma ($n = 4$), and 16.5, 33.1, 331 and 1983 ng/ml in RBCs ($n = 4$) and on replicate determinations of real sample from pharmacokinetic study (320–4645 ng/ml in plasma and 72–235 ng/ml in RBCs).

2.9. Application of the method to a pharmacokinetic study in healthy mice

The experimental protocol was designed in compliance with the recommendations of the European Economic Community (86/609/CEE directive). The animal study was approved by the local Animal Use Committee.

Female mice (body weight 31.6 ± 1.7 g) were fasted overnight (12 h) before drug administration and then weighed. Each mouse received intraperitoneal administration of 5 mg/kg of SAR97276 (expressed in salified compound) over a 2–3 min period. Blood samples were obtained at the following times: 0.083, 0.25, 0.5, 1, 2, 4, 6, 11, 24 and 35 h after dosing. At each time point, nine mice were sampled and one sample per animal was collected. Samples were collected in heparinized polypropylene tubes (0.1 ml lithium heparinate per tube) from the jugular vein after decapitation of the animal. At each sampling time, blood was aliquoted into three polypropylene tubes, each of them corresponding to pooled blood samples collected from three different animals.

Tubes containing blood samples were immediately gently agitated to prevent coagulation then centrifuged (2000 × g) at 4 °C for 10 min. The plasma was separated then immediately stored at −80 °C until assay. RBC samples were washed twice with an equal volume of 0.9% sodium chloride to limit plasma contamination before storage at −80 °C.

Pharmacokinetic analysis was carried out from the average concentration values at each time point using the Pk-fit software [22,23]. Pharmacokinetic parameters were determined from the plasma (or RBCs) concentration–time data using a compartmental approach, and included the total area under the plasma (or RBCs) concentration–time curve ($AUC_{0-\infty}$), elimination half-life ($t_{1/2}$ elim) and total clearance (CL/F). CL was uncorrected for bioavailability (F).

3. Results and discussion

3.1. Selectivity and matrix effect

The analysis of blank matrices from different sources showed the absence of interfering endogenous components at the retention times of the two analytes (Fig. 3).

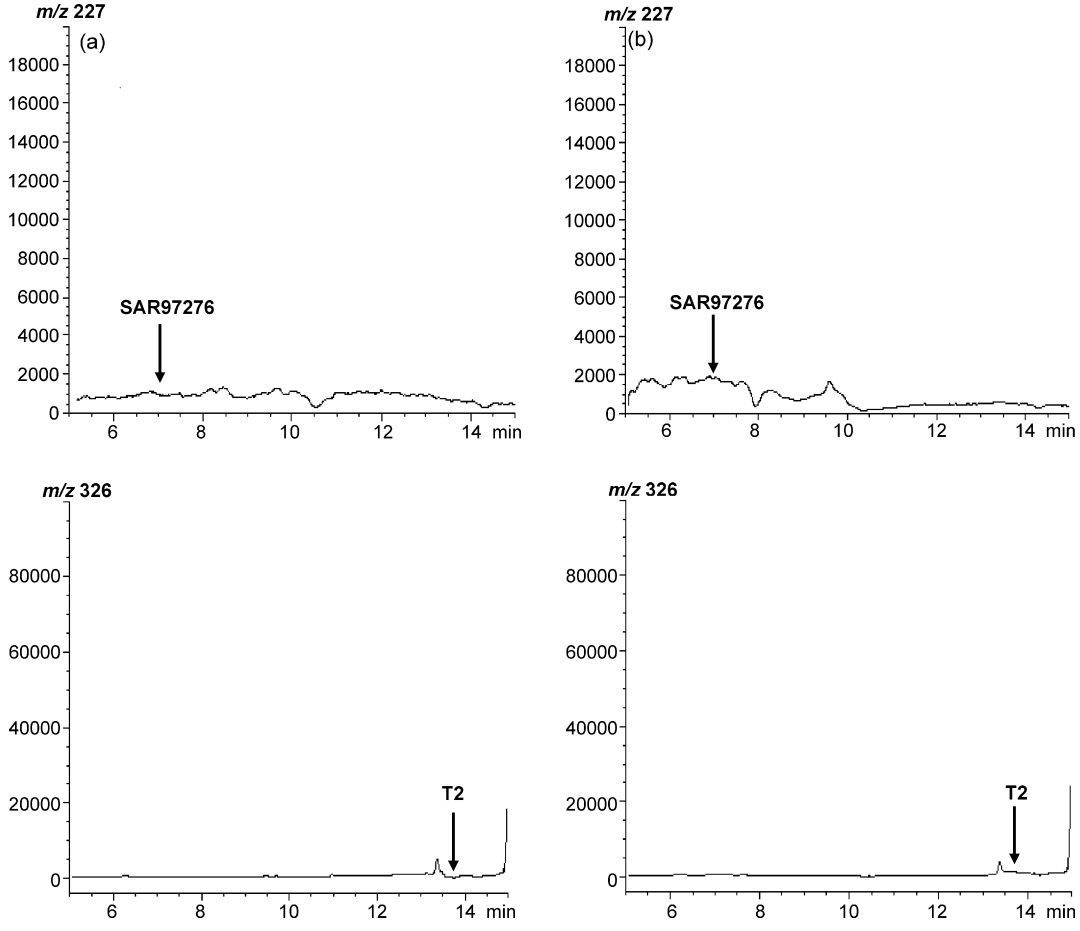


Fig. 3. Typical chromatograms of blank mouse matrices. (a) Plasma; (b) RBCs. For chromatographic conditions see text.

In bio-analytical mass spectrometry, the research of matrix effects is almost inescapable. The mean peak area ratios for SAR97276 and the internal standard (extracted blank matrices fortified with the analytes/reference solutions) ranged from 0.9 to 1.1 (R.S.D., 4–8%). These findings confirmed that the matrix had no influence on the detection of either SAR97276 or the internal standard.

3.2. Relationship between response and nominal concentrations

In LC–MS (or LC–MS–MS), especially with electrospray ionisation, linear calibration curves with a range exceeding two decades are often hard to find. Moreover, the electrospray ionisation does not a priori generate analyte ions by a linear process. In addition, quadratic calibration curves clearly provide an extended measurement interval. In the present study, the unweighted quadratic equation gave the best fit based on the analysis of residuals and the acceptance criteria [16–19] of the concomitant QC samples calculated according the different models applied to a particular calibration set. In doing so, data heteroscedasticity is counteracted and a higher weight would be required to the lower concentrations in the concentration curve, leading to an improved quantification of the low-level concentrations. Unfortunately, the software used to calculate the parameters of the quadratic equation did not allow us to use a weighting factor. Thus, to circumvent this difficulty, two different calibration curves were constructed (1.65–165 and 66.1–1322 ng/ml in plasma; 3.31–264 and 66.1–2644 ng/ml in RBCs). As expected, most of the unknown samples were within these concentration ranges. The use of a linear function introduced accuracy errors. The resulting parameters of the quadratic equation were used to obtain concentration values for that day's QC samples and unknown samples.

Inter-assay repeatability was determined for calibration curves prepared on different days ($n = 10$) in plasma and RBCs. Representative chromatograms are shown in Fig. 4 for plasma and in Fig. 5 for RBCs. During the 4 months of validation, observed retention times were 6.8 min (R.S.D., 0.13%, $n = 10$) for SAR97276 and 13.7 min for T2 (R.S.D., 0.03%, $n = 10$). The k'-value were 2.8 and 5.5, respectively. The peak skew was evaluated using the asymmetry coefficient $A_s = b/a$, where b is the distance after the peak maximum and a is the distance before the peak maximum, both a and b being measured at 10% of the total peak height. The asymmetry coefficients were 1 ± 0.05 for SAR97276 and 0.83 ± 0.04 for T2. Under the chromatographic conditions used, the number of theoretical plates (calculated from the internal standard) was approximately 55,000. The pre-

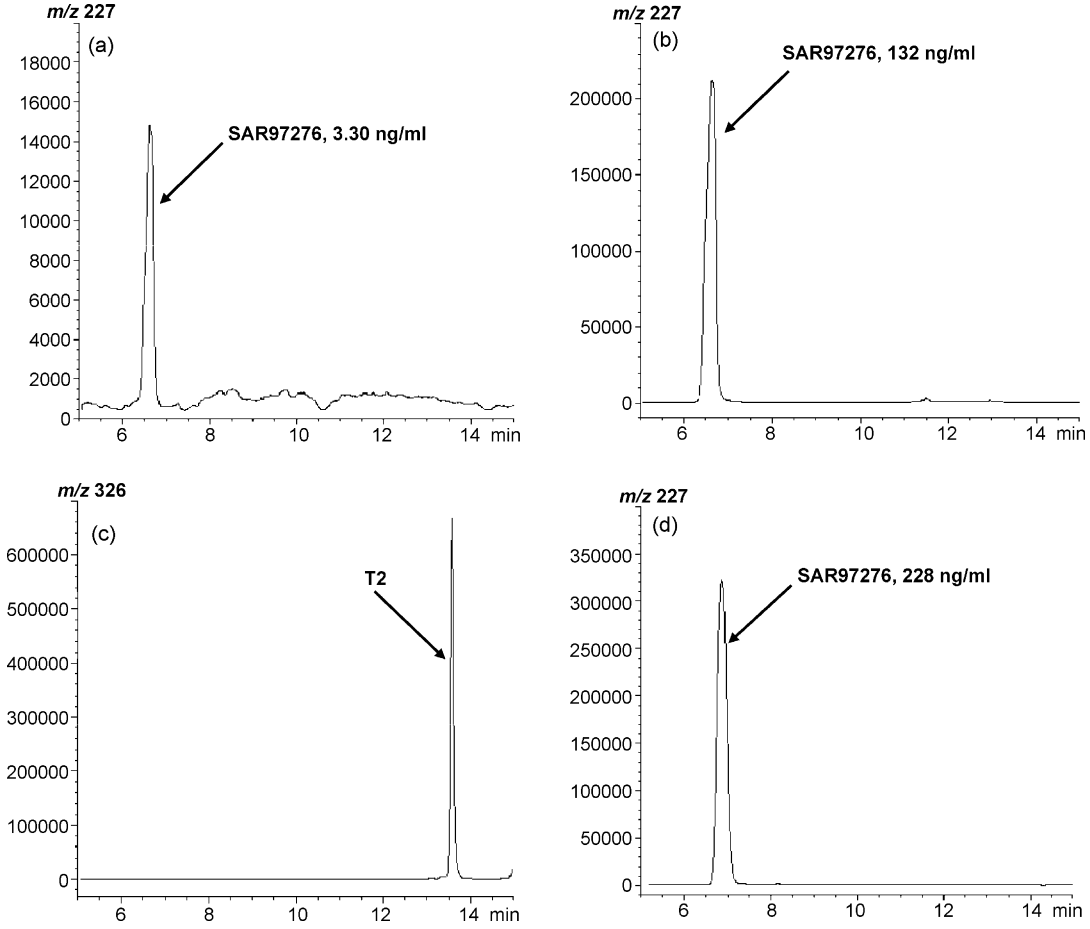


Fig. 4. Selected ion monitoring mode chromatograms for SAR97276 obtained from: (a) and (b) spiked mouse plasma and (d) a real sample from the pharmacokinetic study in mouse; (c) internal standard (T2). For chromatographic conditions see text.

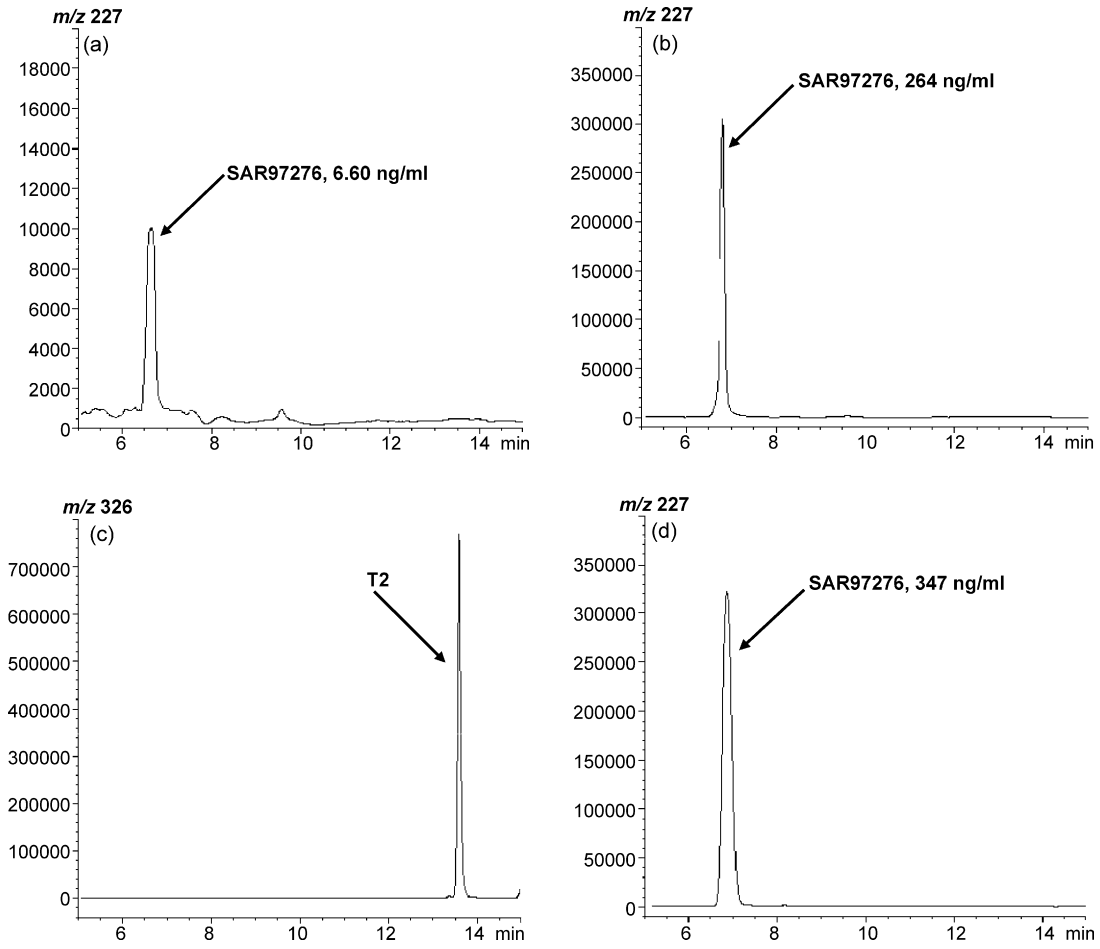


Fig. 5. Selected ion monitoring mode chromatograms for SAR97276 obtained from: (a) and (b) spiked mouse RBCs and (d) a real sample from the pharmacokinetic study in mouse; (c) internal standard (T2). For chromatographic conditions see text.

column was exchanged every 500 sample runs and the column was replaced when the number of theoretical plates decreased by 40%.

Mean parameters of the quadratic equation are presented in Table 1; the coefficient of determination was always higher than 0.997. For each point on the calibration curves, the concentrations were back calculated from the corresponding quadratic equation parameters, and mean ± S.D. values were calculated. The results are presented in Table 2. Back-calculated concentrations computed from quadratic equation parameters of both low and high calibration curves, did not differ more than 1–2%. For concentrations of calibration standards, the R.S.D. around the mean value not exceeded 12%. 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 (slope = 1 and intercept = 0); no significant different was observed; (ii) by studying the frequency distribution histogram of the residuals, which were normally distributed and centered around zero, the number of positive and negative values being approximately equal; (iii) by comparing the bias to zero (3.42 ng/ml for plasma; 2.71 ng/ml for RBCs); a *t*-test showed that this parameter was not statistically different from zero;

Table 1
Mean parameters of the quadratic equation ($n = 10$)

	Mean	Standard deviation	R.S.D. (%)
Plasma, low calibration curves			
a	-1.16×10^{-06}	3.52×10^{-06}	
b	6.85×10^{-03}	3.86×10^{-04}	5.6
c	-1.61×10^{-03}	4.23×10^{-03}	
Plasma, high calibration curves			
a	-5.88×10^{-07}	1.49×10^{-06}	
b	7.12×10^{-03}	7.84×10^{-04}	11.0
c	-1.68×10^{-02}	4.55×10^{-02}	
RBCs, low calibration curves			
a	1.80×10^{-06}	2.51×10^{-06}	
b	5.74×10^{-03}	8.17×10^{-04}	14.2
c	-6.37×10^{-03}	3.39×10^{-03}	
RBCs, high calibration curves			
a	-3.89×10^{-07}	9.36×10^{-08}	
b	6.27×10^{-03}	1.00×10^{-03}	16.0
c	-3.67×10^{-02}	5.05×10^{-02}	

Quadratic regression model: $Y = aX^2 + bX + c$ in which Y is the peak area ratio (analyte/internal standard) and X is the nominal concentration of the analyte. The regression curve was not forced through zero. R.S.D.: relative standard deviation; RBCs: red blood cells; *n*: number of replicates.

Table 2
Relative standard deviation and recovery calculated from back-calculated concentrations from calibration curves performed in plasma and RBCs ($n = 10$)

Theoretical concentration (ng/ml)	R.S.D. (%)		Recovery (%)	
	Plasma	RBCs	Plasma	RBCs
1.65	11.7	–	107	–
3.31	–	9.7	–	107
6.61	7.0	–	92.5	–
13.2	1.8	5.3	99.9	96.1
26.2	–	6.4	–	102
33.1	7.2	–	104	–
66.1	8.2	8.8	100	101
165	4.3	–	96.4	–
264	–	4.0	–	101
331	3.4	–	100	–
661	8.5	1.1	101	99.6
1322	8.2	4.2	95.9	97.3
2644	–	0.8	–	100

R.S.D.: relative standard deviation; RBCs: red blood cells; *n*: number of replicates.

moreover, the 95% confidence interval included the zero value (−1.51, 8.35 for plasma; −0.94, 6.37 for RBCs).

3.3. Accuracy, precision, extraction recovery and lower limit of quantitation (LLOQ)

Precision was below 14% and accuracy ranged from 91.4 to 104%. Individual results are presented in Table 3. These data are well within the generally required validation criteria limits [16–19]. Dilution of the samples had no influence on the performance of the method.

The mean absolute recoveries of SAR97276 from mouse plasma and RBCs, determined with four replicates for each QC level were 95.3% (R.S.D., 8.3%) and 61.2 (R.S.D., 10%), respectively. It was not statistically different over the range of concentrations studied. For the internal standard, recoveries were 88.2% (R.S.D., 8.4%) in plasma and 58.0% (R.S.D., 7.5%) in RBCs.

Table 3
Precision and accuracy of the method

Theoretical concentration (ng/ml)	Precision (%)	Accuracy (%)
Plasma		
3.31 ($n = 10$)	6.6	91.4
16.5 ($n = 10$)	5.9	92.7
132 ($n = 10$)	13.2	95.2
992 ($n = 10$)	9.8	96.9
1300 [1/2] ($n = 9$)	3.6	98.3
1300 [1/5] ($n = 9$)	8.5	94.4
2600 [1/10] ($n = 9$)	1.6	100
Red blood cells		
6.61 ($n = 10$)	8.8	104
33.1 ($n = 10$)	10.4	101
331 ($n = 10$)	8.4	96.1
1983 ($n = 10$)	9.4	95.1
2594 [1/2] ($n = 9$)	13.7	94.8
2594 [1/5] ($n = 9$)	1.9	100
5085 [1/10] ($n = 9$)	6.2	97.8

Values between parentheses are the dilution factor; *n*: number of replicates.

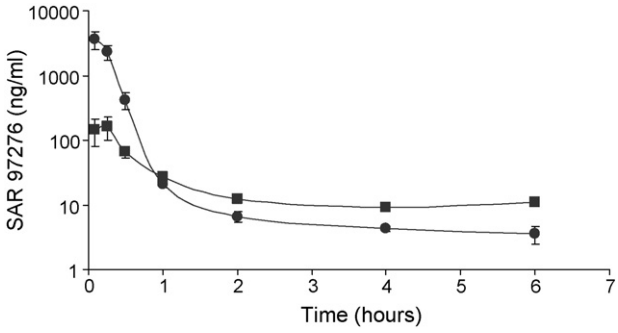


Fig. 6. Mean (±S.D.) plasma (●) and RBC (■) concentration vs. time curves after intraperitoneal administration (5 mg/kg, expressed in salified compound) of SAR97276 in healthy mouse.

The lower limits of quantitation were 1.65 ng/ml in plasma and 3.31 ng/ml in RBCs. At these levels, the precision of determination, expressed as R.S.D. was less than 12%, with adequate assay accuracy [16–19].

3.4. Stability

After storage at 4 °C for 6 h in the refrigerator, no statistical decrease in nominal concentration appeared by comparison with the reference values. In QC and real (from pharmacokinetic study) plasma and RBC samples frozen at −80 °C, SAR97276 was stable for at least 18 months; there was no statistical difference compared to the reference values. Mean recoveries ranged from 88 to 102%. In reconstituted extracts originating from plasma or RBCs (i.e., in the autosampler at 4 °C), no significant losses occurred after 40 h.

3.5. Pharmacokinetic study in healthy mouse

Semilogarithmic plots of the mean (±S.D.) SAR97276 plasma and RBC concentration–time profiles are illustrated in Fig. 6. Maximum RBC concentration was obtained 15 min after drug administration. The elimination half-lives were 4.5 h from plasma and 7.9 h from RBCs. No accumulation of SAR97276 within uninfected RBCs was found. The RBC/plasma AUC ratio was 0.23. Total plasma CL/F was 3.3 l/(h kg).

4. Conclusion

Mass spectrometry detection combined with high-performance liquid chromatography is nowadays increasingly being used in the clinical laboratory and is now accepted as the preferred analytical tool in target compound analysis, specifically in pharmacokinetic studies. In the present paper, a quantitative LC–MS method was presented for the determination of SAR97276, a new compound developed for the treatment of malaria, in plasma and RBCs from mice. Adequate sample clean-up was achieved by a simple and relatively fast SPE. The absence of matrix effects has been verified. In this way, extraction of the analytes from biological matrices using

SPE or liquid–liquid extraction (LLE) should be preferred to simple clean-up procedure such as protein precipitation [21]. Moreover, it has been reported, for several drugs, that after SPE, losses of ESI response were lower than after LLE [24,25]. The SPE procedure described in this paper, with one step each for the sample loading, clean-up, and elution, can be easily automated; the time for sample clean-up and chromatography required approximately 60 min, allowing a fast sample turnover time. This results in a high throughput capability, which proved favorable in view of pharmacokinetic/pharmacodynamic applications. This method was fully validated according to accepted validation standards and was applied to the determination of pharmacokinetic parameters of the SAR97276 compound in the healthy mouse.

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