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Short communication

Rapid resolution liquid chromatography–mass spectrometry determination of SAR97276 in monkey matrices. Pharmacokinetics in rhesus monkey infected by *Plasmodium cynomolgi*

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ABSTRACT

Since several years, we developed a new class of antimalarial drugs targeting the phospholipid metabolism of the *Plasmodium falciparum* malaria parasite. The bis-thiazolium compound, SAR97276, is the lead compound and is now in clinical development. In this paper, we applied the fast rapid resolution liquid chromatography–mass spectrometry technique to the analysis of SAR97276 in monkey matrices. The sample pre-treatment procedure involved an acidic precipitation of proteins followed by solid-phase extraction. The monocationic compound, T2, was used as internal standard. A good separation was achieved on a Zorbax eclipse XDB C8 column (1.8 μm, 50 mm × 4.6 mm) with a mobile phase consisting of acetonitrile–trimethylamine–formate buffer (pH 3) gradient elution. The total run time was 8 min. Inter-assay precisions were <10% in plasma, and ≤12% in blood. Accuracies were 96.6–98.1% (plasma) and 94.5–103% (blood). Mean extraction efficiencies were >85% in plasma, and >75% in blood. The lower limits of quantitation were 3.3 μg/l in plasma and 3.3 μg/kg in blood. No matrix effect was observed. This newly developed method is sensitive, selective, reproducible, and stability indicating. It was used to analyse samples taken during a pharmacokinetic/pharmacodynamic study carried out in infected Rhesus monkey by *Plasmodium cynomolgi* as part of the ongoing development of SAR97276.

1. Introduction

Malaria, one of the world's most common infectious diseases, remains an important public health concern. Up to 500 million clinical cases are reported each year and more than 1 million die annually, causing a devastating impact on the health and economic productivity of afflicted areas. In many parts of the world, the *Plasmodium falciparum* parasites have developed resistance to a number of existing drugs. Artemisinin combination therapies are now the recommended strategy for clinical care and to avoid drug resistance [1,2]. However, experimental resistance to artemisinin and artesunate could be induced in rodent malaria [3]. Thus, despite the availability of several licensed drugs, the emergence of multi-drug resistant strains has dramatically decreased the effectiveness of most antimalarial treatments. The development of new compounds involving novel targets and new mechanisms of action is an urgent priority. During the last 10 years, a new antimalarial pharmacological approach based on inhibition of the plasmodial phospholipid metabolism was identified and developed by our group [4–10]. Mono- [4,11] and bis-quaternary ammonium compounds [7,11,12], and amidine and guanidine [13] compounds have been synthesized. Compounds have shown high efficacy including multi-resistant *Plasmodium falciparum* strains [5,9,10]. The potent antimalarial activity of this class of compounds appears to be related to their capacity (i) to mimic the choline structure which blocks the phosphatidylcholine biosynthesis and (ii) to interact with hemozoin, the malarial hemoglobin-degradation product. The potency of these compounds also seems to be related to their high accumulation inside infected erythrocytes. Their dual mechanism of action should limit the risk of emergence of resistance.

SAR97276 (**T3**, 1,12-bis[5-(2-hydroxyethyl)-4-methyl-1,3-thiazol-3-ium]dodecane dibromide) was emerged as lead compound due to its appropriate pharmacological performances. This compound possesses potent antimalarial activity both in vitro and in vivo. Regulatory preclinical studies have been successfully performed and the compound is currently investigated in phase I

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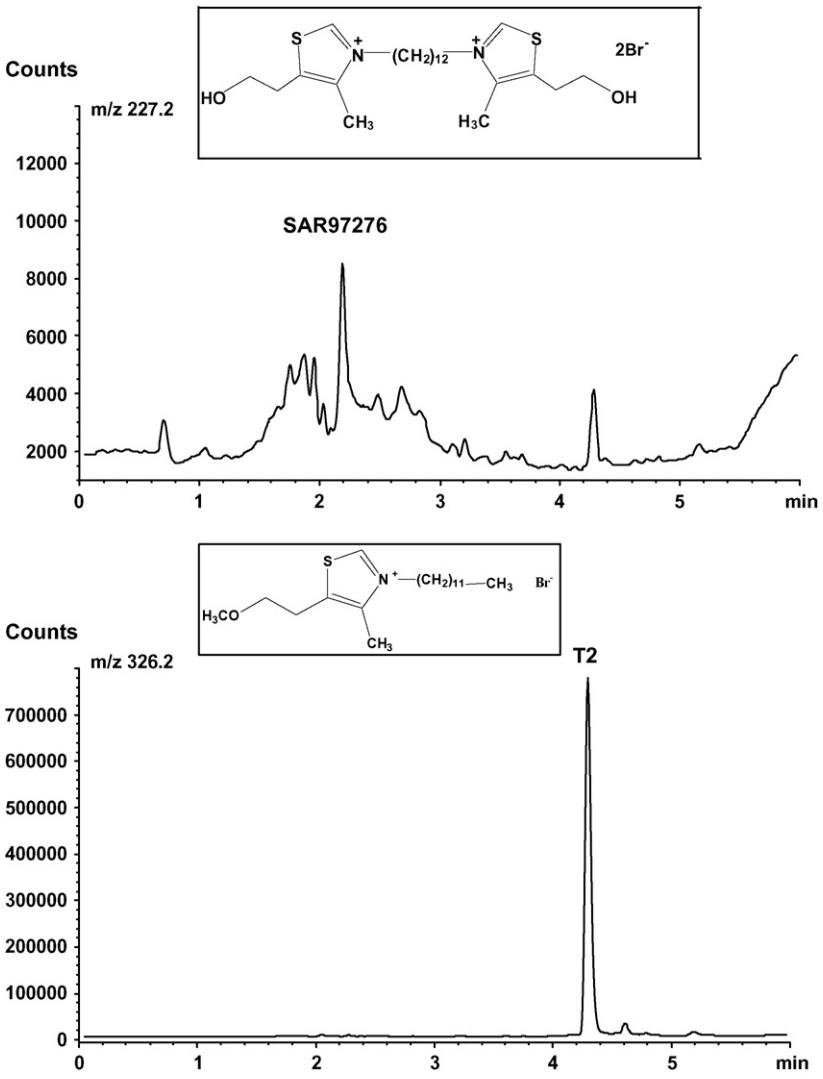

Fig. 1. Typical chromatograms of drug free blood (0.5 g) spiked with SAR97276 at 3.3 μg/kg (LLOQ) and T2 at 308 μg/kg (SIM mode). Structures of the compounds are presented above each chromatogram. See Experimental section for LC–MS conditions.

clinical trials in humans. Conventional LC–MS methods have been developed to quantify this compound in human, rat and mice matrices [14–16].

For an antimalarial drug, in vivo pharmacological studies in primate model are of great interest for clinical assessment. The goal of this paper was to develop a bioanalytical method for quantifying the SAR97276 compound in monkey matrices by rapid resolution liquid chromatography with mass spectrometry (RRLC–MS) detection. This method was used to quantify SAR97276 during a pharmacokinetic/pharmacodynamic study carried out in monkey infected by *Plasmodium cynomolgi*.

2. Experimental

2.1. Chemicals and reagents

SAR97276 (MW 614.7) and T2 (internal standard, MW 406) were synthesized by the Laboratoire des Aminoacides, Peptides et Proteines (UMR 5247, Montpellier I and II Universities) (Fig. 1). All chemicals used were of analytical grade. Trifluoroacetic acid (TFA) and trimethylamine were obtained from Sigma–Aldrich (St. Louis, MO, USA). HPLC-grade acetonitrile was purchased from Carlo Erba (Val de Reuil, France). Formic acid was obtained from Prolabo (Paris, France). Ammonium formate was from Fluka (Vandoeuvre, France). A formate buffer (pH 3) was prepared in purified water (126 mg/l ammonium formate) and adjusted with formic acid. Ultra-high quality water (obtained using a Milli-Q apparatus, Millipore, Bedford, MA, USA) was used throughout. Oasis HLB cartridges (30 mg of sorbent, average particle diameter 30 μm) were purchased from Waters (Saint Quentin, France). Pooled plasma and blood samples from healthy monkeys (EUPRIM, Leibniz institute for primate research, Göttingen, Germany) were collected with lithium heparin.

Individual stock solutions of T2 (45 mg/l) and SAR97276 (65.8 mg/l), expressed in the form of charged compounds, were prepared in purified water. They were further diluted in water to give working solutions of 0.08–32.9 mg/l for SAR97276 and 7.7 mg/l for T2. 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. Stock solutions stored at 4 °C were stable for at least 1 month. Working solutions were extemporaneously prepared.

2.2. LC–MS conditions

All analyses were performed on a LC–MS system from Agilent Technologies (Les Ulis, France) including an Agilent 1200 Series RRLC system equipped with on-line degasser, high-pressure binary pump, autosampler (with temperature-controlled sample

Table 1
Gradient schedule.

Time (min)	% Solvent A: 2 mmol/l ammonium formate buffer	% Solvent B: 130 μl/l trimethylamine in acetonitrile	Flow rate (ml/min)
0	95	5	0.6
4.0	10	90	0.6
4.1	10	90	0.8
4.7	10	90	0.8
5.0	0	100	0.8
5.5	0	100	0.8
6.0	95	5	0.6

compartment) and thermostated column compartment coupled to a quadrupole mass spectrometer type MSD 6140 RRHT (scan rate: 10,000 uma/s). The analytical column was a Zorbax Eclipse XDB C8 (1.8 μm, 50 mm × 4.6 mm from Agilent Technologies); the column compartment temperature was set at 60 °C. The mobile phase consisted of acetonitrile, trimethylamine and formate buffer (pH 3) (Table 1). The flow rate started at 0.6 ml/min (Table 1); the injection volume was 1 μl for blood and 2 μl for plasma.

The LC–MS ionization was performed by electrospray ionization (ESI). The ESI-MS signal was optimized during continuous infusion of a solution of T2 or SAR97276 dissolved in a mixture of water–acetonitrile (50:50, v/v) with 1 ml/l formic acid (data not shown). The optimal sampling cone voltages were 100 V for SAR97276 and 80 V for T2. Optimized operating conditions of the ESI interface in positive mode were as follows: vaporized temperature, 350 °C; nebulizer gas (nitrogen) pressure, 35 psi; drying gas (nitrogen) flow, 12 l/min; and capillary voltage, 4000 V. Quantitative analyses were performed by selected ion monitoring (SIM) at m/z 227.2 ± 0.5 for $[M^{2+}/2]$ ion of SAR97276 and m/z 326.2 ± 0.5 for $[M]^+$ ion of T2.

2.3. Calibration curves and quality control samples

Calibration curves in plasma were prepared from 0.5 ml of control plasma. Calibration curves in blood were prepared from both 0.1 g (diluted with 0.4 ml of purified water) and 0.5 g of control blood in order (i) to cover the wider possible range of concentrations, to limit the number of unknown blood samples to dilute, and (ii) to decrease the lower limit of quantitation (LLOQ) of the method. Calibrators were obtained by adding appropriate volumes of working solutions into the control matrices to achieve 6–7 different concentrations: (i) 3.3–1316 μg/l in plasma and (ii) 3.3–329 μg/kg and 16.5–6581 μg/kg in blood, from 0.1 and 0.5 g, respectively. One calibration curve per day was prepared in each matrix; this procedure was repeated on 8–16 separate occasions to determine inter-assay reproducibility of the method. Calibration curves were constructed by plotting the peak-area ratios (SAR97276/internal

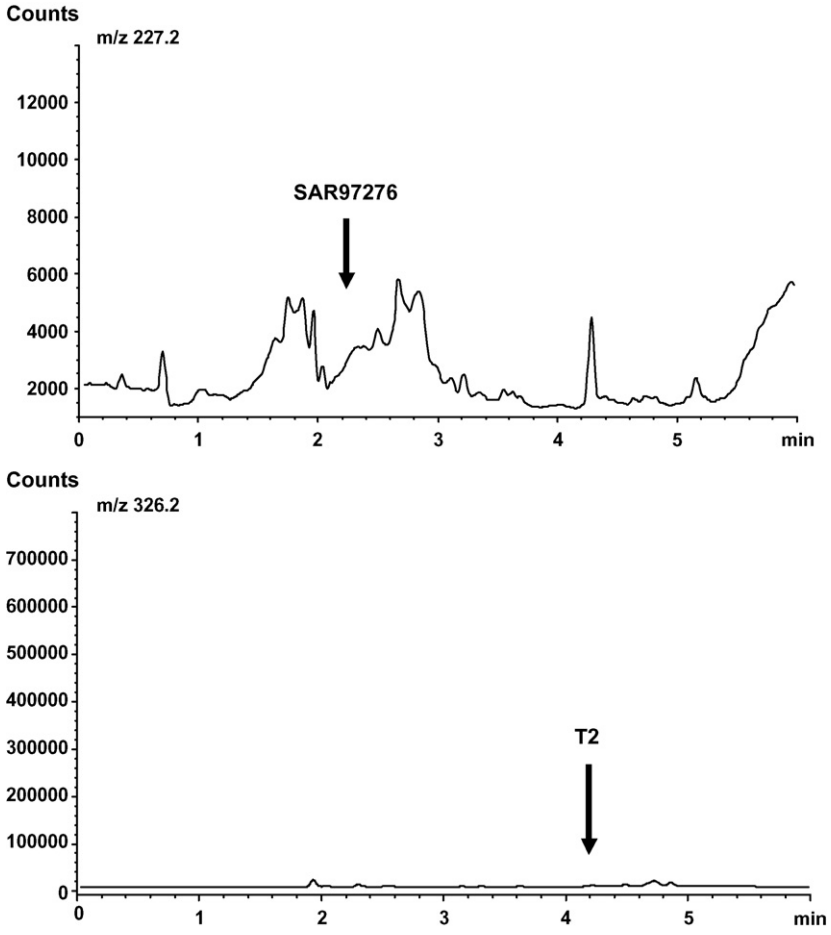

Fig. 2. Representative chromatograms of drug-free monkey whole blood (from 0.5 g). See Experimental section for LC–MS conditions.

standard) against known concentrations. Different models were tested: (a) unweighted or weighted least-squares linear regression and (b) quadratic relationship. The equation parameters were used to back-calculate concentrations that were statistically evaluated.

QC samples were prepared independently in the same way as the calibrators, at the concentrations of: (i) 9.1, 165 and 987 μg/l in plasma; (ii) 9.1, 32.9 and 247 μg/kg, and 45.7, 823 and 4936 μg/kg in blood, from 0.5 and 0.1 g, respectively. To test whether it is possible to apply the described method to plasma samples whose SAR97276 concentrations are higher than the last calibration point, a QC sample spiked at 5265 μg/l in plasma was prepared then diluted to 1/10 with control plasma before dosing.

2.4. Sample preparation

The sample pre-treatment procedure was adapted from the previous publications [14–16]. Briefly, plasma or blood was spiked with the internal standard (T2: 20 μl at 7.7 mg/l), then the mixture was acidified with 0.5 ml of water containing 10 ml/l TFA. After centrifugation (plasma, 3000 × *g*; blood, 19,000 × *g*), the supernatant was loaded onto the cartridge. Purified water (1 ml for plasma; 1.5 ml for blood) as a washing solvent was passed through the sorbent. SAR97276 and T2 were eluted by adding 2 × 1 ml of acetonitrile containing 1 ml/l TFA. After evaporation to dryness under nitrogen stream for 40 min at 40 °C, the dry extract was redissolved in 100 μl of the water–acetonitrile mixture (50:50, v/v) containing 1 ml/l formic acid.

2.5. Validation procedure, performed in agreement with reference [17]

We evaluated the selectivity of the assay by analysis six 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. Moreover, QC samples at the LLOQ were prepared in each batch of each matrix, and then quantified against a qualibration curve. The possible interference by other commonly used antimalarial drugs was also verified.

The matrix effect was assessed by calculating the matrix factor [18]. It was performed by processing six different lots of each drug-free matrix (plasma and blood). For each QC concentration internal standard and SAR97276, working solutions were spiked post extraction in replicates ($n = 6$ per QC concentration). Reference solutions were prepared in the formic acid–acetonitrile–water mixture at the same nominal concentrations. The reconstituted extracts and reference solutions were injected onto the analytical column. The matrix factors were calculated from the peak areas of SAR97276 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 judged acceptable.

Between-day precision and accuracy of the method were assessed by analyzing QC samples at the above-mentioned 10 concentrations against calibration curves. Each QC sample was analysed once a day on 8–16 separate days. Precision was expressed in % R.S.D.; accuracy was evaluated as: [(mean found concentration)/(nominal concentration)] × 100.

The extraction efficiency of SAR97276 was calculated using QC samples prepared as described above ($n = 3$ per concentration). The peak area values of the extracted samples were compared with those of reference samples of the compound prepared in the formic acid–acetonitrile–water mixture at the same concentrations ($n = 3$ per concentration). Extraction efficiencies were also evaluated for the internal standards ($n = 6$).

The LLOQ was defined as the lowest concentration that could be measured with a recovery within 80–120% of the target value and with a precision ≤20%.

2.6. Stability assays

We evaluated the stability of SAR97276 in the matrices by performing replicate analyses of QC samples ($n = 3$) against a calibration curve, immediately after preparation, then after storage at: (i) 4 and 20 °C for 6 h and (ii) −80 °C over a period of 3 months. Analyte stabilities were also assessed for all phases of the analytical process (in the extraction solvent, during the evaporation step and in the autosampler for 36 h). The freeze-thaw stability was evaluated for three cycles of thawing at room temperature followed by re-freezing to −80 °C for 24 h.

2.7. Pharmacokinetics in rhesus monkeys infected by Plasmodium cynomolgi

This research adhered to the "Principles of Laboratory Animal Care" (NIH publication #85-23, revised 1985). The study was approved by the local Animal Use Committee. The experimental protocol was similar to that previously published [9]. Seven *Macaca mulatta* monkeys (7–11 years of age, weighing 4.2–8.5 kg) were infected by intravenous inoculation with *Plasmodium cynomolgi* M strain blood stage parasites. Four of them received intramuscular administration of SAR97276 (2.5 mg/kg, dissolved in 1 ml 0.9% NaCl solution) once a day for 4 consecutive days; the other monkeys received the vehicle. Treatment was initiated on the day when parasitemia was between 0.01% and 0.3%. Blood samples (3 ml, collected in heparinized polypropylene tubes) were drawn daily just prior to drug administration, then 30 min and 12 h after dosing. One milliliter was immediately frozen (−80 °C); the remaining 2 ml were centrifuged (3000 × *g* for 10 min) at 4 °C then plasma was immediately frozen (−80 °C).

3. Results and discussion

In this study, we applied the fast RRLC–MS technique on the short (50 mm × 4.6 mm) column packed with 1.8-μm porous particles to the analysis of SAR97276 in monkey matrices. Compared to the conventional LC method [14–16], excellent peak shape and better baseline resolution of all peaks were obtained, as well as a shorter analysis time (reduced by a factor of 2.5) and a reduction of solvent consumption.

3.1. LC–MS analysis and validation

Fig. 1 illustrates the chromatogram obtained from a drug-free blood (0.5 g) spiked with SAR97276 and T2. Mean observed retention times ($n = 10$) were 2.2 min for SAR97276 and 4.1 min for T2 (R.S.D.s, 0.06–0.79%). The asymmetry coefficients were 0.93 and 1.06 for SAR97276 and T2, respectively. The six different lots of each monkey matrix were chromatographically screened for interfering substances and did not show significant interferences at the retention time of the analytes (Fig. 2). Moreover, precision and accuracy on the analysis of QC samples at the LLOQ, prepared in each lot of each matrix, were <18% and 87–115%, respectively. No interference was found with any tested drugs. The matrix had no influence on the detection of SAR97276 or the internal standards (Table 2).

Quadratic calibration curves were judged to produce the best fit based on (i) the comparison of spiked and back-calculated concentrations (mean relative predictor errors were not statistically different from 0) and (ii) precision and accuracy on the analysis of QC samples. Mean values of the slope *b* were: (i) 27.6×10^{-4} (R.S.D., 25%) in plasma and (ii) 4.0×10^{-4} (R.S.D., 17%) and 22.0×10^{-4}

Table 2
Between-day precision and accuracy of the method and matrix effect.

Number of replicates	Plasma			Blood		
	Added concentration (μg/l)[a]	Precision (% R.S.D.)	Accuracy (%)	Added concentration (μg/kg)[a]	Precision (% R.S.D.)	Accuracy (%)
	Precision and accuracy (0.5 ml)			*Precision and accuracy (0.1 g)*		
$n = 16$	9.1	9.6	97.6	45.7	11	101
	165	7.8	98.1	823	8.4	102
	987	8.7	97.9	4936	7.3	94.5
	5265	6.3	96.6	–	–	–
	–	–	–	*Precision and accuracy (0.5 g)*		
$n = 8$	–	–	–	9.1	11	103
	–	–	–	32.9	12	102
	–	–	–	247	12	101
	Added concentration (μg/l)[a]	R.S.D. (%)	Recovery (%)	Added concentration (μg/kg)[a]	R.S.D. (%)	Recovery (%)
	Matrix effect (0.5 ml)			*Matrix effect (0.1 g)*		
$n = 36$	9.1	2.6	97.8	45.7	2.0	95.5
	165	0.19	90.0	823	5.3	106
	987	1.8	96.5	4936	6.0	108
	T2 (IS)	1.9	93.7	T2 (IS)	2.4	101
$n = 36$	–	–	–	*Matrix effect (0.5 g)*		
	–	–	–	9.1	4.4	105
	–	–	–	32.9	2.1	110
	–	–	–	247	9.2	96.7
	–	–	–	T2 (IS)	2.8	101

[a] Expressed in the form of charged compound; R.S.D., relative standard deviation; IS, internal standard.

(R.S.D., 21%) in blood, from 0.1 and 0.5 g, respectively. Back-calculated concentrations are presented in Supplementary data table; recoveries were 95–107% and R.S.Ds. were ≤15%.

The mean extraction efficiencies were >85% from plasma (SAR97276: 88.7%, T2: 86.4%, R.S.Ds: 8.0–15%) and >75% from blood (SAR97276: 78.8%, T2: 78.1%, R.S.Ds., 5.0–11.5%). Precision and accuracy of the method are presented in Table 2. Dilution of the plasma samples had no influence on the performance of the method. LLOQs were 3.3 μg/l in plasma and 3.3 μg/kg in blood. The LLOQs of SAR97376 in the present method were 2–4 times lower than in human and rat matrices [14,15] but similar to that reported in mouse matrices [16].

3.2. Stability

Stability assays performed in the two matrices indicated that there was no significant loss of SAR97276. Mean recoveries were 93–114% from plasma data (R.S.Ds. < 5%) and 88–113% from blood data (R.S.Ds. ≤ 11%).

3.3. Pharmacokinetics in rhesus monkey

Using a population approach from rich (previous not published study carried out in healthy monkeys after single intravenous administration of SAR97276) and sparse data (present study, 7 data points per animal), total clearances were 0.35–0.69 l/h/kg (standard deviation, S.D., 0.13 l/h/kg) from plasma and 0.60–0.81 l/h/kg (S.D., 0.09 l/h/kg) from blood. Elimination half-lives were 4–13 h (S.D., 4.1 h) and 3–11 h (S.D., 4.0 h), respectively. Bioavailability was 70%. The treatment was extremely effective. After 2 days of treatment, all monkeys were free from detectable blood stage parasites. All doses were rapidly and completely curative; recrudescence only occurred in all untreated animals. SAR97276 treatment was well tolerated.

4. Conclusion

In this work we have developed a highly specific and sensitive method for the RRLC–MS determination of SAR97276 in monkey matrices. This RRLC system coupled to the fast scanning single quadrupole mass spectrometer has the advantage to enable high throughput analyses. The sample extraction and clean-up procedure gave good recoveries consistently and no significant interferences or matrix effects. The limits of quantitation of the assay were 3.3 μg/l in plasma and 3.3 μg/kg in blood. This method has been successfully used for the analysis of samples from a pre-clinical pharmacokinetic study carried out in monkeys as part of the ongoing development of SAR97276.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jpba.2009.02.019.

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