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Development of a SPE/HPLC/DAD method for the determination of antileishmanial 2-substituted quinolines and metabolites in rat plasma

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Abstract

A SPE/HPLC/DAD method was developed for the in vivo monitoring of three new antileishmanial 2-substituted quinolines under study in our laboratory for the development of an oral treatment. Three phase I metabolites were included in this work for the optimization of the method. Trifunctional tC_{18} cartridges (resulting from the reaction of trifunctional silanes with silica surface) were selected among four sorbents tested. Two linear gradients were developed to ensure resolution of metabolites. Recovery of quinolines from rat plasma was comprised between 80.6 and 88.2%. In a drug development perspective, apparent pK_a, lipophilicity and solubility were determined, as well as the extent of plasma protein or albumin binding.

Keywords: Leishmaniasis; Solid phase extraction; 2-Substituted quinoline; Protein binding; Lipophilicity; Solubility

1. Introduction

Leishmaniasis, a parasitic disease caused by several species of *Leishmania* protozoa, is ranked among the six most important tropical infectious diseases by the World Health Organisation, with 350 million people living in endemic zones, 12 million infected people worldwide and 2 million new cases each year [1,2]. It is a proteiform disease, among which the visceral form (VL) is lethal. Endemic zones being mostly in developing countries, more than 90% of the patients with VL die untreated. Indeed, classical treatments are expensive and require parenteral administration. Furthermore, they are highly toxic and show high percentage of resistance. Therefore there is an evident need to develop new per os treatments.

We previously isolated a series of 2-substituted quinolines from a Bolivian tree, *Galipea longiflora* (Rutaceae), traditionally used topically to cure ulcerations of cutaneous leishmaniasis by the native Chimane Indians [3]. These 2-substituted quinolines seem to be specific of the Galipea genus [4–7]. In 1994, it has been demonstrated that these compounds also exhibit antileishmanial properties after oral administration in an in vivo mouse model of VL [8]. In addition to their antileishmanial activity, 2-substituted quinolines isolated from Galipea genus also exhibit antitrypanosomal [9,10] antiretroviral [9–12], nematocidal [13], trichomonacidal [14], antibacterial [7], molluscicidal [6] and antiplasmodial activities [10,8]. Recently, we synthesized the most active of these compounds, compound **1**, as well as two of its derivatives, compounds **2** and **3** (Fig. 1) [9,13]. They all showed in vivo antileishmanial properties [14,15], and have then been chosen as new leads for an oral treatment of VL.

In a previous collaborative work, the pharmacokinetic study of **1** has been already carried out in mouse by HPLC but with no metabolite determination [16]. It has also been demonstrated that **1** inhibits intestinal P-glycoprotein on rat everted gut sacs and human intestinal Caco-2 cell lines [17]. Furthermore, three major phase I metabolites of **1** (α- and γ-hydroxylated quinolines

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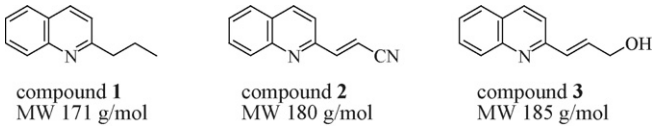

Fig. 1. Antileishmanial 2-substituted quinolines: **1**, originally natural propylic quinoline, **2** and **3**, propenyl-nitric and -alcoholic derivatives.

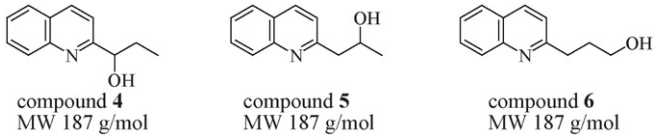

Fig. 2. Metabolites of **1**: **4**, **5** and **6** α-, β- and γ-hydroxylated compounds.

and an aromatic epoxide biotransformed into dihydroxylated metabolite) were identified in vitro using human liver microsomes [18]. Very recently, we studied the in vitro effects of human and rat liver microsomes on synthetic compounds **1**, **2** and **3**. We found the α- and the γ-hydroxylated and also the epoxide phase I metabolites of compound **1** in accordance with previous results [18], but the dihydroxylated compound was not observed. However, a new β-hydroxylated metabolite was identified under our experimental conditions [19].

In order to study the pharmacokinetics of compounds **1**, **2** and **3** in rat plasma, a reliable and efficient method of separation is needed, allowing the detection of their putative metabolites. To optimize the analytical conditions (HPLC separation and SPE extraction), the identified in vitro phase I metabolites of compound **1** (**4**, **5** and **6**, α- β- and γ-hydroxylated quinolines, Fig. 2) were included in this work.

We present here a SPE/HPLC/DAD method, allowing the quantification of compounds **1**–**3** in rat plasma. The objective of this work is also to allow the detection of putative metabolites, and to confirm the in vivo formation of the metabolites identified in vitro. If these metabolites are found in rat plasma after oral administration, complete validation of the method will be done in future work. For the development of this method, physicochemical parameters were determined. This method was also applied to determine the extent of albumin and plasma protein binding.

2. Experimental

2.1. Chemicals, reagents and biological samples

Methanol and acetonitrile (ACN) were of HPLC grade (VWR International, Fontenay-sous-Bois, France). Ether was of Rectapur grade (VWR International, France). Propyl paraben (WINTHROP laboratory, Dijon-Logvic, France) was used as internal standard (I.S.). All other reagents were used without further purification.

Quinolines **1**–**6** were synthesized according to our previous reports [9,13], and isolated as hydrochloride salts. Briefly, 6 mmol of quinoline were dissolved in 5 mL of ether, and dry HCl was bubbled through the solution for a few minutes, until apparition of a precipitate. The supernatant was then discarded, and the precipitate was dissolved in methanol and dried under vacuum. Yield was around 85%.

Whole blood of adult male Wistar rats (Janvier, Le Genest-Isle, France), anaesthetized with subcutaneous pentobarbital was drawn via the carotid vein into EDTA Vacutainer tubes. All experiments were conducted according to local institutional guidelines for the care and use of laboratory animals. Plasma and erythrocytes were separated after centrifugation (1000 × *g*, 10 min). Plasma was frozen at −20 °C until analysis or used for daily experiments.

Bovine serum albumin (BSA) (Acros Organics, Fisher scientific, New Jersey, USA) was dissolved in phosphate buffer saline (PBS) pH 7.4 from Gibco™ (Invitrogen, Cergy-Pontoise, France) to yield plasmatic concentration of 40 mg/mL.

2.2. Instrumentation

2.2.1. Solid phase extraction

Solid-phase extractions were carried out using a J.T. Baker Inc. extraction manifold (Mallinckrodt Baker, Paris, France). Different sorbents (1 mL, 100 mg) were tested: octadecyl bonded silica: Sep-Pak® tC_{18} (Waters, Saint-Quentin en Yvelines, France), octyl bonded silica: Bond Elut® C_8, cyanopropyl bonded silica: Bond Elut® CN, propylsulfonic acid bonded silica: Bond Elut® PRS (Varian, Les Ulis, France).

2.2.2. High performance liquid chromatography

A Waters LC system was used, consisting of a 600-model pump, an in-line degasser, a 717 plus autosampler and a 996 photodiode array detector. Instrument monitoring and data acquisitions were performed using Empower® software (Waters, France). The dwell volume of this system is 6 mL. Injection volumes were 10 μL.

A Sunfire™ C_{18} analytical column 150 mm × 4.6 mm i.d., 5 μm (Waters, France) protected with a Sunfire™ C_{18} 20 mm × 4.6 mm i.d., 5 μm guard column both thermostated at 30 °C were used. The UV absorption spectrum was recorded between 210 and 400 nm, and the chromatograms were extracted at 233 nm for **1**, **4**, **5**, and **6**, 264 nm for **2**, 249 nm for **3** and 257 nm for I.S.

2.3. Lipophilicity

Lipophilicity of quinolines was determined between *n*-octanol and buffer solution at two levels of pH (7.4 and 2.0), by small-scale shake flask experiments [20]. Briefly, solvents were mutually saturated on a mechanical shaker overnight, at ratio 1:1 (v/v), after which each phase was separated. In a glass tube containing 100 μg of the studied quinoline, 1 mL of each phase was added and the system was gently shaken until equilibrium. Concentrations of quinoline in each phase were determined by HPLC after appropriate dilution in mobile phase. The partition coefficient (*P*) was calculated as follows:

$$P = \frac{[C]_{\text{octanol}}}{[C]_{\text{buffer}}} \quad (1)$$

where $[C]_{octanol}$ and $[C]_{buffer}$ are the quinoline concentrations in *n*-octanol and buffer, respectively. Results are expressed as log *P*. Means and standard error of the mean (S.E.M.) were calculated on the basis of three experiments in duplicate ($n = 6$).

2.4. Solubility

Solubility was studied in pH 7.4 and pH 2.0 buffer solutions with an excess of quinoline. Suspensions were agitated for 24 h at 25 °C, then filtered through a 0.45 μm Durapore® membrane filter (HVLP01300, Millipore). The concentration of solubilized quinoline was determined by HPLC after appropriate dilution in mobile phase. Means and S.E.M. were calculated on the basis of 3 experiments in duplicate ($n = 6$).

2.5. Method

2.5.1. Sample preparation

Spiked plasma samples were extracted by Solid Phase Extraction (SPE) on a tC_{18} cartridge (resulting from the reaction of trifunctional silanes with silica surface [21]) using the following procedure: (1) conditioning with 1 mL methanol followed by 1 mL of water–methanol 95:5 (v/v); (2) loading the plasma sample diluted to 1/10th in pH 2.5 phosphoric acid–methanol 95:5 (v/v) containing 20 μg/mL of I.S.; (3) clean-up in two steps: 0.5 mL 2% NH_4OH–methanol (80:20, v/v), then 0.5 mL HCl 10^{-2} M; (4) elution of quinoline with 0.5 mL methanol.

2.5.2. Analytical conditions

In order to determine the physico-chemical parameters of quinolines and to develop the SPE step, as well as to characterize protein binding, we used an isocratic mobile phase consisting in a mixture of 10^{-2} M pH 4.0 acetate buffer and methanol (40:60, v/v), with a flow rate of 1 mL/min. The influence of pH (from 2.0 to 7.0) was studied with appropriate mixtures of 10^{-2} M phosphate–citrate buffer solutions and methanol (40:60, v/v).

For the determination of quinolines and metabolites in rat plasma, a binary linear gradient was developed. It was composed of phosphoric buffer 10^{-3} M pH 5.2 and ACN and consisted of 4 min with 25% ACN, increasing to 80% within 19 min, and 5 min with 80% ACN. The flow rate was set at 1.5 mL/min.

2.6. Method validation

Stock solutions of quinolines and I.S. were prepared in methanol (5 mg/mL) and stored at 4 °C until use. They proved to be stable at least 5 months. Working solutions were prepared daily by diluting stock solutions in pH 7.4 PBS.

Calibration curves were prepared in aqueous phase and in drug-free plasma spiked with the above working solutions. Concentration ranges were 0.78–50 μg/mL. Aqueous solutions were directly injected into HPLC, while plasma standards were extracted by SPE, as described previously, prior to HPLC analysis. Recovery from plasma was calculated from the straight-line regression of calibration curves in mobile phase.

2.7. Determination of protein binding by ultrafiltration

Protein binding was investigated by ultrafiltration technique [22], using Ultrafree-MC centrifugal filter device with a molecular cut-off of 30,000 Da (Millipore). In our experiments, no adsorption of **1**–**3** on the filter was observed. Plasma samples or albumin solutions containing 20 μg/mL of quinoline were allowed to equilibrate for 20 min at 37 °C. Samples were then diluted to 1/10th in PBS, to avoid filter plugging and ultrafiltrated at 2800 × *g* for 20 min at room temperature. Plasma ultrafiltrates were cleaned-up by SPE prior to HPLC analysis, whereas albumin ultrafiltrates were directly injected in HPLC. Percentage of unbound quinoline (f_u%, free fraction) was calculated with the following equation:

$$f_u(\%) = \left(\frac{[C]_{unbound}}{[C]_{total}}\right) \times 100 \quad (2)$$

where $[C]_{unbound}$ and $[C]_{total}$ are quinoline concentration measured in the ultrafiltrate and total concentration measured in the aqueous standard, respectively.

3. Results and discussion

3.1. Optimization of the HPLC conditions

3.1.1. Isocratic mobile phase

Rapid analytical conditions were needed for the determination of the physico-chemical parameters and the SPE optimization, as well as for the characterization of protein binding. For this purpose, a reversed stationary phase was chosen since it is commonly used for the separation of basic compounds. The composition of the mobile phase described in previous work [16] was investigated in order to optimize the separation.

The influence of strong solvent percentage (methanol) was studied in order to minimize the retention factors of **1**, **2** and **3**. At pH 5.5, where the three quinolines are, as expected, under their molecular forms, proportion of methanol higher than 60% led to an unsuitable retention factor (<1) for the most polar compound **3**. Thus, the influence of the aqueous phase pH-value on retention factors was studied with 60% methanol. As can be seen in Fig. 3, the retention factor of **1** could be diminished of

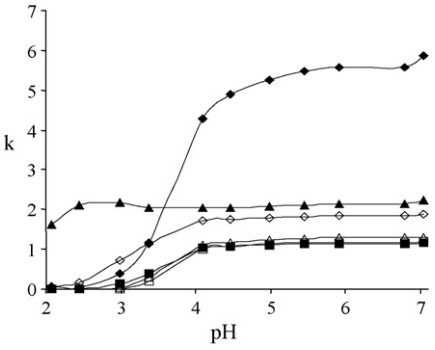

Fig. 3. Influence of the aqueous mobile phase pH-value (aqueous phase/methanol 40:60 (v/v)), on retention factors of **1** (♦), **2** (▲), **3** (■), **4** (◊), **5** (△), and **6** (□), leading to the determination of the apparent pK_a.

1 unity at pH 4.0 as compared to pH 5.5, with no modification for compounds **2** and **3**. Then, the selected mobile phase was as follow: acetate buffer 10^{-2} M pH 4.0 and methanol (40:60, v/v), retention factors being: 1.05, 1.15, 1.12, 1.82, 2.10 and 5.48 for **6**, **5**, **3**, **4**, **2** and **1**, respectively.

Studying the influence of pH (2.0–7.0) on retention factors led to the determination of apparent pK_a. As shown in Fig. 3, compounds **4**, **5**, **6**, **3** and **1** have apparent pK_a between 3.3 and 3.7, while it is <2 for **2**. Thus, compounds **1**–**6** appear to be in their basic non-ionic forms at physiological pH.

3.1.2. Gradient elution conditions

With the isocratic mobile phase containing methanol, **6** and **5** were not resolved (retention factors 1.05 and 1.15, respectively) that is unsuitable for their identification. The effect of temperature was evaluated from 20 to 40 °C. Retention factors decreased with increasing temperature, but the resolution was not improved. Thus we replaced methanol with ACN. The influence of ACN content was studied with acetate buffer pH 4.0 or phosphate buffer pH 5.2. Using ACN instead of methanol allowed us to achieve the resolution between **5** and **6**. At pH 4.0 with 30% ACN, retention factor of **6** was still <1, while it could be increased to 1.7 using pH 5.2. However, under these conditions, **1** had a high retention factor (18.1) leading to 40 min analysis. A linear gradient was then developed. Analyses were achieved within 18 min, with resolution and sufficient retention under the following conditions: 0–4 min 25% of ACN; then ACN is increased to 80% in 19 min and a plateau of 5 min with 80% ACN to eliminate apolar impurities. Under these conditions the retention times were: 5.74, 6.51, 6.88, 11.37, 15.08, 16.27 and 16.89 min for **6**, **5**, **3**, **4**, I.S., **2** and **1**, respectively (Fig. 4).

Another gradient, also allowing resolution between metabolites **5** and **6** but leading to higher retention of quinolines, could be used as a control in the visualization of unknown more hydrophilic in vivo metabolites. It consisted of methanol and acetate buffer 10^{-2} M pH 4.0 and was: 20% methanol

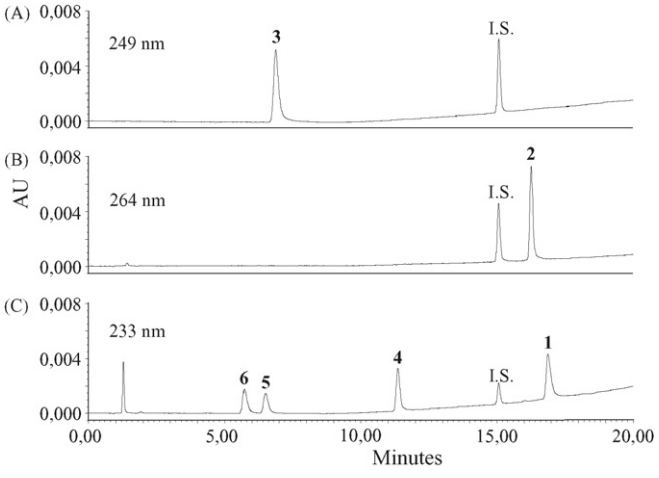

Fig. 4. Chromatograms of quinolines with internal standard (I.S., propylparaben) extracted at corresponding wavelength, using the linear gradient (0–4 min 25% of ACN, at 23 min 80% ACN, at 28 min 80% ACN); sodium phosphate buffer 10^{-3} M pH 5.2 and ACN. (A) Compound **3**, (B) compound **2** and (C) compound **1** with its metabolites **4**, **5** and **6**.

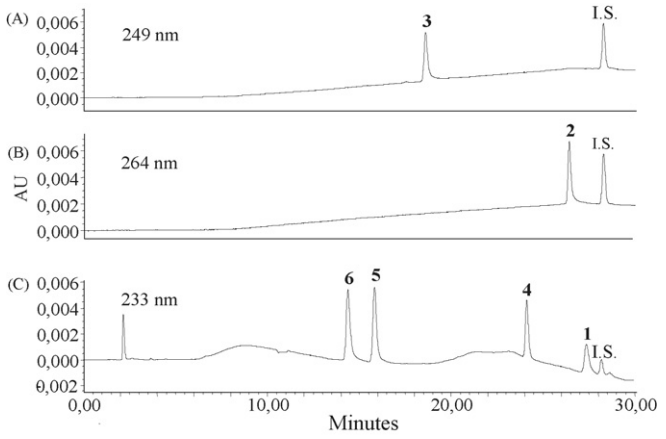

Fig. 5. Chromatograms of quinolines with internal standard (I.S., propylparaben) extracted at corresponding wavelength, using the linear gradient (methanol increased from 20 to 60% in 20 min, and 10 min with 60% methanol); acetate buffer 10^{-2} M pH 4.0 and methanol. (A) Compound **3**, (B) compound **2** and (C) compound **1** with its metabolites **4**, **5** and **6**.

increased to 60% within 20 min, and a plateau of 10 min with 60% methanol to elute more apolar compounds (**4**, **2** and **1**), leading to retention times of: 14.4, 15.9, 18.6, 24.2, 26.4, 27.4 and 28.3 min for **6**, **5**, **3**, **4**, **2**, **1** and I.S., respectively (Fig. 5).

3.2. Lipophilicity and solubility of *1–3*

In a drug development process, lipophilicity and solubility are the parameters to be determined the first, as indications for putative bioavailability [23,24]. Thus, only compounds **1**–**3** were included in the study. These parameters were also used for the development of the analytical procedure.

Lipophilicity of **1**–**3** were determined upper and under the apparent pK_a of **1** and **3**. Table 1 shows that at physiological pH, the three quinolines **1**–**3**, in their molecular form, are highly lipophilic, increasing from **3** to **1**. At pH 2.0, protonated compounds **1** and **3** appear hydrophilic, while **2** is still lipophilic, as expected, due to the predominance of the molecular form of the latter at this pH. These results are in accordance with the determination of the apparent pK_a, and indicate suitability for passive absorption by enterocytes, as previously observed with experiments on caco-2 cells (unpublished results), and as indicated by *p.o.* administration during in vivo experiments [8,14,15].

The rate and extent of absorption is related to the solubility of a drug, because this process can only occur after drugs enter solution after oral administration [25]. It is therefore linked

Table 1
Lipophilicity (log *P*) and solubility (mg/mL) of quinolines **1–3** in HCl 10^{-2} M (pH 2.0) and in PBS pH 7.4 ($n = 6$)

	log *P* ± S.E.M.		Solubility ± S.E.M.	
	pH 2.0	pH 7.4	pH 2.0	pH 7.4
1	−0.56 ± 0.05	3.19 ± 0.16	2.39 ± 0.09	[a]
2	1.48 ± 0.03	2.30 ± 0.07	1.10 ± 0.03	0.25 ± 0.02
3	−1.20 ± 0.02	2.11 ± 0.10	2.13 ± 0.17	1.10 ± 0.11

[a] Discarded value due to the oily state of **1**.

with therapeutical effects [26]. Results in Table 1 predict an adequate solubility of the three compounds (**1**–**3**) in gastro intestinal fluids, allowing oral administration. Considering their lipophilicities, the solubility of compound **1** was expected to be lower than that of **2** and **3** at both pH 2.0 and 7.4. However, unexpected results (high apparent solubility) were observed at physiological pH, probably due to the oily state of **1**. Its solubility being undeterminable at pH 7.4 with our protocol, the experimental value was discarded.

3.3. Optimization of the extraction procedure

In the original method reported by Iglarz et al. [16], **1** was extracted from plasma by liquid-liquid extraction (LLE). We attempted to use this procedure for the extraction of **2** and **3** from plasma, but our optimization efforts proved to be unsuccessful. A solid-phase extraction (SPE) was then developed, reducing time for sample preparation and allowing good recovery.

Optimization of the following experimental parameters was investigated: nature of the sorbent, pH of the aqueous phase of the loading solution, aqueous and hydro-organic clean-up solutions, and volume of the eluting solvent. Conditioning of the cartridges was always performed with 1 mL of methanol, and 1 mL water–methanol 95:5 (v/v), except for the optimization of the organic clean-up were they were preconditioned with the studied hydro-organic solution. The flow rate being an important parameter for retention and elution, vacuum was set at 30 kPa producing a flow rate of 1–2 mL/min.

3.3.1. Effect of sorbent, loading solution, and aqueous clean-up on the retention of *1–3*

Considering the lipophilicity and the apparent pK_a of quinolines, their retention was studied on various bonded silicas, leading to different selectivity (C_8, C_{18}, CN and PRS). To analyze the influence of quinolines' ionic state on their retention, the influence of the pH was studied at different steps of the SPE process. Loading solutions containing **1**, **2** or **3** (1 mL, 50 μg/mL in water–methanol 95:5, v/v) were prepared with aqueous phase at pH 2.5, 3.5 or 4.5, and clean-up step was evaluated with 1 mL of HCl 10^{-2} M versus pure water. To achieve procedure evaluation, three successive 1 mL volumes of eluting methanol were analysed. Results are presented in Fig. 6. Elution from the PRS cartridges was achieved with methanol containing 2% NH_4OH, to disrupt electrostatic interactions.

The CN cartridges were chosen for their ability to exhibit π–π interaction, which was expected to occur with the quinoline ring and would give a particular selectivity. Unfortunately, they proved to be not satisfying, because acidic pH did not enable sufficient retention for the three compounds during the clean-up step. Moreover, the most polar compound **3** was not even retained during loading at pH 2.5.

The C_8 sorbent was evaluated, comparatively to the C_{18} sorbent, for its moderate non-polar interaction. As the studied quinolines and their metabolites have different lipophilicity, a shorter length of the bonded alkyl chain would lead to different selectivity. With the C_8 sorbent, acidic clean-up, leading to quinoline protonation, required higher eluting volumes to obtain

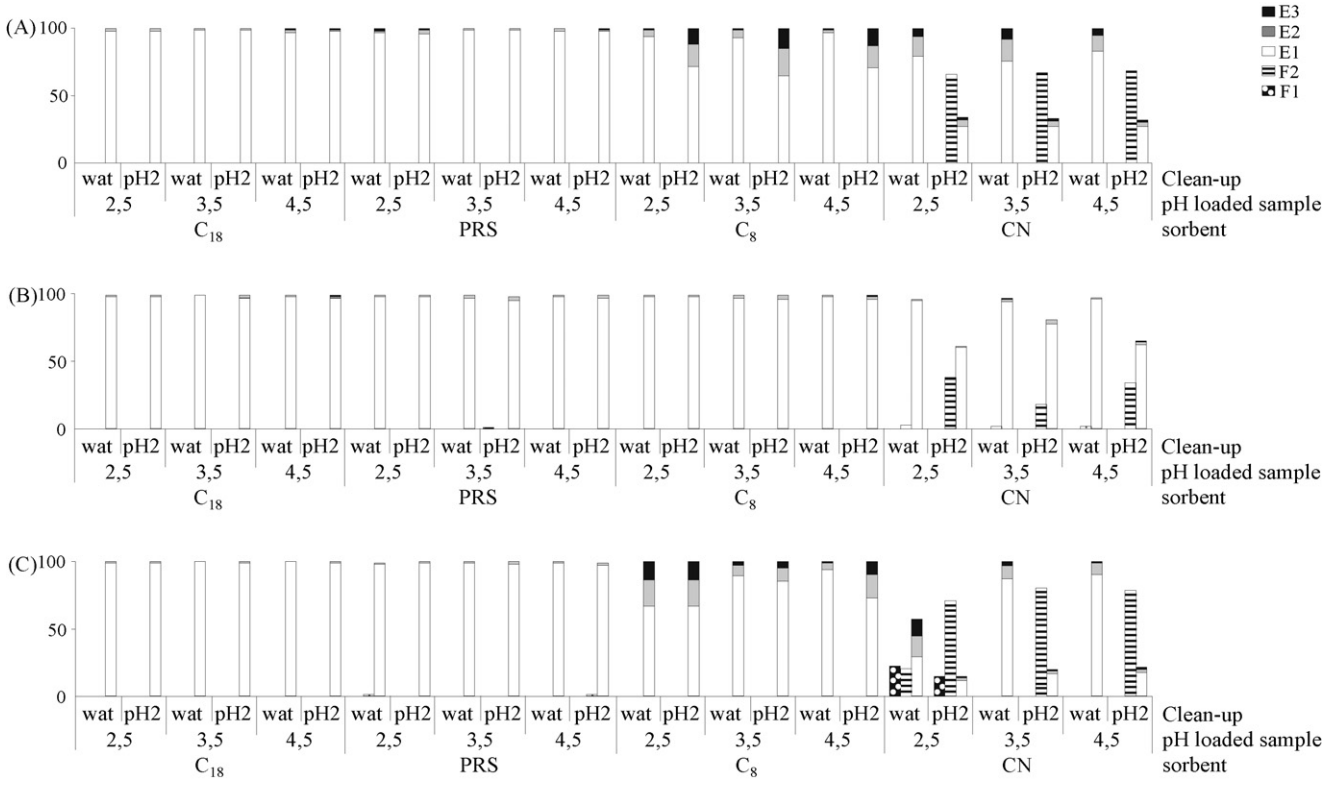

Fig. 6. Percentage of quinoline eluted during SPE optimization process. F1: loading, F2: clean-up, E1: first 1 mL, E2: second 1 mL, E3: third 1 mL of eluting solution (methanol, containing 2% NH_4OH for the PRS). (A) compound **1**, (B) compound **2**, (C) compound **3**.

complete elution of **1** and **3**, as compared with a neutral clean-up. This unexpected higher retention of protonated compounds **1** and **3** with C_8 sorbent, in regard to tC_{18}, might be explained by the involvement of electrostatic interactions between the ionized "free" silanols and the protonated quinolines, which are minimized with the tC_{18} sorbent.

The C_{18} sorbent, the most widely used reversed-phase sorbent, was studied for its capability of high non-polar interaction, and was a good candidate considering the lipophilicity of quinolines at physiological pH. The PRS sorbent which is a cationic-exchanger, was chosen for the strong electrostatic interaction expected with protonated quinolines. The tC_{18} and PRS sorbents both permitted good retention for the three quinolines during loading and clean-up, as well as elution with minimal volumes. However, since PRS sorbent did not yield good recoveries of **2** from spiked plasma in further studies (around 30%, data not shown), the tC_{18} cartridges were finally selected for the extraction procedure of quinolines from plasma.

Since all loading-pH tested afforded good retention of quinolines on the tC_{18} cartridges, pH 2.5 phosphoric acid–methanol (95:5, v/v) was chosen in order to have **1** and **3** in their protonated form, minimizing interactions with plasma proteins. Proteins elimination was facilitated by acidic clean-up (HCl 10^{-2} M), but as chromatograms presented interferences, the development of a hydro-organic clean-up appeared necessary.

3.3.2. Optimization of the hydro-organic clean-up. Studies with *1–6*

Quinolines retention on the C_{18} sorbent was studied with hydro-organic solutions containing various percentages of methanol (5, 20, 40, 60 and 80%) with acidic (HCl 10^{-2} M) or alkaline (2% NH_4OH) aqueous phase. The sorbent was preconditioned with 1 mL of methanol and 1 mL of the hydro-organic solution. Quinolines (50 μg/mL in the studied hydro-organic solution) were loaded on the sorbent by 100 μL (bed volume). The quinolines were eluted with the hydro-organic solution, and eluate aliquots (100 μL to 1000 μL) were analyzed by HPLC. Breakthrough volume, elution volume and recovery volume were determined [27].

In accordance with apparent pK_a, **1**, **3**, **4**, **5** and **6** had smaller elution volumes with acidic aqueous phase as compared with alkaline one (at least 20 times with 20% methanol and 5 times with 40%), while there was no difference for **2**. The pH of the hydro-organic clean-up was then chosen to be alkaline, allowing the application of higher percentage of methanol without eluting quinolines. With alkaline solutions, the elution order was: **6**, **5**, **3**, **4**, **2** and **1** which is also in accordance with lipophilicity and retention factors. Breakthrough, elution and sample volumes of quinolines obtained with 20 and 40% of methanol and alkaline aqueous phase are reported in Table 2. Breakthrough volumes were inferior to 1 mL for **4**, **5** and **6** with 40% of methanol, while 5 mL (50× bed volume) of 20% methanol did not elute any quinolines. The organic clean-up was then further achieved with an alkaline hydro-organic phase containing 20% of methanol.

Finally, the SPE procedure was as described in Section 2; the alkaline hydro-organic clean-up being achieved before the aqueous acidic one, in order to prevent elution (2–3%) of compounds **4–6** during the hydro-organic clean-up which occurs when acid water is flushed before.

Table 2
Elution volumes from SPE C_{18} cartridges during semi-organic clean-up (methanol–2% NH_4OH) ($n = 3$)

	Volume (mL)	
	20% methanol	40% methanol
1		
Breakthrough volume	>10.1	5.1
Elution volume	>10.1	>10.1
Sample volume	>10.1	>10.1
2		
Breakthrough volume	6.10	2.1
Elution volume	>10.1	3.1
Sample volume	>10.1	5.1
3		
Breakthrough volume	6.1	1.1
Elution volume	>10.1	1.6
Sample volume	>10.1	3.1
4		
Breakthrough volume	9.1	0.8
Elution volume	>10.1	1.9
Sample volume	>10.1	4.1
5		
Breakthrough volume	6.1	0.7
Elution volume	>10.1	1.4
Sample volume	>10.1	3.1
6		
Breakthrough volume	5.1	0.7
Elution volume	>10.1	1.8
Sample volume	>10.1	3.6

3.3.3. Eluting volume of methanol on tC_{18} cartridges

In order to reduce sample dilution, the eluting volume of methanol was studied with compound **1**, the most retained on C_{18}. Under our conditions 0.5 mL methanol was sufficient for the elution of quinoline **1** with 98.4% recovery.

3.4. SPE/HPLC/DAD method validation

Drug-free plasma submitted to SPE was analyzed with the linear gradient and chromatograms extracted at quinolines wavelengths did not show any interfering peaks at the retention times of the six studied quinolines (**1–6**).

The validation of the method was carried out only for compounds **1–3** because the aim of this work was to quantificate these compounds in rat plasma for the determination of their pharmacokinetic parameters.

The straight-line regression equations, describing the calibration curves of **1–3** in aqueous phase and spiked plasma, were constructed by plotting peak area ratio of quinoline to I.S. (y) versus quinoline quantity (x) (Table 3). Good linearity between peak area ratios and concentrations within the studied concentration range (0.78–50 μg/mL), with correlation coefficient >0.9997 were obtained for both mobile phase and plasma standards.

Table 3
Calibration curves for the quantitation of quinolines in mobile phase and plasma standards

Standard	Slope (±S.D.)	Intercept (±S.D.)	r^2 (±S.D.)
Mobile phase			
1	0.0796 ± 0.0022	−0.0094 ± 0.0831	0.9999 ± 0.0001
2	0.0903 ± 0.0016	−0.0080 ± 0.0677	0.9999 ± 0.0001
3	0.1171 ± 0.0013	−0.0169 ± 0.0453	0.9999 ± 0.0001
Plasma			
1	0.0653 ± 0.0013	−0.0222 ± 0.0843	0.9997 ± 0.0001
2	0.0773 ± 0.0013	−0.0285 ± 0.1050	0.9998 ± 0.0001
3	0.1008 ± 0.0058	0.0129 ± 0.1263	0.9998 ± 0.0001

S.D.: standard deviation.

The recoveries of quinolines from spiked plasma, calculated with the calibration curve of standards in aqueous phase, are shown in Table 4. They were satisfactory for all substances and concentrations tested with values ≥80.6% for **1**, ≥84.1% for **2**, and ≥84.0% for **3**. These results revealed the suitability of the SPE procedure.

Three concentrations of **1**–**3** corresponding to the low, medium and high ranges of the standard calibration curve (0.78, 3.12 and 12.5 μg/mL), were analysed on each day of a 3-day validation ($n = 5$ for each concentration). The mean measured quantity of standards in mobile phase and plasma were calculated using the corresponding calibration curves and the standard deviations (S.D.) were calculated. The repeatability (intra-day precision) and reproducibility (inter-day precision) are presented in Tables 5 and 6. The precision was expressed as the relative standard deviation (RSD%), and the relative error (RE%) of the measurement was used to express accuracy. Results indicated that the precision was comprised between 1.1 and 3.6% for the mobile phase standards and 1.0 to 5.2% for the plasma standards. The accuracy of the assay was from 97.7 to 101.5% for the mobile phase measurements and 98.4 to 104.7% for the plasma measurements. The obtained values were within recommended limits.

The limit of detection (LOD, signal-to-noise ratio ≈3) and the limit of quantitation (LOQ, signal-to-noise ratio ≈10), were comprised between 12 and 20 ng/mL for **1**; 8 and 20 ng/mL for **2** and 5 and 10 ng/mL for **3**.

3.5. Determination of protein binding using ultrafiltration

Ultrafiltration is a widely used method for the determination of protein binding because it has the advantage of its simplicity and rapidity. Determination of unbound (free) fraction of drug in plasma is essential for therapeutic monitoring of drugs, as it influences both pharmacokinetic and pharmacodynamic parameters. Albumin, the most abundant plasma protein, binds and transports various endogenous and exogenous compounds.

Table 7 shows the unbound fraction of **1**–**3** in rat plasma and albumin solution. It demonstrates that compounds **1** and **2** are largely bound to plasma proteins (89.4 and 71.3%, respectively), and mainly to albumin. Compound **3** also binds to plasma proteins, in a lesser extent (46.8%), also involving albumin. The

Table 5
Mean measured concentration[a], intra- and inter-day precision and inaccuracy for the quantification of quinolines in mobile phase standards

Injected concentration (μg/mL)	Mean measured concentration ± S.D. (μg/mL)		R.S.D.% (precision)		RE% (accuracy)	
	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day
1						
0.78	0.786 ± 0.021	0.791 ± 0.025	2.7	3.2	0.6	1.2
3.125	3.131 ± 0.034	3.137 ± 0.034	1.1	1.1	0.2	0.4
12.50	12.389 ± 0.448	12.327 ± 0.334	3.6	2.7	−0.9	−1.4
2						
0.78	0.788 ± 0.018	0.795 ± 0.028	2.4	3.6	0.9	1.8
3.125	3.136 ± 0.048	3.144 ± 0.037	1.5	1.2	0.4	0.6
12.50	12.465 ± 0.239	12.522 ± 0.234	1.9	1.9	−0.3	0.2
3						
0.78	0.793 ± 0.009	0.795 ± 0.009	1.2	1.1	1.5	1.8
3.125	3.055 ± 0.040	3.063 ± 0.035	1.3	1.1	−2.3	−2.0
12.50	12.532 ± 0.436	12.361 ± 0.330	3.5	2.7	0.3	−1.1

S.D.: standard deviation; R.S.D.%: relative standard deviation; RE%: relative error of the measurement.
[a] Calculated with the calibration curve of standards in mobile phase.

Table 4
Recoveries of quinoline from spiked plasma after SPE extraction ($n = 3$)

	Concentration of quinolines spiked in plasma (μg/mL)						
	0.78	1.56	3.12	6.25	12.5	25	50
1	83.2 ± 4.0	82.0 ± 3.4	84.1 ± 3.2	80.6 ± 2.7	80.6 ± 2.0	82.7 ± 1.6	82.0 ± 1.6
2	86.8 ± 3.7	85.0 ± 2.1	86.8 ± 1.6	84.1 ± 1.6	84.1 ± 1.3	86.2 ± 1.1	85.5 ± 1.4
3	88.2 ± 2.3	86.2 ± 1.4	86.7 ± 2.3	84.0 ± 1.2	85.2 ± 2.2	84.0 ± 1.0	84.6 ± 1.6

Values are recovery (%) ± S.D. (standard deviation).

Table 6
Mean measured concentration[a], intra- and inter-day precision and inaccuracy for the quantification of quinolines in plasma standards

Injected concentration (μg/mL)	Mean measured concentration ± S.D. (μg/mL)		R.S.D.% (precision)		RE% (accuracy)	
	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day
1						
0.78	0.812 ± 0.042	0.787 ± 0.032	5.2	4.0	4.0	0.7
3.125	3.224 ± 0.137	3.161 ± 0.101	4.2	3.2	3.2	1.1
12.50	12.294 ± 0.340	12.458 ± 0.239	2.8	1.9	−1.6	−0.3
2						
0.78	0.818 ± 0.037	0.793 ± 0.028	4.6	3.6	4.7	1.5
3.125	3.193 ± 0.063	3.215 ± 0.070	2.0	2.2	2.2	2.9
12.50	12.311 ± 0.215	12.311 ± 0.125	1.7	1.0	−1.5	−1.5
3						
0.78	0.796 ± 0.023	0.798 ± 0.019	2.9	2.3	1.9	2.2
3.125	3.144 ± 0.092	3.133 ± 0.085	2.9	2.7	0.6	0.3
12.50	12.376 ± 0.355	12.345 ± 0.199	2.9	1.6	−1.0	−1.2

S.D.: standard deviation; R.S.D.%: relative standard deviation; RE%: relative error of the measurement.
[a] Calculated with the calibration curve of standards in plasma.

Table 7
Free fraction (f_u) in rat plasma and bovine serum albumin (BSA) solution ($n = 6$)

	f_u% ± S.E.M.	
	Rat plasma	BSA
1	10.6 ± 1.0	26.0 ± 1.7
2	28.7 ± 0.6	38.5 ± 3.5
3	53.2 ± 7.7	69.4 ± 6.1

protein binding is correlated with lipophilicity (log P) of quinolines at physiological pH, suggesting hydrophobic interaction. Furthermore, considering the high extent of quinolines bound to plasma protein, the good recoveries from plasma, using SPE, showed that this procedure permits the disruption of protein binding.

4. Conclusions

This work allowed us to define the physico-chemical parameters of three antileishmanial quinolines under pre-clinical study for the development of an oral treatment of VL. A SPE/HPLC/DAD method for their quantification in rat plasma was developed, affording precision and accuracy. This method was also optimized to allow the determination of more hydrophilic derivatives, expected to be in vivo phase I metabolites. This simple and effective method, we applied here to characterize the extent of plasma protein and albumin binding of the three drug candidate, will be further used for the establishment of essential pharmacokinetic parameters.

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