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Determination of sinefungin in rat plasma by high-performance liquid chromatography

Christine Tharasse-Bloch[a,*], Philippe Brasseur[b], Loïc Favennec[b], Jacques Marchand[a]

[a]*Laboratoire de Pharmacochimie de Biopharmacie, U.F.R. de Médecine-Pharmacie, B.P. 97, Avenue de l'Université, 76803 Saint Etienne du Rouvray Cedex, France*

[b]*Laboratoire de Parasitologie expérimentale, U.F.R. de Médecine-Pharmacie, Saint-Etienne-du-Rouvray, France*

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Abstract

A reversed-phase high-performance liquid chromatographic method for the determination of sinefungin, a new antiprotozoal drug, in rat plasma has been developed and validated. Sample preparation was performed at 4°C by deproteinization with acetonitrile. Vidarabine was used as an internal standard. Both sinefungin and vidarabine were separated on a C_{18} column with a mobile phase of ammonium dihydrogenphosphate–acetonitrile (95:5, v/v) and detected by ultraviolet absorbance at 260 nm. Recoveries of sinefungin from plasma were $75 \pm 3.2\%$ and $81 \pm 4.8\%$ following dosage at concentrations of 10 μg/ml and 30 μg/ml, respectively. Using 250 μl of rat plasma, the limit of quantitation was 1 μg/ml sinefungin, and the assay was linear from 1 to 30 μg/ml. This method appears sensitive enough to be used in further pharmacokinetic studies of sinefungin in animal models.

Keywords: Sinefungin

1. Introduction

Sinefungin is a natural nucleoside antibiotic produced by *Streptomyces griseolus* and *Streptomyces incarnatus* (see Fig. 1 for structure) [1]. It has been reported to be effective as an antiprotozoal drug in vitro for the control of *Plasmodium falciparum*, *Leishmania* spp. and *Entamoeba histolytica*, and in vivo for the control of *Trypanosoma* spp. and *Toxoplasma gondii* [2,4]. In addition, sinefungin exhibits unique

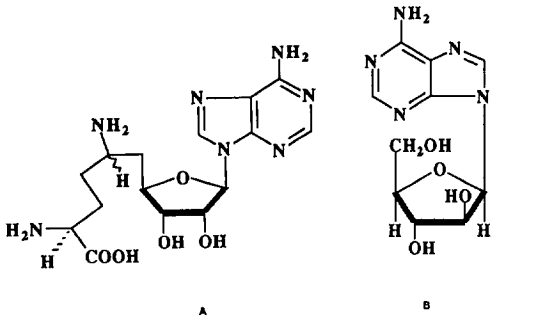

Fig. 1. Structure of (A) sinefungin and (B) the internal standard, vidarabine.

* Corresponding author.

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curative and preventive activity in vitro towards *Cryptosporidium parvum* infection [3–5]. *Cryptosporidium parvum* is a coccidial protozoan that causes life-threatening diarrhea in immunocompromised patients, especially those with AIDS. In these patients, *C. parvum* is considered one of the most important enteric opportunistic infections.

For pre-clinical testing a sensitive assay was needed to determine preliminary disposition and bioavailability of the drug in animals.

In this paper we report the first quantitative assay for sinefungin in rat plasma [1] using high-performance liquid chromatography (HPLC).

2. Experimental

2.1. Chemicals and reagents

Sinefungin produced by *Streptomyces incarnatus* was obtained from Rhône-Poulenc-Rorer [2] (Vitry-sur-Seine, France). The internal standard, vidarabine (9-β-D-arabinofuranosyladenine), was supplied by Sigma (Saint-Louis, MO, USA). See Fig. 1 for structure. Ammonium dihydrogenphosphate buffer was purchased from Prolabo (France). Acetonitrile was of liquid chromatographic grade and supplied by Chromanorm Prolabo (France). Distilled water used for all procedures was obtained from Biosedra (Malakoff, France).

2.2. Instrumentation

The HPLC system consisted of an isocratic solvent delivery pump (110A Beckman pump), a sample injector (Rheodyne Model 7125, Latek, Eppelheim, Germany) equipped with a 20-μl loop, a reversed-phase column (Beckman Ultrasphere Octadecyl, 250 mm × 4.6 mm I.D., 5 μm particle size, Fullerton, CA, USA) protected by a guard cartridge packed with the same material (Beckman, 4.5 cm × 4.6 mm I.D.), and a variable-wavelength UV detector (Beckman Model 166, San Ramon, CA, USA). The data recording system consisted of an IBM personal computer PS/2 Model 8550.Z with System Gold software (version 5.1, Beckman).

2.3. Chromatographic conditions

The mobile phase consisted of acetonitrile–0.05 *M* ammonium dihydrogenphosphate buffer adjusted to pH 6.0 by 0.1 *M* sodium hydroxide (5:95, v/v), and was delivered at a flow-rate of 1.0 ml/min. The optimum wavelength for detection was determined by a UV scan of sinefungin and the internal standard in the mobile phase: both exhibit maxima at 210 nm and 260 nm. In view of the biological matrix interferences observed when UV absorbance detection at 210 nm is used, a less sensitive but more specific detection at 260 nm was preferred.

2.4. Stock solutions

A stock solution of sinefungin (1 mg/ml) was prepared in water and stored at 4°C. This solution was analysed periodically: sinefungin was eluted as a single peak with a constant peak area over a period of four months under these conditions.

A working stock solution was prepared by diluting primary stock solution with water to a concentration of 100 μg/ml. This stock solution was stable for at least four months when stored at 4°C.

An internal-standard stock solution (1 mg/ml) was prepared in water and stored at 4°C. This solution was further diluted to obtain the working stock solution (300 μg/ml).

2.5. Preparation of rat plasma standards

The standard solution of sinefungin (100 μg/ml) was diluted with water to obtain spiking solutions. Plasma standards were freshly prepared by spiking aliquots of the standard solutions into the blank rat plasma to give the following final concentrations: 0.5, 1, 1.5, 2, 4, 5, 10, 20, 30 μg/ml.

2.6. Preparation of samples

Rat blood samples were collected in heparinized tubes and were immediately cooled to 4°C. Plasma was separated by centrifugation at 4°C (13 000 *g*, 5 min) and stored at −20°C until analysis. It was very important that the samples should be frozen and stored quickly since, at room temperature, the sinefungin concentration in blood specimens was found to fall rapidly (to about 10% after 15 min). Specimens were kept at 4°C since adenosine deaminase is not active at this temperature [6,7]. An assay was performed with specimens kept for 15, 30, and 60 min at 4°C without any decrease in the sinefungin concentration. At −20°C, in vitro deamination by adenosine deaminase was also inhibited, and no degradation of sinefungin was detected for three months.

In a 5-ml glass hemolyse tube maintained at 4°C, 50 μl of internal standard stock solution (300 μg/ml) was added to 250 μl for the sample (or plasma standard) to yield a final concentration of 50 μg/ml. The tube was vortexed for 5 s. Proteins were precipitated by the addition of 1 ml acetonitrile during vortexing. The tube was then centrifuged for 5 min at 13 000 *g* and 4°C.

The supernatant was removed, placed into a 5-ml glass tube and evaporated to dryness at 40°C under nitrogen. The dried sample was reconstituted in 400 μl of mobile phase and vortexed for 30 s; 20 μl was injected into the LC system.

The method described above was used to analyse the plasma from rats that had received either a 10 mg/kg or a 30 mg/kg dose of sinefungin subcutaneously, or a single oral dose of 100 mg/kg.

2.7. Method validation

As the HPLC column aged and the retention time of sinefungin decreased, the composition of the mobile phase was adjusted to compensate for the change in retention and the amount of acetonitrile was reduced from 5% to 3%. The service life of the column depended on the R_s value and the column was removed as soon as the R_s value fell below 1.

Calibration curves were obtained by plotting the area ratios of sinefungin/vidarabine. Injections of a series of rat plasma standards ranging from 1 to 30 μg/ml were performed in triplicate. The recovery of sinefungin from plasma was determined by comparing peak areas for rat plasma samples spiked with sinefungin in quintuplicate with those for aqueous standard solutions at two concentrations: 10 and 30 μg/ml. The intra-day precision was obtained by quintuplicate injections of plasma samples spiked with sinefungin at 1, 10 and 30 μg/ml on the same day. The inter-day precision was determined by injections of 5 μg/ml and 20 μg/ml samples daily for five days.

2.8. Pharmacological application

The HPLC method described above has been used for the assessment of the plasma concentrations of sinefungin in non-immunosuppressed Sprague Dawley rats, each weighing 250 g. The experiments were divided into four groups.

In group 1, four rats each received sinefungin subcutaneously at a dosage of 10 mg/kg and in group 2, two rats each received 30 mg/kg. Blood specimens were collected every 15 min for 120 min. In group 3, two rats each received a single dose of 100 mg/kg administered orally and blood specimens were collected every 15 min for 360 min. In group 4, two rats each received 35 mg/kg (8.75 mg) sinefungin intravenously. Blood specimens were collected 15 and 270 min after injection; 270 min after injection a bile specimen was also collected. In addition, sinefungin concentrations were evaluated in the urine samples collected during the 270 min of the experiment.

3. Results

3.1. Chromatograms

Typical chromatograms of sinefungin and the internal standard are shown in Fig. 2. Parameters characterizing the HPLC separation of sinefun-

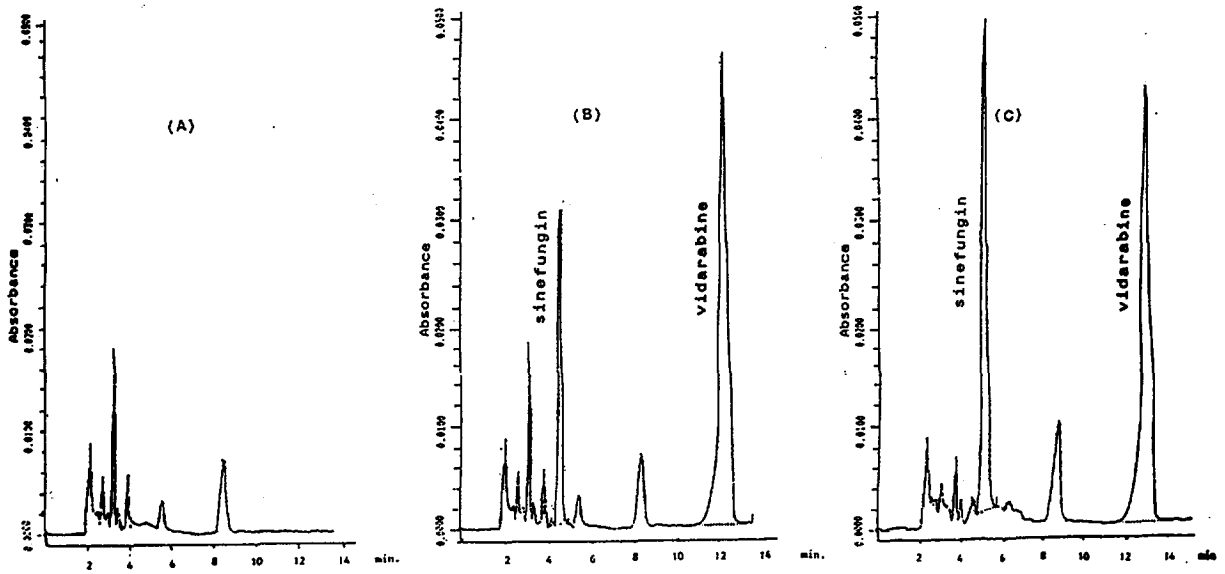

Fig. 2. (A) Chromatogram of extracted blank rat plasma. (B) Chromatogram of drug-free rat plasma spiked with 10 μg/ml sinefungin (4.5 min) and 50 μg/ml vidarabine (12 min). (C) Chromatogram of rat plasma sample 120 min after a 30 mg/kg subcutaneous dose of sinefungin. Measured concentration: 19.7 μg/ml.

gin and vidarabine are summarized in Table 1. Retention times for sinefungin and vidarabine were 4.5 min and 12 min, respectively; symmetrical peaks were obtained.

Fig. 2A shows a chromatogram of extracted blank rat plasma. Fig. 2B represents a chromatogram of drug-free plasma spiked with 10 μg/ml sinefungin and 50 μg/ml vidarabine. Fig. 2C is a chromatogram obtained from a rat plasma sample in which the concentration of sinefungin determined was 19.7 μg/ml.

3.2. Standard curves and assay validation

The sinefungin-to-internal-standard chromatographic peak-area ratio was directly proportional to the drug concentration over the range 1–30 μg/ml in 250 μl plasma with a linear regression equation of $y = 1.37x - 0.42$ and a correlation coefficient of 0.998.

The recoveries of sinefungin from plasma were found to be 75 ± 3.2% and 81 ± 4.8% at concentrations of 10 μg/ml and 30 μg/ml, respectively. Intra-day and inter-day precision are shown in Table 2. The results are expressed as the coefficient of variation (C.V., %). The limit of detection was defined as the minimum drug concentration corresponding to twice the signal-

Table 1
Parameters characterizing HPLC separation of sinefungin and vidarabine

Compound	t_R	k'	$W_{1/2}$(min) [a]	R_s [b]
Sinefungin	4.5	1.5	0.1	2.4
Vidarabine	12	5.5	0.3	3.8

[a] $W_{1/2}$ (min), bandwidth at half height.
[b] R_s, resolution of the chromatographic band.

Table 2
Reproducibility of sinefungin concentration

Spiked concentration (μg/ml)	Mean calculated concentration (μg/ml)	C.V. (%)
Intra-day (n = 5)		
1	0.97 ± 0.037	3.8
10	12.0 ± 0.34	2.8
30	30.3 ± 0.42	1.4
Inter-day (n = 5)		
5	4.85 ± 0.14	2.8
20	20.5 ± 0.59	2.9

Table 3
Plasma concentrations of sinefungin evaluated using the HPLC method at different times after subcutaneous injection in rats of 10 (group 1) and 30 (group 2) mg/kg

Time (min)	Concentration (μg/ml)	
	Group 1	Group 2
15	6 ± 0.6	14.6 ± 1.1
30	8 ± 0.2	35 ± 1.4
45	10 ± 0.4	33 ± 0.4
60	12.7 ± 0.4	26.8 ± 3
90	8.9 ± 0.8	21.8 ± 0.5
120	5.4 ± 0.6	19.7 ± 0.3

to-noise ratio. It was evaluated as 1 μg/ml in 250 μl plasma.

3.3. Pharmacokinetic study of sinefungin in rats

The results (Table 3) showed that in group 1, mean plasma concentration peaked at 12.7 μg/ml, 60 min after subcutaneous injection of 10 mg/kg of sinefungin, and in group 2 at 35 μg/ml, 30 min after subcutaneous injection of 30 mg/kg (Fig. 3). The plasma concentrations obtained in group 1 and in group 2 were found to be correlated to the dosage of sinefungin injected. The ratio of the areas under the curves of plasma concentrations over a period of 120 min after subcutaneous injection of 10 mg/kg and 30 mg/kg (Fig. 3) is 0.343. In group 3, sinefungin concentrations ranged between 1.06 and 2.1 μg/ml. In group 4, the mean plasma concentration of sinefungin was 0.88 μg/ml and 1 μg/ml, 15 and 270 min after intravenous injection, respectively. The bile concentration was less than 0.5 μg/ml. After 270 min the quantity of sinefungin excreted in urine was 4.05 ± 0.15 mg (46.2% of the sinefungin injected).

4. Discussion

The information gathered in these preliminary experiments using an HPLC method to evaluate sinefungin concentrations suggests that absorption of the drug throughout the digestive tract is at a very low level. When sinefungin is injected intravenously, the blood clearance time is less than 270 min and 50% of sinefungin is eliminated by excretion in the urine. The plasma concentrations obtained following intravenous injection are higher than those obtained using the subcutaneous route. Following the injection of 10 or 30 mg/kg subcutaneously, the ratio of areas under the curves of plasma concentrations was 0.343 and is consistent with a high dose proportionality, since the ratio would be theoretically equal to 0.33. The bile concentration was found to be very low; however, the bile samples should

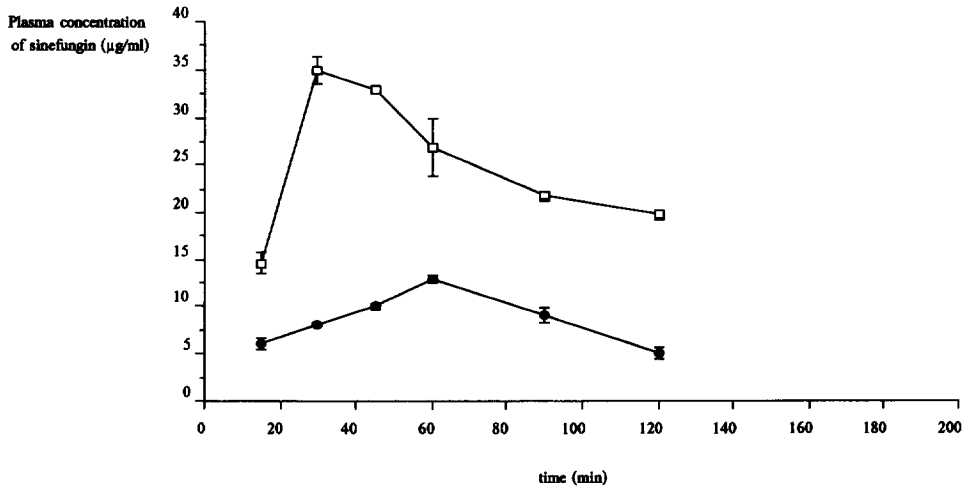

Fig. 3. Plasma concentrations of sinefungin (μg/ml) evaluated using the HPLC method at different times after subcutaneous injection in rats of 10 (●) and 30 (□) mg/kg.

be collected rather earlier than 270 min after injection of the compound to provide useful information.

No data on the metabolites of sinefungin are available as yet. A deamination of adenosine in hypoxanthine could be expected. This would not be a problem, as hypoxanthine would not interfere with the detection of sinefungin since the two compounds do not have the same lipophilia. In addition, we have found that, in rat plasma specimens maintained at 4°C for 60 min, the concentrations of sinefungin did not decrease. This suggests that adenosine deaminases are not active at this temperature.

This HPLC method could also be used to evaluate sinefungin concentrations in the faeces and in different parts of the digestive tract, providing information that would be useful in the setting up of procedures for sinefungin therapy.

5. Conclusion

This preliminary study shows that HPLC method is sensitive and accurate enough to be applied to further pharmacokinetic studies of sinefungin in animal models.

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