Journal of Nanomedicine & Biotherapeutic Discovery

Azevedo et al. J Nanomedic Biotherapeu Discover 2011, 1:2
DOI: 10.4172/2155-983X.1000101

Research Article Open Access

Prolonged Blood Circulation Time of Antimony in Dogs with Visceral Leishmaniasis from Liposomes with 175-nm Diameter

Erly Guilherme Azevedo[1], Raul Rio Ribeiro[2], Cláudio dos Santos Ferreira[3], Sydnei Magno da Silva[4], Dante Aligheiri Schettini[5], Marilene Suzan Marques Michalick[4], Cynthia Demicheli6 and Frédéric Frézard[1]*

[1]*Department of Physiology and Biophysics, Institute of Biological Sciences, Federal University of Minas Gerais, Av Antonio Carlos 6627, Pampulha, 31270-901 Belo Horizonte, MG, Brazil*
[2]*Department of Veterinary Medicine, Center for Agricultural Sciences, Environmental and Biological Sciences, Federal University of Bahia Recôncavo, Campus of Cruz das Almas, Centre, 44380-000 Cruz das Almas, Bahia, Brazil*
[3]*Instituto of Exact Sciences and Technology, Forest Campus, University of Viçosa, LMG 818 km Highway 06, Forest 35690-000, MG, Brazil*
[4]*Departament of Parasitology, Institute of Biological Sciences, Federal University of Minas Gerais, Av Antonio Carlos 6627, Pampulha, 31270-901 Belo Horizonte, MG, Brazil*
[5]*D. West Campus Center Lindu, Federal University of Sao Joao del Rei, Av Sebastian 400 Gonçalves Coelho, Chanadour, Divinópolis 35501-296, MG, Brazil*
[6]*Department of Chemistry, Institute of Exact Sciences, Federal University of Minas, Gerais, Av Antonio Carlos 6627, Pampulha, 31270-901 Belo Horizonte, MG, Brazil*

Abstract

The achievement of parasitological cure of dogs with visceral leishmaniasis (VL) remains a great challenge, since dogs act as main reservoir for transmission of *Leishmania infantum* to humans and respond poorly to conventional drugs including pentavalent antimonials. Liposome-encapsulated antimonials are hundreds of times more effective than the free drugs against VL based on parasite suppression in the liver. However, complete parasite elimination in dogs seems to depend on the ability of liposomes to reach less accessible infection sites such as the bone marrow and the skin. Recently, the reduction of liposome size from 1200- to 400-nm diameter was found to improve the targeting of Sb to the bone marrow of dogs with VL. In the present work, the influence of further reduction of vesicle diameter from 400- to 175-nm on the pharmacokinetics of Sb in dogs with VL and on the distribution of Sb in the liver, spleen and bone marrow were investigated. For this purpose, two liposome formulations of meglumine antimoniate with the same lipid composition but different mean hydrodynamic diameters were prepared. The formulations were given to mongrel dogs with VL as a single intravenous bolus injection and Sb concentrations were determined by graphite furnace atomic absorption spectroscopy. Surprisingly, much more prolonged blood levels of Sb were achieved from small size (175 nm) than medium size (400 nm) liposomes. Small size vesicles were also less effective than medium size ones in targeting Sb to the liver. On the other hand, similar Sb concentrations were achieved in both spleen and bone marrow. In conclusion, the prolonged blood circulation time of liposomes with 175-nm diameter makes this nanosystem suitable for passive drug targeting to the less accessible infection sites in dogs with VL.

Keywords: Liposomes; Size; Pharmacokinetics; Dogs; Antimony; Visceral leishmaniasis

Introduction

The leishmaniases are a group of diseases produced by invasion of the mononuclear phagocyte system (MPS) of a mammalian host by a parasite of the genus *Leishmania (L.)*. This parasite is found as a motile promastigote in the sandfly and transforms into an amastigote when engulfed by host macrophages. Visceral leishmaniasis (VL) is the most severe form of the disease, causing death of humans if not treated [1].

The achievement of complete cure of dogs with VL, or at least the blockade of infectivity to the sandfly vector, is currently a great challenge since dogs act as main reservoir for transmission of *L. infantum* to humans and respond poorly to conventional drugs including pentavalent antimonials [2].

In the 1970s, a major advance occurred when it was found that liposome-encapsulated antimonial drugs were hundreds of times more effective than the free drugs against experimental VL based on parasite suppression in the liver [3]. This effect of liposome encapsulation was attributed to the drug sustained release property of liposomes and to their natural tendency to be cleared from the circulation by the fixed macrophages of the liver and spleen, which are major sites of parasite infection. In this context, much effort has been devoted to the search for effective liposomal formulations in dogs with VL [4]. However, complete parasite elimination in dogs seems to depend on the ability of liposomes to reach less accessible infection sites, such as the bone marrow and the skin.

Recently, liposome size reduction from 1200- to 400-nm diameter was found to improve the targeting of Sb to the bone marrow of infected dogs [5]. High anti-leishmanial activity of medium size (400 nm) liposomes containing meglumine antimoniate drug was also reported after treatment of infected dogs with four doses of 6.5 mg of Sb/kg body weight [6]. Significant parasite suppression was found in the spleen and liver of dogs, however, blockade of infectivity of dogs to the sandfly was not achieved, indicating the presence of parasite in the skin of treated animals. These results suggest that improvement of the

***Corresponding author:** Frédéric Frézard, Department of Physiology and Biophysics, Institute of Biological Sciences, Federal University of Minas Gerais, Av Antonio Carlos 6627, Pampulha, 31270-901 Belo Horizonte, MG, Brazil. Tel. +55 31 34092940; Fax: +55 31 34092924; E-mail: frezard@icbufmg.br

Received November 21, 2011; **Accepted** December 08, 2011; **Published** December 13, 2011

Citation: Azevedo EG, Ribeiro RR, Ferreira CS, da Silva SM, Schettini DA, et al. (2011) Prolonged Blood Circulation Time of Antimony in Dogs with Visceral Leishmaniasis from Liposomes with 175-nm Diameter. J Nanomedic Biotherapeu Discover 1:101. doi:10.4172/2155-983X.1000101

actual liposome formulation, and more specifically the prolongation of its blood circulation time, is needed to more effectively target drugs to less accessible infected sites.

As an attempt to prolong the blood circulation time of our liposome formulation, vesicle diameter was reduced from 400- to 175-nm. The impact of vesicle size reduction on the blood pharmacokinetics of Sb in dogs with VL and on the metal distribution in the liver, spleen and bone marrow were investigated.

Even though the influence of the size of conventional liposomes on their *in vivo* fate is well established for comparison between very small liposomes (SUVs, with diameter < 100 nm) and larger ones (LUVs, with diameter > 100 nm) [7], the effect of size reduction from medium size liposomes (200 nm < diameter < 500 nm) to smaller LUVs (150 nm < diameter < 200 nm) has not yet been reported. Furthermore, most studies about the influence of vesicle size on liposome pharmacokinetics were carried out in the rat or mouse model, but those may not apply to dogs [8].

The present study shows that reduction of liposome diameter from 400- to 175-nm resulted in more prolonged blood Sb levels and a marked reduction of Sb concentration in the liver of dogs with VL. The prolonged blood circulation time of liposomes with 175-nm diameter makes this nanosystem suitable for passive drug targeting to the less accessible infection sites in dogs with VL.

Materials and Methods

Materials

Cholesterol (CHOL) and dicetylphosphate (DCP) were purchased from Sigma Co. (St. Louis, MO, USA). Distearoylphosphatidylcholine (DSPC) was obtained from Lipoid (Ludwigshafen, Germany). N-methyl-D-glucamine and antimony pentachloride ($SbCl_5$, 99%) were obtained from Aldrich Chemical Co. (Milwaukee, WI, USA).

Animals

Male mongrel dogs (weighing 5–15 kg), naturally infected with *Leishmania (L.) infantum chagasi* and destined to euthanasia, were obtained from the Centro de Zoonoses of the Prefeitura Municipal de Santa Luzia (MG, Brazil). Animals were found to be positive according to the following tests for *L. infantum*: indirect immunofluorescence (IFAT), complement fixation test (RFC) and enzyme-linked immunosorbent assay (ELISA), demonstration of *Leishmania* amastigotes in Giemsa-stained bone marrow aspirates and polymerase chain reaction (PCR) using specific oligonucleotide primers for the amplification of a repetitive DNA sequence of *L. infantum*.

Synthesis of meglumine antimoniate

Meglumine antimoniate was synthesized, as previously described [10, 11], from equimolar amounts of N-methyl-D-glucamine and pentavalent antimony oxyhydrate. The resulting product contained 28% of Sb by weight.

Preparation and characterization of meglumine antimoniate-containing liposomes

Small unilamellar vesicles (SUVs) were prepared by ultrasonication of a suspension of multilamellar vesicles made from DSPC, CHOL and DCP (molar ratio of 5:4:1) in deionized water, at the final lipid concentration of 55 g/L. After filtration through sterile 0.22 μm membrane, the SUVs suspension was mixed with an aqueous sucrose solution at a sugar/lipid mass ratio 3:1 (Lip 400) or 1:1 (Lip 175). The resulting mixture was immediately frozen in liquid nitrogen and subsequently lyophilized (freeze-dryer 4.5 L, Labconco, UK).

Rehydration of the dried powder was performed with an aqueous meglumine antimoniate solution (Sb concentration of 80 g/L) as follows: 50% of the original SUVs volume of meglumine antimoniate solution was added to the lyophilized powder and the mixture was vortexed and incubated for 45 min at 60°C. Only Lip 175 was further extruded across two stacked 200-nm polycarbonate membrane at 60°C in order to reduce the mean diameter of vesicles. In both formulations, the same volume of phosphate buffer saline (PBS: 0.15 mol/L NaCl, 0.01 mol/L phosphate, pH 7.4) was then added and the mixture was vortexed and incubated for 15 min at 60°C. Drug-containing liposomes were separated from the non- encapsulated drug by centrifugation (20,000×g, 45 min). The liposome pellet was then washed and finally resuspended in PBS at final Sb concentration of 10 g/L.

The amount of Sb was determined in the resulting liposome suspension by graphite furnace atomic absorption spectroscopy (Analyst AA600, Perkin Elmer Inc., MA, USA), after digestion of the sample with nitric acid [5].

The size of the vesicles in suspension was investigated by photon correlation spectroscopy at 25°C using particle size analyzer (Zetasizer S90, Malvern, UK). The mean hydrodynamic diameter and polydispersity index were determined.

Kinetics of release of antimony from liposomes

Liposomal formulation was diluted 1:10 in PBS and incubated at 37°C under constant stirring. After different times of incubation, an aliquot was centrifuged (20,000×g, 45 min), the pellet was recovered and Sb was determined as described above.

Pharmacokinetics and tissue distribution of antimony in dogs

Animals were divided into three groups which received different formulations of meglumine antimoniate, as intravenous bolus injection. The first group (5 dogs) received meglumine antimoniate-containing liposomes with 400-nm mean diameter (Lip 400) at 4.2 mg Sb/kg of body weight. The second group (4 dogs) received meglumine antimoniate- containing liposomes with 175-nm mean diameter (Lip 175) at 6.5 mg Sb/kg of body weight. A third group (5 dogs) received a meglumine antimoniate solution (Sb concentration of 0.66 mol/L) at 100 mg Sb/kg of body weight. Blood samples were collected from the jugular vein at the following time intervals: 5, 20, 60,150 min 6, 12 and 24 h. All animals were sacrificed 24 h after administration. Liver and spleen were recovered, homogenized and frozen at −20°C. In the case of the bone marrow, samples were obtained by aspiration from both the sternal bone and iliac crest.

A barbituric drug, sodium thiopental, was used to perform the humane euthanasia of the dogs, as described previously [12] and routinely used at the Veterinary Hospital of the Federal University of Minas Gerais. The present research adhered to the Principles of Laboratory Animal Care (NIH publication #85-23, revised in 1985) and received approval from the Ethics Committee in Animal Experimentation of the Federal University of Minas Gerais (123/05 protocol).

Antimony was determined in the tissues by graphite furnace atomic absorption spectroscopy after digestion of the tissues with nitric acid, as described previously [5]. The proportion of total Sb dose recovered from the liver and spleen were calculated using the actual weight of

the organs. In the case of bone marrow, tissue weight was estimated as 2.2% of the total body weight [8].

Pharmacokinetic analysis: Pharmacokinetic parameters were determined using compartmental analysis. Iterative weighted nonlinear least-squares regression with the Rstrip 4.03 computer program was used and model selection was guided by Akaike's information criterion [13]. Experimental blood concentration–time data were best fitted by a mono-compartment open model with i.v. bolus input. Fitted parameters included the blood half-life ($t_{1/2}$), the volume of distribution at steady state (V_{SS}), the total body clearance (CL) and the mean residence time projected to infinity ($MRT_{0-\infty}$).

Statistical analysis: Comparison of the levels of Sb between different tissues was performed using Kruskal- Wallis test (followed by Dunn's multiple comparison test) or by Mann-Whitney test. A two-tailed P value of <0.05 was considered statistically significant.

Results

Characterization of the different liposome formultions of meglumine antimoniate

Two different liposome formulations of meglumine antimoniate were obtained: Lip 400 whose vesicle size was controlled by the use of cryoprotective sugar [5] and Lip 175 that was further extruded through 200-nm pore polycarbonate membrane to achieve vesicle size reduction.

Lip 400 and Lip 175 exhibited mean hydrodynamic diameters of 410 ±75 and 175±25 nm and drug entrapment efficiencies of 40±4 and 34±3%, respectively. The polydispersity index in the case of both formulations was lower than 0.3, indicating monodisperse vesicle dispersions.

Figure 1 shows the kinetic of release of Sb from Lip 175 in isotonic saline for 24 h at 37°C.

No significant release was detected during the first 3 h of incubation, however, release of Sb was observed from 6 to 24 h, reaching a value of about 40%. This percentage of drug release is greater than that reported previously for Lip 400 in the same conditions [5]. In the latter case, 12% of encapsulated drug was released after 24 h.

Blood pharmacokinetics and tissue distribution of antimony in dogs

Lip 175 and Lip 400 were given to dogs with VL as intravenous bolus injections of 6.5 mg Sb/kg and 4.2 mg Sb/kg of body weight, respectively. The free drug was also given intravenously at a therapeutic dose of 100 mg Sb/kg for comparison purpose.

Figure 2 displays the pharmacokinetics of Sb in the blood of dogs from the free drug and their liposome formulations. The elimination of Sb from blood circulation occurred within 6 hours for the free drug and Lip 400, whereas significant levels of Sb were still detected

After 24h in the case of Lip 175. Data were consistent with monoexponential eliminations. Table 1 summarizes the main pharmacokinetic parameters determined for the three formulations. Interestingly, the values of elimination half-life and mean residence time obtained for small size liposomes (Lip 175) were at least 5-fold greater than those of medium size ones. On the other hand, Lip 400 and the free drug exhibited similar values of these parameters. The lower value of CL from Lip 175 is also consistent with the more prolonged blood levels of Sb from these liposomes.

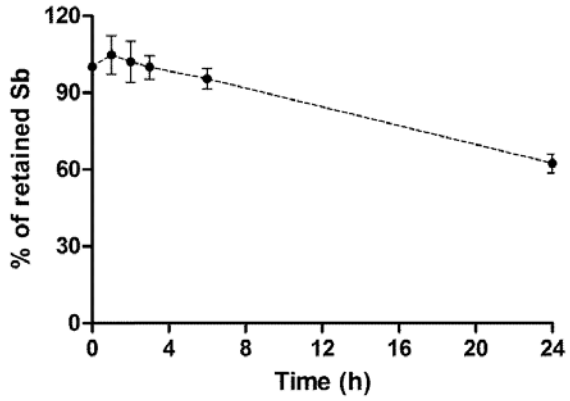

Figure 1: Kinetic of release of Sb from small size liposomes (Lip 175) in isotonic buffer at 37°C. Liposome formulation was diluted at 1 g of Sb/L in PBS and incubated at 37°C under constant stirring. After different times of incubation, an aliquot was centrifuged, the pellet was digested with nitric acid and Sb was determined by graphite furnace atomic absoption spectroscopy. Data are given as mean ± SD (n = 3).

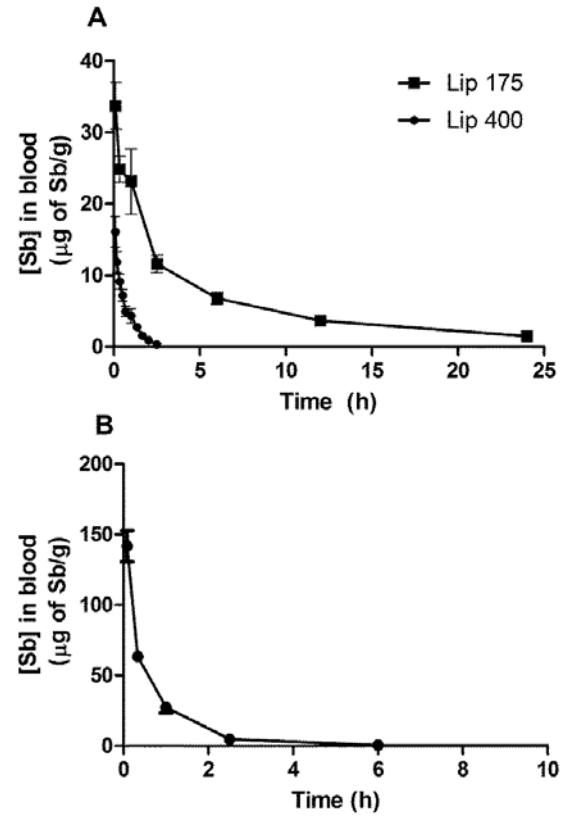

Figure 2: Pharmacokinetics of Sb in the blood of dogs with VL after intravenous bolus injection of liposomal (A) or free (B) meglumine antimoniate. Dogs received small size liposomes (Lip 175, 6.5 mg Sb/kg), medium size liposomes (Lip 400, 4.2 mg Sb/kg) or the free drug (100 mg Sb/kg). After 24 h, organs were collected, weighed, homogenized and submitted to digestion with nitric acid. Antimony was determined by graphite furnace atomic absorption spectroscopy. Data are given as mean ± SD (n = 4-5).

Drug/ Parameters	*C*max (mg/L)	$t_{1/2}$ (min)	$MRT_{0-\infty}$ (min)	V_{ss} (L/kg)	$AUC_{0-\infty}$ (g.min/L)	*CL* (L/h/kg)
Lip 175	31.6 ± 6.3	127 ± 25	184 ± 35	0.21 ± 0.04	5.7 ± 1.2	1.2 ± 0.3
Lip 400	17.3 ± 5.4	26.5 ± 7.9	38.3 ± 11.5	0.26 ± 0.07	0.63 ± 0.14	7.0 ± 1.6
Free Drug	172 ± 37	15.2 ± 9.8	25.5 ± 9.8	0.60 ± 0.16	4.1 ± 0.5	24.4 ± 2.5

[a]Dogs (n= 4-5) received small size liposomes (Lip 175, 6.5 mg Sb/kg body weight), medium size liposomes (Lip 400, 4.2 mg Sb/kg) or free drug (100 mg Sb/kg). Blood samples were collected at different time intervals for determination of Sb by graphite furnace atomic absorption spectroscopy. Pharmacokinetic parameters were determined using Rstrip 4.03 computer program. C_{max}, maximum blood concentration of Sb; $t_{1/2}$, half- life of blood elimination of Sb; $MRT_{0-\infty}$, mean residence time of Sb projected to infinity; V_{ss}, volume of distribution at steady state; $AUC_{0-\infty}$, area under the blood concentration–time curve projected to infinity; *CL*, total body clearance of Sb.

Table 1: Blood pharmacokinetics parameters[a] (mean ± SD) in dogs with VL after intravenous bolus injection of different formulations of meglumine antimoniate.

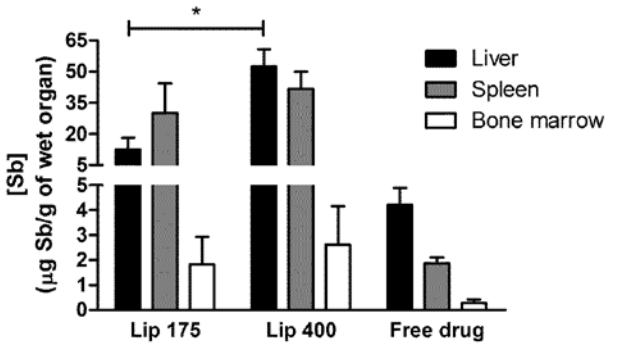

Figure 3: Concentrations of Sb determined in the liver, spleen and bone marrow of dogs with VL, 24 h after intravenous administration of liposomal or free meglumine antimoniate. Dogs received small size liposomes (Lip 175, 6.5 mg Sb/kg), medium size liposomes (Lip 400, 4.2 mg Sb/kg) or the free drug (100 mg Sb/kg). Blood samples were collected at different time intervals for determination of Sb by graphite furnace atomic absorption spectroscopy. Data are given as mean ± SD (n = 3-5). *P<0.05 for comparison of Sb levels in liver between Lipo 175 and Lipo 400 (Mann-Whitney test). In the spleen and bone marrow, no significant difference was found between both liposome formulations. The free drug showed significantly lower Sb levels in all organs when compared to Lipo 400.

Figure 3 shows the concentrations of Sb determined in the liver, spleen and bone marrow of dogs 24 h after administration. Despite the higher dose of Sb given with Lip 175 compared to Lip 400, a 4-fold lower Sb concentration was found in the liver. On the other hand, the concentrations of Sb in the spleen and bone marrow did not differ significantly between both liposome formulations. Interestingly, the concentration of Sb achieved in the liver after a therapeutic dose of the free drug was 3-fold and 12-fold lower than those achieved after Lip 175 and Lip 400, respectively, given at 15- and 20-fold lower doses of Sb.

The proportions of total dose of Sb recovered from the liver, spleen and bone marrow were also calculated (Table 2) to compare the targeting effectiveness of both liposome formulations. Strikingly, whereas 50% of the amount of administered Sb was found in the liver and spleen 24 h after Lip 400, only 10% was encountered in the same organs after Lip 175. Accordingly, medium size liposomes targeted more effectively the liver than small size liposomes. On the other hand, similar targeting effectiveness was observed towards the bone marrow and spleen.

Discussion

In the present study, two liposome formulations of meglumine antimoniate with the same lipid composition, but differing in their mean vesicle diameter, were prepared and characterized in order to investigate the influence of vesicle size on the blood pharmacokinetics and tissue distribution of Sb in dogs with VL.

The choice of this experimental model is justified by its relevance in the field as the main reservoir of VL and by the fact that liposome pharmacokinetics is expected to depend on the animal species [8] and on the infected state of organs involved in liposome capture.

As main results of this work, reduction of liposome size from 400- to 175-nm resulted in more prolonged blood levels of Sb in infected dogs and a marked reduction of Sb concentration in the liver.

The marked increase of elimination half-life and reduction of hepatic uptake of Sb following liposome size reduction strongly suggests a less effective liposome capture by the Kupffer cells, the main cell involved in removal of vesicles from the circulation.

Both vesicle size and lipid dose are known critical parameters that strongly influence the rate of blood clearance of conventional liposomes [7, 14]. It has been proposed that liposomes of differing size and surface characteristics may attract different arrays of plasma proteins, called "opsonins", the content and conformation of which may account for the different pattern in the rate and site of vesicle clearance from the blood [15]. In this regard, it is clearly established that reduction of vesicle size from LUV (diameter > 100 nm) to SUV (diameter < 100 nm) results in more prolonged blood circulation time of the vesicles. However, the influence of curvature in terms of changes to the lipid packing may apply to radii of less than 150 nm, but differences are not expected above this size [14]. Thus, the increased blood residence time of Sb, as reported here, is probably not due to a direct size effect.

Even though Lip 175 was administered at about 2-fold greater lipid dose (60 mg lipid/kg body weight) when compared to Lip 400 and this dose may approach the saturation threshold of the liver, this factor cannot account solely for the marked pharmacokinetic changes observed. The increased total exposed surface area of Lip175 as a result of size reduction may be the main contributing factor. Accordingly, possible consequences of the increased surface area may be that the pattern of bound opsonins was altered reducing the rate of vesicle capture by Kupffer cells and/or the amount of exposed lipid reached the saturation threshold of the liver. Interestingly, this proposal is consistent with the fundamental nanotechnology concept that significantly modified properties of a system are observed, when the size of this system is reduced from to the nanometer range, as a result of increased surface area.

In conclusion, liposomes with reduced diameter (175-nm), in

	Spleen	Liver	Bone Marrow
Lip 175	3.3 ± 1.7	7.7 ± 2.9*	0.62 ± 0.38
Lip 400	6.1 ± 0.8	49.9 ± 8.7*	0.85 ± 0.50
Free drug	0.060 ± 0.017	0.51 ± 0.12	0.021 ±0.013

Dogs (n= 3-5) received intravenous bolus injection of either small size liposomes (Lip 175,6.5 mg Sb/kg body weight), medium size liposomes (Lip 400, 4.2 mg Sb/kg) or free drug (100 mg Sb/kg). After 24 h, organs were collected, weighed, homogenized and submitted to digestion with nitric acid. Antimony was determined by graphite furnace atomic absorption spectroscopy. *P<0.05, for comparison between both liposome formulations (Mann- Whitney test).

Table 2: Proportions (%, mean ± SD) of the total dose of Sb recovered from liver, spleen and bone marrow of dogs with VL, 24 h after dosing with liposomal and free meglumine antimoniate.

addition to high drug encapsulation efficiency and retention, promote prolonged Sb levels in the blood of dogs with VL. This property makes this nanosystem suitable for passive drug targeting to the less accessible infection sites in dogs with VL.

Acknowledgements

We acknowledge the Brazilian agencies CNPq, FAPEMIG and CAPES for financial support. R.R.R. and C.S.F. were recipients of postdoctoral fellowship from FAPEMIG and CNPq, respectively.

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