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TISSUE DISTRIBUTION OF RADIOLABELED PHOSPHATIDYLSERINE-CONTAINING LIPOSOME IN MICE

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ABSTRACT

Liposomes are used as drug delivery systems to modify pharmacokinetic of drugs and also to improve their action in target cells. Liposomes containing phosphatidylserine are efficiently eliminated from the blood by cells of the mononuclear phagocytic system (MPS), predominantly Kupffer cells in the liver. In this way, this is a valuable approach to treat infectious diseases involving MPS, especially leishmaniasis. Leishmaniasis is a severe parasitic disease, caused by intramacrophage protozoa *Leishmania* sp., and is fatal if left untreated. *Leishmania* resides mainly in the liver and the spleen. Antileishmanial agents containing-liposomes showed more effective therapies with reduction of toxicity and adverse side effects. The purpose of this study was to investigate the tissue distribution of radioactive meglumine antimoniate encapsulated in phosphatidylserine-containing liposome. Meglumine antimoniate was neutron irradiated inside the IEA-R1 nuclear reactor to produce antimony radiotracers, ^{122}Sb and ^{124}Sb, and encapsulated in liposome. Healthy mice received a single intraperitoneal dose of the radiolabeled drug. Analysis of the mean radioactive tissue concentration-time data curves showed that liver and spleen had the highest levels of radioactivity. In addition these levels of drug remained for more than 48 hours. The dominant route of elimination was via biliary excretion with slow rate. Small fraction of the drug was found in the kidneys with very fast elimination. In conclusion, the phosphatidylserine-containing liposome showed to be a very useful tool to target antileishmanial agents to MPS and to sustain the drug levels for longer times. Besides, radiolabeled liposome is the easiest approach to perform biodistribution evaluation.

1. INTRODUCTION

Leishmaniasis is a group of protozoan diseases caused by several *Leishmania* spp and is transmitted to human beings and animals by sand flies. It is endemic in the tropics and neotropics. The most important clinical manifestation is Visceral Leishmaniasis that is a systemic disease and fatal if left untreated. Despite its increasing worldwide incidence, leishmaniasis has become one of the so-called neglected diseases, with little interest by financial donors, public-health authorities, and professionals to implement activities in order to research, prevent, or control the disease [1]. The main treatment against all forms of leishmaniasis is based in the use of pentavalent antimonials, as meglumine antimoniate (Glucantime®), although having toxic side-effects, high resistance in some parts of the world

and unclear knowledge about their mode of action. Available drugs are limited in number and each has various shortcomings and a vaccine currently does not exist. The high prevalence of leishmaniasis and the emergence of resistance to conventional drug demonstrate the need to develop new, less toxic and more efficient treatment. An alternative is to carry antileishmanial agents using drug delivery systems, such as liposomes [2].

Liposomes are defined as vesicles in which an aqueous volume is entirely surrounded by a phospholipid membrane. Liposome properties have been extensively investigated and can vary substantially with the desired size, lipid composition, surface charge and method of preparation. Liposomes have been shown to improve the efficacy and reduce the systemic toxicity of drugs, specifically as carriers for antifungal, anticancer, antibiotics and others [3]. As liposomes are naturally taken up by the mononuclear phagocytic system (MPS), predominantly by the macrophages of the liver and spleen, the main reservoirs of parasite in visceral leishmaniasis, the use of liposomes represents a logical strategy to target these tissues to more efficiently treat this parasitic infection. Antimonial agent containing-liposome was found to be several times more active than non-encapsulated drug, thus confirming the potential of liposome system [4].

Liposomal encapsulation offers opportunities for the improvement of pharmacological properties of drugs. However, the mechanisms involved in the elimination of liposomes from blood compartment are still not fully understood. In the development of liposomal drug delivery systems, pharmacokinetic, biodistribution, and cellular uptake of such systems are major issues to be known. To investigate the fate of liposomes *in vivo*, a large variety of liposomal markers are available. Radiolabels provide a sensitive and powerful tool to determine liposome biodistribution [5].

IPEN has a Research Reactor, so called IEA-R1m, that nowadays operates at 3.5MW for 64 hours continuously, which allows the production of many radioisotopes used in the routine production and also for research in the radiopharmacy field [6]. The purpose of this study was to investigate the tissue distribution of radioactive meglumine antimoniate encapsulated in phosphatidylserine-containing liposome employing the Sb radiotracers, ^{122}Sb and ^{124}Sb, produced at the Reactor.

2. MATERIALS AND METHODS

2.1. Animals

Female BALB/c mice (20-24g) were supplied by the Animal Breeding Facility at the Faculty of Medicine of Sao Paulo University and maintained in sterilized cages under a controlled environment, with free access to food and water. Animal procedures were performed with the approval of the Research Ethics Commission, in agreement with the Guide for the Care and Use of Laboratory Animals from the National Academy of Sciences.

2.2. Production of Radioactive Meglumine Antimoniate (IMA)

In order to obtain radioactive antimony, samples of meglumine antimoniate (Glucantime®; Aventis, SP, Brazil) in clean polypropylene tube were placed inside the aluminum container

and irradiated at a thermal neutron flux of 0.8-1.0 x 10^{12} n/cm^2.s for 15 minutes, inside the IEA-R1m nuclear reactor (IPEN/CNEN-SP). The tracers were produced through the reactions ^{121}Sb(n,γ)^{122}Sb and ^{123}Sb(n,γ)^{124}Sb. Radionuclidic purity was determined by γ-spectrometry, using an HPGe detector (Canberra Company) coupled to the Genie-PC program. Radioactive concentration was also measured with the same system after efficiency calibration with the standard ^{60}Co, ^{137}Cs and ^{152}Eu sources [6].

2.3. Radiolabeled Liposome Preparation (FDEL-IMA)

To perform the FDEL-IMA, liposome was prepared as described by Schettini *et al.* [7] with some modifications. The lipid mixture from phosphatidylserine, cholesterol and phosphatidylcholine in the molar ratio 1:4:5 was dissolved in an organic solvent and dried under vacuum at 55 °C by a rotary evaporation. The dry lipid film was hydrated with isotonic buffer and the lipid suspension was sonicated in an ultrasonic bath. The solution was frozen and then freeze-dried. The rehydration of the dried powder was performed with an aqueous radioactive meglumine antimoniate (IMA) solution by gentle stirring and incubation. Non-encapsulated drug was removed by overnight dialysis at 4 °C against a dialysis buffer. The determination of radioactive antimony concentration was done by γ-spectroscopy, using an HPGe detector coupled to the Genie-PC program, as described before. Encapsulation efficiency was determined by measuring the Sb in the liposomal dispersion before and after separation to remove all non-encapsulated drug. Values were calculated as the percentage of drug encapsulated into liposome by the following equation:

$$\text{Encapsulation Efficiency (\%)}: (Sb_{encapsulated}/Sb_{total}) \times 100$$

Where Sb_{total} and $Sb_{encapsulated}$ mean the amount of antimony (^{122}Sb) in the solution before and after separation, respectively.

2.4. Biodistribution of Radiolabeled Liposome

Biodistribution studies of FDEL-IMA were performed in healthy female BALB/c mice (20-24g) (n=5). Groups of mice were injected, by intraperitoneal route, with 0.08 mg Sb^{+5}/100µL with activity approximately of 1 x 10^3 Bq ^{122}Sb /100µL (0.03 µCi) and 2.5 x 10^2 Bq ^{124}Sb /100µL (0.006 µCi). After 5, 15, 30, 60, 120, 300, 1080, 1440 and 2880 minutes, mice were sacrificed by cervical dislocation with blood sampling. The organs were excised and activity was measured in a NaI(Tl) scintillation counter (Cobra Auto-Gamma - Canberra Company). The data analysis was conducted with Graph Pad Prism 5.02 software.

3. RESULTS AND DISCUSSION

3.1. Production of Radioactive Meglumine Antimoniate (IMA)

Radioactive antimony was produced by the neutron irradiation of meglumine antimoniate drug. This procedure allowed to obtain a compound with high radionuclidic purity, producing

only two radioisotopes ^{122}Sb and ^{124}Sb, where ^{122}Sb ($t_{1/2}$ = 2.7 days) gamma peaks were observed in 563.99 keV (69.3%) and 692.94 keV (3.78%) and ^{124}Sb ($t_{1/2}$ = 60.2 days) in 602.66 keV (97.8%), 645.77 keV (7.38%), 668.87 keV, 709.26 keV, 713.73 keV, 722.75 keV (10.76%), 790.46 keV, 968.31 keV, 1045.15 keV, 1368.43 keV (2.62%) and 1691.51 keV (47.34%). Good specific activity was verified corresponding to 9.6 MBq (260 μCi)^{122}Sb/mL of meglumine antimoniate and 0.18 MBq (4.8 μCi)^{124}Sb/mL of meglumine antimoniate. These results confirm the usefulness of antimony radioisotopes to elucidate some questions about antimonial agents properties, as they exhibit several specifications in order to fulfill the application requirements, such as good specific activity, high radionuclidic purity and adequate total activity of the radionuclide, as described previously by Borborema *et al*. [8]. The most relevant role of nuclear reactors in medical applications is the production of radionuclides, the key component of any radiopharmaceutical. Moreover, this work emphasizes the peaceful application of nuclear energy in biomedical fields.

3.2. Radiolabeled Liposome Preparation (FDEL-IMA)

Preparations of radiolabeled liposome were analyzed by γ-spectroscopy technique to determine the amount of antimony. The determination of antimony radioactivity in the samples was performed in several steps of the preparations. Table 01 shows the results of the evaluation of encapsulation efficiency of radioactive antimony in FDEL liposome. The encapsulation efficiency of FDEL-IMA was approximately 10%. The same evaluation was performed by other techniques, such as ICP-OES, and the results were not reproducible due to problems in the preparation and digestion of the samples. The technique used presented no such problems because there was no need to digest the samples. These results proved the accuracy and reliability of the γ-spectroscopy method for the determination of trace amounts of antimony in different samples, besides being easier and faster.

Table 01. Evaluation of Efficiency Encapsulation (Ee) of antimony in FDEL liposome. FDEL-IMA= freeze-dried empty liposome rehydrated with radioactive meglumine antimoniate. Data are given as mean ± standard deviation (n=4).

Sample	Sb/LP ratio (w/w)	Sb mass (mg)	Recovery (%)	Ee (%)
FDEL-IMA	0.6	6.0 ± 2	98 ± 9	10 ± 2

The FDEL method was a simple and easy procedure, moreover it showed very good recovery of the drug, near 100%. These findings suggest that the FDEL method is very useful for preparation of liposome containing radiolabeled drugs, thus it could reduce the amount of drug used and decrease of waste of the radiolabeled agents. In addition, a significant technological advantage of this method over conventional ones is that the liposomal formulation may be stored as freeze-dried empty liposomes and that rehydration may be performed just before use.

3.3. Biodistribution of Radiolabeled Liposome

The use of radioisotopes as labels for molecules involved in biological structure and dynamics is a very important way to know the drug concentration and its distribution in a body. This tool allowed to study the fate of liposomes *in vivo* and determine their pharmacokinetic in animal model. The radiolabeled liposomal formulation of meglumine antimoniate was evaluated in healthy mice. For this purpose, the liposomal drug was given in a single intraperitoneal dose. Fig. 01 shows the pharmacokinetics of antimony in the blood. Data are consistent with a biexponential open model. The absorption of radioactive meglumine antimoniate-containing liposomes, after intraperitoneal administration, showed to be fast, occurring in the first 30 minutes. The elimination of antimony from blood occurred within 5h after administration, showing a first rapid phase followed by a second slower phase. The first of these hypothetical kinetic compartments represents a central compartment which includes the blood, volume into which the drug is absorbed after intraperitoneal injection and from which the drug is excreted through the urine. The second compartment may represent a peripheral compartment into which the drug is distributed and finally eliminated via biliary excretion. These findings corroborate with previously data from the group [9] using non-encapsulated radioactive antimonial agent.

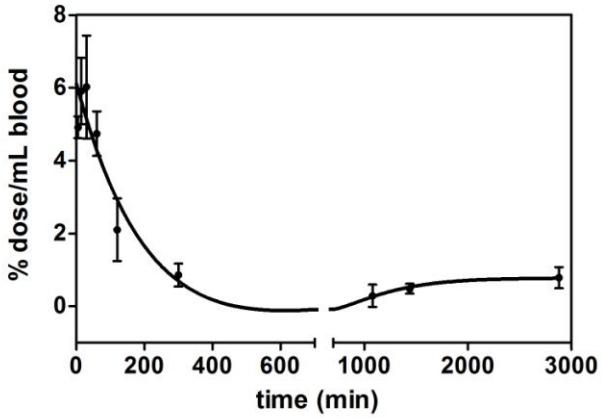

Figure 01. Pharmacokinetics of antimony in the blood of healthy mice, after intraperitoneal injection of radioactive meglumine antimoniate-containing liposome (FDEL-IMA). Data are given as means ± standard deviation (n = 4 animals).

Fig. 02 shows the distribution of antimony in the MPS, organs which are known to harbor *Leishmania* parasites and also for their rapid uptake of liposomes, at different times after injection of the liposomal drug.

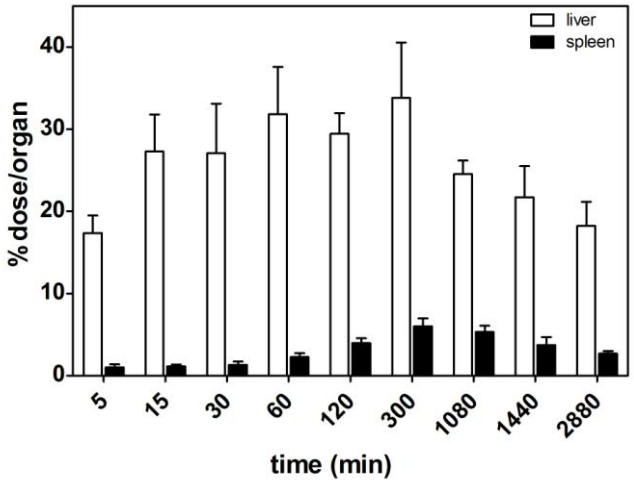

Figure 02. Pharmacokinetics of antimony in the liver and spleen of healthy mice, after intraperitoneal injection of radioactive meglumine antimoniate-containing liposome (FDEL-IMA). Data are given as means ± standard deviation (n = 4 animals).

High levels of antimony were found in the liver and spleen of animals. The highest level of drug was verified 5h after injection. In addition these levels of drug were maintained for more than 48 hours. The dominant route of elimination was via biliary excretion with slow rate. Small fraction of the drug was found in the kidneys with very fast elimination. According to this data, the liposomal drug promoted a marked targeting of antimony to MPS tissues, besides the use of encapsulated drug in liposome maintained the high doses in the organs for prolonged period. At least one of the two observed blood phases should correspond to the capture of liposomes by MPS organs, because more than 45% of injected antimony was presented in the liver and spleen. In addition, it was verified an increase in the MPS organs at the same time that there was a reduction of the amount of drug in the blood. These same results were also encountered by Schettini *et al.* [7] in infected dogs. It has been showed that after parenteral administration, liposomes are taken up by macrophages of the spleen and the liver, especially phosphatidylserine-containing liposomes that are more efficiently eliminated from blood by cells of the MPS, predominantly Kupffer cells and hepatocytes in the liver [10]. It is known that meglumine antimoniate encapsulated in phosphatidylserine-containing liposomes promotes macrophage recognition by scavenger receptors followed by increase in the uptake and internalization of the liposome [4]. These findings could explain the results presented in this work and also emphasize the great feasibility of encapsulating the standard drug to treat leishmaniasis in phosphatidylserine-containing liposomes.

3. CONCLUSIONS

In conclusion, meglumine antimoniate encapsulated in phosphatidylserine-containing liposomes enhances the uptake of the drug by MPS organs and it may represent a better approach to target macrophages in the leishmaniasis. These results could lead to numerous developments in pharmacology and improvement in therapeutic field.
This study also made possible to choose the most appropriate conditions in terms of neutron irradiation of antimonial agent, as well as to evaluate the encapsulation efficiency of this drug in liposome formulations, moreover it was possible to establish the pharmacokinetic profile

of the standard drug used to treat all forms of leishmaniasis phosphatidylserine containing-liposome. In addition, this study illustrates the opportunities to enhance the utilization of nuclear reactor facilities for cooperative investigations in the production of several tracers, such as ^{122}Sb and ^{124}Sb, in biomedicine and the provision of therapeutic radioisotopes for research, radiopharmaceutical development and further clinical applications. Basic research on drug delivery systems and direct pharmacological applications of these new research tools could be conducted using this model.

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