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Formulation and evaluation of oil-in-water emulsions of piperine in visceral leishmaniasis

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Present studies are aimed to find out the utility of oil-in-water emulsions also known as lipid nanospheres (LN) or fat emulsions for delivering piperine for the treatment of visceral leishmaniasis. Lipid nanosphere formulations of piperine were prepared using soybean oil, egg lecithin, cholesterol, stearylamine and phosphatidylethanolamine distearylmethoxypolyethyleneglycol (DSPE-PEG) by homogenization followed by ultrasonication of oil and aqueous phases. Antileishmanial activity of all the formulations was assessed in BALB/c mice infected with *Leishmania donovani* AG83 for 60 days. A single dose (5 mg/kg) of piperine, or lipid nanospheres of piperine (LN-P), or lipid nanosphere of piperine with stearylamine (LN-P-SA) or pegylated lipid nanospheres of piperine (LN-P-PEG) was injected intravenously. Mice were sacrificed after 15 days of treatment with piperine or formulations and Leishman Donovan Unit (LDU) is counted. Toxicity of formulations and pure piperine was assessed in normal mice. The size distribution of formulations ranged from 200 to 885 nm. Piperine reduced the parasite burden in liver and spleen by 38% and 31% after 15 days post infection respectively. LN-P reduced the parasite burden in liver and spleen by 63% and 52% after 15 days post infection, respectively. LN-P-PEG reduced the parasite burden in liver and spleen by 78% and 75% after 15 days post infection, respectively. LN-P-SA reduced the parasite burden in liver and spleen by 90% and 85% after 15 days post infection, respectively. LN-P, LN-P-PEG, LN-P-SA treated mice did not show any significant changes in the serum levels of SGOT, ALP, creatinine and urea compared to normal mice. Stable and sterile formulations of lipid nanospheres of piperine were developed. A single dose of 5 mg/kg of lipid nanospheres of piperine could significantly reduce the liver and splenic parasite burden.

1. Introduction

The powder of dried seeds of black pepper (*Piper nigrum* Lin, Piperaceae) has been used not only as a seasoning spice but also as a useful drug in Indian medicine (Krishnamurthy 1969). In folk medicine, the vapors of pepper species are used in treating epilepsy, common cold, headache (Atal et al. 1975) and pepper also has been used as abortifacient in few areas of India (Chandhoke et al. 1978; Kholkute et al. 1979). There have been studies on the antimalarial activity (Shelef 1983), antifertility (Piyachaturawat et al. 1982) and neurogenic effect (Szolesanyi 1983) of piperine. Piperine has also been shown to enhance the bioavailability of many drugs by inhibiting their metabolism (Atal et al. 1985; Bano et al. 1991; Shoba et al. 1998; Reen et al. 1996; Zutshi et al. 1985). The pharmacology and clinical use of piperine and its derivatives in the treatment of epilepsy has been reviewed by Yin Quan Pei (1983). Piperine is also reported to inhibit aflatoxin B_1-induced oxidative stress (Khajuria et al. 1998) and to exhibit analgesic activity against tail-clip pressure and anti-inflammatory activity against carragenin-induced edema in rats (Lee et al. 1984). Antifungal (Madhyastha et al. 1984) and anti-amoebic activities (Ghoshal et al. 1996) of piperine have been reported. Regular oral feeding of piperine did not show any adverse effect in weaning rats (Bhat et al. 1986) and caused increased food uptake in rats and an increase in liver weight mainly due to higher total and neutral lipid contents (Srinivasan et al. 1981). Acute, subchronic and chronic toxicity studies have shown that at pharmacologically effective doses, piperine did not cause any abnormality in the general growth pattern, body to organ weight ratio and clinical pathology (Piyachaturawat et al. 1983). Piperine determination in serum and tissue homogenates and pharmacokinetics of piperine following i.v. bolus have been published by Sunkara et al. (2001).

Leishmaniasis is a complex disease with visceral, cutaneous, and mucocutaneous pathologies caused by up to 15 different species of the protozoan parasite leishmania. The visceral form of the disease, caused by *Leishmania donovani, Leishmania infantum*, or *Leishmania chagasi*, can be potentially fatal if untreated (Aden et al. 2000). Visceral leishmaniasis (VL) is found in tropical and subtropical regions of the World and has a worldwide annual incidence of up to 500,000 cases (Boelaert et al. 2000). Co-infections with *Leishmania infantum* and human immunodefi-

ciency virus (HIV) have been a growing problem in mediterranean countries and have indicated that this parasite is also an opportunist (Herwaldt 1999). Pentavalent antimonials, sodium stibogluconate (pentostam) and deglumine antimoniate (glucantime) have been in clinical use for leishmaniasis for more than 50 years. Other recommended drugs include amphotericin B and paromomycin (Berman 1997; Berman et al. 1998).

Piperine is a potent inhibitor of *Leishmania donovani* promastigotes in vitro (Kapil 1993). Piperine, an amide alkaloid, isolated from the Indian medicinal plant, *Piper nigrum* exhibited antileishmanial properties when tested in experimental leishmaniasis models. Recently it has been observed that piperine inhibits type I DNA topoisomerase, a vital enzyme of *Leishmania donovani*. A liposomal formulation of piperine has shown significant antileishmanial activity compared to free piperine (Barnini et al. 1999). Present investigations are aimed at developing oil-in-water emulsions or lipid nanospheres of piperine (LN-P) and their evaluation *in vivo*. We prefer to use the term lipid nanosphere since the systems contain lipid spheres of nano range.

2. Investigations and results

The average particle size and zeta potential before sterilization and afters sterilization of LN-P were 214.9 $\pm$ 1.8 nm, -42.0 ± 0.5 mV, and 213.1 $\pm$ 1.9 nm, -40.8 ± 0.1 mV, respectively. The average particle size and zeta potential of formulations of piperine are presented in Table 1. The retention time of piperine is found to be 7.4 min. No detectable peak was observed at 7.4 min in samples obtained from ultracentrifugation of LN-P. The assay values of all formulations were found to be 0.92 mg/ml. Hence the entrapment efficiency of the systems were found to be 100%.

The parasite burden in the liver and spleen of untreated (control) mice was 973.8 ± 94.9 and 58.9 ± 5.6 Leishman Donovan Unit (LDU), respectively. Highest antileishmanial activity was found with LN of piperine with stearylamine (LN-P-SA) (Table 2).

Table 1: Size and Zeta potential of lipid nanosphere formulations after sterilization

Group	Size (nm)	Zeta Potential (mV)
LN-B	196.0 ± 1.7	-35.6 ± 0.2
LN-P	214.9 ± 1.8	-42.0 ± 0.5
LN-PEG	287.2 ± 3.0	-43.7 ± 0.5
LN-P-PEG	297.1 ± 4.4	-39.1 ± 0.2
LN-SA	711.9 ± 14.6	$+22.1 \pm 1.0$
LN-P-SA	884.6 ± 11.9	$+24.2 \pm 0.4$

LN-B (LN without piperine), LN-P (LN with piperine), LN-PEG (LN with PEG), LN-P-PEG (LN with piperine and PEG), LN-SA (LN with stearylamine) and LN-P-SA (LN with piperine and stearyl amine) were measured by Malvern Zeta Sizer. Results are shown as mean $\pm$ S.E (n = 3). nm-nanometers, mV-millivolts.

Table 2: Leishman Donovan units (LDU) in liver and spleen

Group	15th Day	
	Liver	Spleen
Control	973.8 ± 94.9	58.9 ± 5.6
Piperine	601.4 ± 1.2	40.4 ± 7.8
LN-P	364.7 ± 51.6	28.1 ± 3.2
LN-B	884.5 ± 88.4	50.7 ± 4.6
LN-PEG	798.5 ± 137.3	44.2 ± 7.3
LN-P-PEG	214.2 ± 22.0	14.7 ± 3.8
LN-SA	847.2 ± 104.3	44.7 ± 8.6
LN-P-SA	97.4 ± 6.7	8.8 ± 1.5

LN-P (LN with piperine), LN-B (LN without piperine), LN-PEG (LN with PEG), LN-P-PEG (LN with piperine and PEG), LN-SA (LN with stearylamine) and LN-P-SA (LN with piperine and stearyl amine) were measured by Malvern Zeta Sizer. Results are shown as mean $\pm$ S.E (n = 6).

Toxicity studies were conducted in normal BALB/c mice. Phosphate buffer solution (PBS), piperine, LN-P, LN-P-SA, and LN-P-PEG were injected intravenously (equivalent to 5 mg piperine/kg body weight). Serum levels of enzymes (alkaline phosphatase (ALP) and serum glutamine pyruvate transaminase (SGPT) and biochemical markers (creatinine and urea) were estimated on 15th day following administration. The levels of SGPT, ALP, creatinine and urea are within the normal range (Table 3).

3. Discussion

Piperine is a highly lipid soluble drug and a suitable candidate for LN formulation. Fat emulsions with particle size below 1 μm were found to be stable. According to Takamura et al. (1984), a lecithin concentration of more than 1.2% is essential for the formation of LN. In the formulation of LN, cholesterol in sufficient quantity was used to increase the rigidity of lipid monolayer and glycerol was used to maintain the isotonicity of the formulation. It is reported that as the number of shear applications increase, the mean diameter of the emulsion droplets decreased sharply and reached a minimum constant value at ten cycles (Ishii et al. 1990). In our study, as the homogenization time increased the particle size decreased sharply and then a slight increase in particle size was observed. During initial stages of homogenization, the concentration of larger particles is considerably high and there was less frequency of collision between the particles. As the number of shear applications increased the larger particles size was reduced and the number of smaller particles increases and reaches ideal size. Further homogenization produces higher incidence of particle collision and causes increase in particle size by coalescence.

Table 3: Serum data of BALB/c mice that received lipid nanospheres of piperine formulations

Sample	SGPT (u/ml)	ALP (kA/dl)	Urea (mg/dl)	Creatinine (mg/dl)
Control	24.83 ± 16.54	24.05 ± 12.09	58.36 ± 5.18	0.26 ± 0.04
Piperine	13.33 ± 0.57	35.36 ± 5.03	37.99 ± 6.36	0.34 ± 0.09
LN-P	11.33 ± 2.08	22.10 ± 14.64	33.68 ± 6.28	0.18 ± 0.04
LN-P-PEG	20.53 ± 2.18	14.34 ± 4.04	56.18 ± 5.00	0.20 ± 0.02
LN-P-SA	22.43 ± 0.81	16.00 ± 0.26	29.23 ± 1.50	0.20 ± 0.02

SGPT, serum glutamine pyruvate transaminase; ALP, alkaline phosphatase.
Control (PBS), LN-P (LN with piperine), LN-P-PEG (LN with piperine and PEG) and LN-P-SA (LN with piperine and stearyl amine) were injected intravenously (5 mg/kg of piperine) into the BALB/c mice and scarified on 15th day. Results are shown as mean $\pm$ S.E (n = 6).

Stearylamine was used to impart positive charge to the lipid nanospheres. Pegylated LN were prepared using DSPE-PEG to increase the circulation time of nanospheres in the biological system. Several reports on pegylated liposomes revealed that the circulation times were increased due to avoidance of uptake by reticuloendothelial system (RES) (Papahadjopoulos et al. 1991).

For analysis of the particle size distribution of LN, formulations were appropriately diluted with their aqueous phase before the measurements. It was found that there was no significant effect of dilution on zeta potential or particle size. The average diameter and particle size distribution of LN-P after 20 min sonication was 214.9 ± 1.8 nm and 200–400 nm, respectively.

A coarse-emulsion (with globule size <10 μm) can be subjected for ultrasonication to obtain globule size in the range of 200–800 nm (Yamaguchi et al. 1994). Ultrasonication time was optimized at 20 min by analyzing the particle size at different time intervals of sonication. It was found that even after 20 min sonication there was no significant change in average particle size but the particle size distribution was narrowed with time and it was between 200–885 nm (for formulations containing stearylamine) after 20 min sonication. All other formulation's size range was between 190 and 400 nm.

One of the challenging tasks in the colloidal drug delivery system is the determination of the fraction of drug in the continuous phase of the formulation. Dipali et al. (1996) found that among all available methods, dialysis and ultrafiltration methods are most efficient. Ultrafiltration of LN yielded 100–150 μl of aqueous phase, which was diluted and analyzed by HPLC. The entrapment efficiency of lipid nanosphere piperine is 100%. The saturation solubility of piperine in water at 25 °C was found to be 10 μg/ml. This indicates that piperine is totally accommodated in LN, which can also be deduced from increase in size of LN when compared with blank formulation. Generally, parentral emulsions are sterilized by autoclaving at 121 °C for 15 min. Groves and Herman (1993) reported that the phospholipids rapidly relocate during autoclaving from aqueous phase to oil phase, forming a cubic liquid crystalline phase, the bulk oil which is converted to a lamellar phase on cooling and that this organization of interfacial material accounts for the enhanced stability of phospholipid emulsions after heat sterilization. From a physical standpoint, however, the particle size of the disperse phase droplets remains unchanged or decreases after sterilization (Lee et al. 1981; Hansrani et al. 1983). It was observed that after sterilization of LN, there was no significant change in appearance, zeta potential and particle size.

HPLC analysis shows that drug entrapment efficiency of lipid nanospheres is unaffected after sterilization. The poor water solubility and excellent lipid solubility of piperine make it an ideal candidate for the preparation of LN.

The results show that piperine entrapped in lipid nanospheres (a single dose of 5 mg piperine/kg body weight) could significantly reduce the liver and splenic parasite burden indicating efficient localization in liver and spleen. LN-P- SA reduced the parasite burden in liver and spleen by 90% and 85% respectively 15 days after infection. The positive charge and bigger size (than other formulations) of LN-P-SA may be responsible for high localization of the nanospheres in liver and spleen. Further, stearylamine containing liposomes without any antileishmanial drug has shown activity when tested *in vitro* (Tuhina et al. 2000) and *in vivo* (Farhat et al. 2001). The leishmanicidal activity of stearylamine-phosphatidylcholine (SA-PC) liposomes on intracellular amastigotes may be due to the preferential uptake of stearylamine bearing positively charged liposomes by peritoneal macrophages in comparison to neutral and negatively charged vesicles (Nakanishi et al. 1997).

Toxicity studies were conducted to know whether the entrapment of piperine in LN is safe or not. The enzyme levels ALP and SGPT in serum of BALB/c mice treated with LN-P were normal indicating the normal functioning of liver. Piperine entrapped in LN has not shown any nephrotoxicity.

4. Experimental

4.1. Materials

Piperine, egg lecithin, stearylamine and DSPE-PEG were purchased from Sigma (USA) (PC: 60), soybean oil was purchased from Cargil Foods (India) and cholesterol of analytical grade was purchased from Qualigens Fine Chemicals (India). Methanol (HPLC grade) is purchased from Rankem chemicals Limited (India). All other chemicals were commercial products of reagent grade. CENTRISART filters (Molecular weight Cut off: 20,000 dalton) are purchased from Sartorius (Germany).

4.2. Preparation of lipid nanospheres of piperine

Piperine, egg lecithin (1.8%) and cholesterol (0.25%) were dissolved in oil phase (10%), heated to 70 °C on a water bath, and stirred until the piperine was dissolved. Glycerol (2.21%), sucrose (1.8%) and sodium oleate (0.2%) were dissolved in sufficient amount of distilled water and the aqueous phase was added to the oil phase at the same temperature (70 °C). A coarse emulsion was prepared by homogenization (Remi homogenizer, model: L56–3 C.ExNo: GKU-3504, at 6000 rpm). The coarse emulsion so formed was subjected to ultrasonication using Bandelin sonoplus (Bandelin electronic, GmbH & Co., Germany), a probe type ultrasonicator with 12T probe at 100 W to get the LN drug delivery system for 20 min. The blank LN formulation (LN-B) was prepared in similar manner without drug. Stearylamine was used to prepare positively charged LN (LN-P-SA) and DSPE-PEG was included to prepare pegylated LN. All the formulations were filled in 5 ml injection vials and sterilized at 121 °C for 15 min by autoclaving.

4.3. Determination of particle size and zeta potential

Particle size and zeta potential of LN were measured using Malvern Zeta Sizer, which is based on the principle of Photon Correlation Spectroscopy (PCS) at a fixed angle of 90 and a temperature of 25 °C. The field strength used was 29 V/cm. Sterilized and unsterilized samples were diluted with double distilled water and subjected for size and charge analysis. The zeta meter (Malvern) measures the zeta potential based on Smoluchowski equation.

$$\xi = \eta\mu/\varepsilon_r\varepsilon_o \quad (1)$$

Where as ξ is zeta potential (mV), μ is electrophoretic mobility ($m^2\ Volt^{-1}\ S^{-1}$), η is the viscosity of the medium (Pa.s), $\varepsilon_{r\,I}$ is the relative permitivity of the medium ($N/Volt^2$), ε_o is the permitivity of free space ($N/Volt^2$).

4.4. Entrapment efficiency of the system

The free drug in the aqueous phase was estimated by the ultrafiltration method (Ishii et al. 1990). Centrisart, an ultrafiltration unit, consists of a sample recovery chamber and support base tube was used. Filter membrane is (Molecular weight cut off: 20000 dalton) present at the base of the sample recovery chamber. A 500 μl of undiluted sample was placed in the support base and the sample recovery chamber placed on top of the sample. The unit was centrifuged at 3500 rpm for 15 min. The LN along with encapsulated drug remained in the support base and aqueous phase moved into the top sample recovery chamber through membrane. The formulations were subjected for ultrafiltration and the amount of the drug in the samples was estimated by HPLC.

4.5. Assay

0.1 ml of formulation was diluted to 1 ml with chloroform : methanol (1 : 1) solution. The final dilution was made with mobile phase and piperine content was estimated by HPLC.

4.6. Leishmania donovani infection in BALB/c mice and evaluation of effect of lipid nanospheres of piperine.

BALB/c mice (4 to 6 weeks old) were each infected intravenously (I.V.) with 2.5×10^7 amastigotes. The mice were maintained for 60 days. A sin-

gle dose (5 mg/kg body weight) of piperine or formulations was injected intravenously. Six mice from each group were sacrificed 15 days after injection and parasite load was determined by the weights and microscopic examination of Giemsa-stained impression smears of liver and spleen (Afrin et al. 1997). The parasite load was expressed as Leishman Donovan Units (LDU) and calculated using the formula: number of amastigotes per 1000 cell nuclei x organ weight (mg) (Stauber et al. 1958).

4.7. Preparation of piperine solution

Piperine was solubilized in a solvent system consisting of water, propylene glycol and ethanol (50 : 40 : 10) as described by Sunkara et al. (2001).

4.8. HPLC analysis of samples

The HPLC method reported by Sunkara et al. (2001) was used in the study. HPLC analysis was carried out on a Shimadzu LC-6A model equipped with a spectrophotometric detector (SPD-6AV model) and with 20 µl injection port. A C_{18} column (250 mm, 4.6 mm I.D, 5 µm particle size) was used for the analysis. A mobile phase consisting of methanol : water (75 : 25) was pumped through the column at a rate of 1 ml/min and the eluent was monitored at 343 nm. 20 µl samples were injected from standard solutions of piperine (1, 2, 3, 4, 5 and 6 µg/ml) and the peak areas and peak heights were noted.

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