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ORIGINAL PAPER

Ivermectin-loaded lipid nanocapsules: toward the development of a new antiparasitic delivery system for veterinary applications

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Abstract Ivermectin (IVM) is probably one of the most widely used antiparasitic drugs worldwide, and its efficacy is well established. However, slight differences in formulation may change the plasma kinetics, the biodistribution, and in consequence, the efficacy of this compound. The present study focuses on the development of a novel nanocarrier for the delivery of lipophilic drugs such as IVM and its potential application in antiparasitic control. Lipid nanocapsules (LNC) were prepared by a new phase inversion procedure and characterized in terms of size, surface potential, encapsulation efficiency, and physical stability. A complement activation assay (CH50) and uptake experiments by THP-1 macrophage cells were used to assess the stealth properties of this nanocarrier in vitro. Finally, a pharmacokinetics and biodistribution study was carried out as a proof of concept after subcutaneous (SC) injection in a rat model. The final IVM-LNC suspension displayed a narrow size distribution and an encapsulation rate higher than 90 % constant over the evaluated time (60 days). Through flow cytometry and blood permanence measurements, it was possible to confirm the ability of these particles to avoid the macrophage uptake. Moreover, the systemic disposition of IVM in the LNC administered by the SC route was higher ($p < 0.05$) (1367 ng h/ml) compared to treatment with a commercial formulation (CF) (1193 ng.h/ml), but no significant differences in the biodistribution pattern were found. In conclusion, this new carrier seems to be a promising therapeutic approach in antiparasitic control and to delay the appearance of resistance.

Keywords Drug delivery systems · Ivermectin · Lipid nanocapsules · Pharmacokinetic · Tissue distribution

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Introduction

Ivermectin (IVM) is a semi-synthetic avermectin broad-spectrum agent active against endoparasites and ectoparasites and widely used in veterinary and human medicine. This compound is commercially available as injectable and pour-on formulations for use in cattle and oral suspensions for small ruminants, dogs, and humans. IVM is a large and highly lipophilic molecule, relatively insoluble in water despite possessing two sugar rings and two hydroxyl groups (González Canga et al. 2009).

Pharmacokinetic disposition of IVM in different animal species may be affected by formulation design since the vehicle in which these compounds are formulated may influence their absorption and the resultant drug concentration profiles achieved in the bloodstream and at the target tissues of parasite location (Lo et al. 1985; Wicks et al. 1993; Lifschitz et al. 2000). After the introduction of the first approved IVM formulation (propylene glycol/glycerol formal 60:40), different pharmaceutical modifications have been assayed to extend its persistent endectocide activity in cattle since the duration of

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the antiparasitic effect has been considered a relevant attribute in parasite control programs in livestock.

These long-acting IVM 3.15 or 3.5 % preparations were introduced to the veterinary pharmaceutical market, and their pharmacokinetic behavior was evaluated (Lifschitz et al. 2007). These long-acting endectocide formulations are currently used for the advantage of persistent anthelmintic efficacy in strategic programs for controlling nematodes and ectoparasites. Nevertheless, the intensive use of these broad-spectrum antiparasitic compounds has led to the emergence of high levels of resistance mainly in nematodes of sheep and goats but also in gastrointestinal (GI) parasites of cattle (Kaplan 2004; Demeler et al. 2009). The traditional and long-acting injectable preparations are irritating after subcutaneous administration; this accounts for the switch to oral administration of IVM to dogs as tablets and chewable formulations (Eraslan et al. 2010).

This high level of resistance to IVM in ruminants and the limitations observed with the injectable formulations applied to other species have encouraged the search for strategies to optimize the enormous potential of IVM as an antiparasitic agent (Kaplan 2004; Yates and Wolstenholme 2004). The most promising approach for improving formulation lies in innovative delivery systems using carriers with defined physicochemical properties. Over the last 20 years, nanomedicines offer new insight for the improvement of existing management strategies with better treatment outcomes and cost effectiveness (Kawashima 2006; Petrak 2006; Teli et al. 2010) These carriers will increase both the solubility and stability of drugs which is critical for pharmaceutical systems intended for parenteral administration.

Among drug carrier systems, lipid nanocapsules (LNC) emerge as one of the most promising systems prepared according to an original phase-inversion method (PIM) avoiding the use of organic solvents, thus constituting an attractive alternative to liposomes (Heurtault et al. 2002). Structurally, the lipophilic drug is solubilized into the central lipid core, which is surrounded by a membrane of lecithins and pegylated hydroxystearate (HS-PEG) in order to provide stealth properties (Vonarbourg et al. 2006). They are also able to reduce toxicity and modify its pharmacokinetic parameters by prolonging circulation time and improving its tissue distribution. Besides, LNC have the potential to promote increased drug internalization by phagocytic cells of the mononuclear phagocytic system/reticulo-endocytosis system, which are reservoirs of intracellular parasites or co-localized in the infection site. This fact would lead to increased efficacy in peripheral infection-targeting sites (Lifschitz et al. 2000). LNC formulations have resulted in increased therapeutic efficacy for a variety of substances in such areas as cancer, (Lamprecht and Benoit 2006; Peltier et al. 2006) pain treatment, (Lamprecht et al. 2004), and radiopharmaceutical (Ballot et al. 2006), but they have not been fully investigated in the veterinary field (Pensel et al. 2015).

In the present work, the ability of these lipid nanocapsules to encapsulate IVM was assessed in order to obtain antiparasitic delivery systems compatible with systemic administration. The resulting nanocapsules were characterized in terms of size, zeta potential, and long-term stability and evaluated with respect to their pharmacokinetic behavior and biodistribution in rats in an attempt to gain insights into the possible role of the carrier on overall performance and its potential as an alternative in the anthelmintic therapy.

Materials and methods

Materials

IVM was purchased from Parafarm (Buenos Aires, Argentina). Labrafac WL® 1349 (caprylic-capric acid triglycerides) and Captex® 8000 (tricaprylin) were gifts from Gattefossé S.A. (Saint-Priest, France) and Abitec Corp (Columbus, OH, USA), respectively. Lipoid® S75-3 (soybean lecithin at 70 % of phosphatidylcholine and 10 % of phosphatidylethanolamine) and Solutol® HS 15 (mixture of free polyethylene glycol 660 and polyethylene glycol 660 hydroxystearate) were provided by Lipoid Gmbh (Ludwigshafen, Germany) and BASF (Ludwigshafen, Germany), respectively. Due to the complex composition of these products, they will henceforth be referred to by their brand names in the following text. Sodium chloride (NaCl) was obtained from Prolabo (Fontenay-sous-Bois, France) and water was from Milli RO System (Millipore®, Paris, France). Culture reagents and Nile Red were purchased from Lonza (Verviers, Belgium) and Sigma-Aldrich (Saint-Quentin Fallavier, France), respectively. All other chemical reagents were purchased from Fisher Scientific (Elancourt, France) and were of analytical grade.

LNC preparation

The overall study was made on 50-nm-diameter LNC prepared according to the previously described original process (Heurtault et al. 2002). Briefly, Solutol®, Lipoïd®, Labrafac® or Captex® 8000, NaCl, and water were mixed and heated, with magnetic stirring, to 90 °C. Three cycles of progressive heating and cooling between 90 and 60 °C were then performed and followed by an irreversible shock induced by dilution with 12.5 ml of 0 °C deionized water added to the mixture at 75 °C. For the formulation of IVM-LNC, the drug (at final concentrations of 0.29, 0.57, and 1.14 mg/g, respectively) was first solubilized in Labrafac® or Captex 8000®. Then, the other excipients were added to the mixture and the LNC were produced as described above. Finally, the nanoparticle suspensions were filtered by 0.22-μm filters (Sartorius®, Goettingen, Germany) to remove the aliquot of drug that

remained non-encapsulated and that precipitated as large crystals in the aqueous medium. The corresponding batches of fluorescent LNC were obtained by using a lipophilic marker (Nile Red), which was previously dissolved in acetone (0.6 %, *w*/*w*) and incorporated into the Labrafac phase (1:10, w/w) as described elsewhere (Garcion et al. 2006).

Size and zeta potential measurements

The size and surface charge distribution were analyzed by Photon Correlation Spectroscopy (PCS) using a Malvern Zetasizer Nano series DTS 1060 (Malvern Instruments S.A., Worcestershire, UK). The average hydrodynamic diameter, the polydispersity index (PI), and the zeta potential (ZP) were determined at 25 °C in triplicate. The nanoparticle suspensions were diluted (1:400) in distilled water to ensure a convenient scattered intensity on the detector.

Atomic force microscopy

Atomic force microscopy (AFM) was carried out in a Bruker Innova Atomic Force Microscope (Santa Barbara, USA) with an Autoprobe CP fitted with a 2-μm cantilever and a monocrystalline silicon tip Ultra-lever UL020 (Park Scientific Instrument, Geneva, Switzerland). Droplets of steady volume (20 μl of the final suspension) were deposited onto freshly cleaved mica. Then, the drop was dried and the contact mode was used at room temperature. The measurements were performed in triplicate in different sample locations.

Drug payload and encapsulation efficiency

IVM concentration was measured in the supernatant by HPLC in triplicate experiments, and the rate of encapsulation was calculated by considering the initial amount of drug added. The setup conditions were as follows: Waters 717plus Autosampler, Waters 600 Controller, and Waters 2487 Dual Absorbance Spectrometer (Waters® SA, Saint-Quentin-en-Yvelines, France), with a ZORBAX Eclipse XDB C18 Rapid Resolution column (4.6 × 150 mm, 3.5 μm), were used. The UV detector was set at 245 nm. The mobile phase consisted of acetonitrile/methanol/water mixture (64:30:6 *v*/*v*/*v*) at a flow rate of 1 ml/min. The assays were performed in triplicate, and the mean ± SD of IVM loading was calculated (mg of IVM/g of LNC dispersion).

Stability studies

The stability of IVM-loaded LNC dispersion was evaluated after storage at 4–6 °C for 2 months. The particle size distribution, ZP, and drug payload of samples were determined after filtration using a Minisart 0.22-μm filter (Sartorius®, Goettingen, Germany).

Measurement of complement activation: CH50 assay

Complement consumption was assessed in normal human serum (NHS) (provided by the Etablissement Francais du Sang, CHU, Angers, France) by measuring the residual hemolytic capacity of the complement system after contact with different particles (Kazatchkine and Carreno 1988). The technique consisted in determining the amount of serum able to lyse 50 % of a fixed number of sensitized sheep erythrocytes with rabbit anti-sheep erythrocyte antibodies (CH50), according to the procedure described elsewhere (Vonarbourg et al. 2006). Complement activation was expressed as a function of the surface area in order to compare particles with different mean diameters. Nanoparticle surface areas were calculated using the equation $S = n4\pi r^2$ and $V = n(4/3)(\pi r^3)$ leading to $S = 3\ m/r\rho$ where S is the surface area (cm^2) and V the volume (cm^3) of n spherical beads of average radius r (cm), m the weight (μg), and ρ the volumetric mass ($\mu g/cm^3$) (Passirani et al. 1998). All experiments were performed in triplicate, and a *t* test of non-matched samples was used to test for the statistical significance of the results.

Macrophage uptake evaluation

THP-1 cells (human monocyte/macrophage cell line obtained by ATCC, Manassas, VA, USA) were grown in suspension at 5 % CO_2 and 37 °C in ATCC medium. The experiment was performed on differentiated cells, and the differentiation was induced in the same medium by adding 200 mM of phorbol 12-myristate 13-acetate (PMA, Sigma, Saint-Quentin Fallavier, France) for 24 h to allow their adherence (Tsuchiya et al. 1982). Cells (0.6×10^6/ml) were grown in 24-well cell culture plates for 24 h at 37 and 4 °C. After 24 h, the suspensions of Nile Red-loaded (fluorescent) LNC were deposited on the cell monolayer and incubated for 90 min at either temperature. The amount of phagocytized nanoparticles was determined quantitatively by a fluorescence-activated cell sorter (FACS).

In vivo experiments

Experimental animals, treatment, and sampling

Thirty female Wistar rats weighing 250 to 350 g were used in this experiment. The management of experimental animals was performed in accordance with institutional and internationally accepted welfare guidelines (Canadian Council on Animal Care 1980; American Veterinary Medical Association 2001, EC Directive 86/609/EEC for animal experiments). The animals were kept under controlled conditions of temperature and cycles of darkness/light. Animals in group A received commercial formulation (CF) of IVM at 200 μg/kg (Ivomec®; Merial, Argentina) by the subcutaneous route (SC). Rats in group B

received ivermectin-loaded LNC dispersions (IVM-LNC) by SC at the same dosage.

Under anaesthesia (ketamine hydrochloride 100 mg/kg and ether purchase from Holliday-Scott, Buenos Aires, Argentina), animals from each experimental group were sacrificed at 6, 12, 24, 48, and 96 h post-treatment. The animals were also observed daily, and reduced mobility and significant weight loss (20 %) were considered as the limit point for tolerance determinations.

The extraction of IVM from plasma, liver, lungs, and intestinal wall was carried out following the technique described earlier (Alvinerie et al. 1993). Samples (0.5 ml or g) were fortified with 40 ng/ml of abamectin (used as internal standard) and 0.25 ml of acetonitrile, plus 1.25 ml of deionized water. The preparation was mixed (Multi-Tube Vortexer; VWR Scientific Products, West Chester, PA, USA) for 15 min, sonicated in an ultrasonic bath for 10 min (Transsonic 570/H; Laboratory Line Instruments Inc., Melrose Park, IL, USA), and the solvent-sample mixture was centrifuged at 2000*g* for 10 min. The supernatant was manually transferred into a tube, and the procedure was repeated one more time for tissue samples.

The supernatant was applied to a conditioned Supelclean LC-18 Cartridge (Supelco, Bellefonte, PA). After washing with 1 ml of deionized water followed by 1 ml of water/methanol (4:1 *v*/*v*), the cartridges were dried for 5 min and the sample was eluted with 1.5 ml of methanol, which was collected. After solid phase extraction, the eluate was evaporated to dryness under a gentle stream of dry nitrogen at 60 °C in a water bath. The sample was subjected to a derivatization described elsewhere (De Montigny et al. 1990). After completion of the reaction, an aliquot (100 μl) of each sample was injected directly into the chromatographic system. The IVM concentrations were determined by HPLC using a Shimadzu 10A HPLC system with autosampler (Shimadzu Corporation, Kyoto, Japan). HPLC analysis was undertaken using a reverse phase C_{18} column (Kromasil, Eka Chemicals, Bohus, Sweden, 5 μm, 4.6 mm × 250 mm) and an acetic acid 0.2 % in water/methanol/acetonitrile (0.5/60/39.5) mobile phase at a flow rate of 1.5 ml/min at 30 C. IVM was detected with a fluorescence detector (Shimadzu, RF-10 Spectrofluorometric Detector, Kyoto, Japan), reading at 365 nm (excitation) and 475 nm (emission wavelength). IVM concentrations were determined by the internal standard method using the Class LC 10 Software version 1.2 (Shimadzu Corporation, Kyoto, Japan). The peak area ratios were considered to calculate drug concentrations in spiked (validation) and experimental plasma samples. There was no interference of endogenous compounds in the chromatographic determinations. The solvents (Baker, Phillipsburg, NJ, USA) used during the extraction and drug analysis were of HPLC grade.

Pharmacokinetic and statistical analyses

The concentration versus time curves obtained for each fluid and tissue analyzed were fitted using the PK Solutions 2.0 computer software (Summit Research Services, Ashland, OH, USA). Pharmacokinetic parameters were determined using a non-compartmental method. Peak concentration (C_{max}) was read from the plotted concentration-time curves. The area under the concentration-time curve (AUC) was calculated by the trapezoidal rule (Gibaldi and Perrier 1982). Mean pharmacokinetic parameters for IVM obtained after its administration, alone or loaded into LNC, were statistically compared using Student's *t* test. The assumption that the data obtained after treatments have the same variance was assessed. A log transformation was used where significant differences among standard deviations were observed. The statistical analysis was performed using the InStat 3.0 Software (Graph Pad Software, CA, USA). A value of $P < 0.05$ was considered statistically significant.

Results

Formulation and characterization of IVM-LNC

In order to evaluate the feasibility for IVM incorporation into the lipidic core of LNC, we previously checked that IVM was soluble enough in the selected lipids (Labrafac® and Captex® 8000), which suggested that this drug could be formulated directly using the regular preparation process (data not shown). The PIM described by Heurtault et al. (2002) was easily reproduced, and the physicochemical characteristics of LNC are summarized in Table 1. Thus, all LNCs presented a monomodal size distribution (50–55 nm) and a low PI (≤0.2).

Moreover, the presence of IVM at different concentrations in the lipid affected neither the efficiency of the method nor the physico-chemical characteristics of the final suspension. From this point of view, any of the studied lipids could be used as oil core, although their influence on IVM-LNC biopharmaceutical behavior exceeded the scope of this work and should

Table 1 Particle size, polydispersity index (PI), zeta potential (ZP), and encapsulation efficiency (EE%) of different lipid core and IVM loaded LNC

	Particle size [nm]	PI	ZP [mV]	EE [%]
IVM 0.29 mg/ml				
Labrafac®	53.1 ± 0.4	0.039	−17.8 ± 6.2	98 ± 1 %
Captex® 8000	54.2 ± 0.2	0.049	−19.9 ± 3.8	96 ± 3 %
IVM 0.57 mg/ml				
Labrafac®	55.4 ± 1.5	0.075	−15.8 ± 6.8	95 ± 4 %
Captex ® 8000	54.8 ± 1.8	0.052	−16.7 ± 3.1	90 ± 4 %
IVM 1.14 mg/ml				
Labrafac®	52.4 ± 1.1	0.048	−15.8 ± 4.0	93 ± 3 %
Captex® 8000	53.9 ± 1.1	0.048	−15.3 ± 1.6	92 ± 4 %

be further evaluated. Regarding this, we choose the IVM-LNC with Labrafac® as oil core for further investigations.

Figure 1 shows the resulting AFM images obtained from a suspension of LNC with fixed doses of IVM. The diameter observed in the images differed slightly from the values of PCS size determinations, since LNC flattened during the drying step in sample preparation. This observation seems to occur frequently in the study of LNC by this technique (Lamprecht et al. 2004).

Table 2 Stability of IVM-LNC. Mean size, polydispersity index (PI), zeta potential (ZP), and drug encapsulation efficiency (EE%) after 60 days storage at 4 °C

	Particle size [nm]	PI	ZP [mV]	EE [%]
Blank LNC	57.1 ± 2.8	0.063	−10.9 ± 0.9	–
IVM 0.29 mg/ml	55.6 ± 0.6	0.048	−13.3 ± 3.1	94 ± 4 %
IVM 0.57 mg/ml	56.5 ± 1.5	0.066	−13.47 ± 2.7	91 ± 4 %
IVM 1.14 mg/ml	55.1 ± 1	0.081	−12.17 ± 0.9	90 ± 2 %

Physical stability

The stability of IVM-LNC was assessed upon storage at 4–6 °C for up to 8 weeks. The parameters determined at different time points were particle size, PI, ZP, and encapsulation efficiency (EE%). Similar in all batches, LNC had a low PI (0.04-0.08) and showed a monomodal particle size distribution, which accounts for its homogeneity, and qualified the carrier system for intravenous administration while the negative ZP is critical for cellular uptake. In addition, the EE% was higher than 90 % and did not change over time, another indication of the physical stability of the systems (Table 2).

Complement system activation and macrophage uptake

The CH50 consumption was expressed as a function of the nanoparticle surface area (cm^2) representing an increase in nanocapsule concentration. The results are depicted in Fig. 2.

In this test, the classical complement pathway of NHS is activated after the contact with sensitized sheep erythrocytes, leading to the lysis of erythrocytes and hemoglobin release. When human serum is in the presence of activating nanoparticles, less complement proteins are available in the serum to lyse the sheep erythrocytes, causing a reduction of CH50 units (Vonarbourg et al. 2006).

Both uncharged and loaded nanoparticles caused the activation of complement proteins, up to a value of 35 % at 1400 cm^2/ml, despite the small sizes (50 nm) and low zeta potential of these carriers. In a previous study (Passirani et al. 1998), the 50-nm LNC determined a low activation of complement proteins at low surface values (until 1000 cm^2/ml) demonstrating that the activation of complement proteins by LNC is size-dependent and the presence of a PEG layer on the LNC surface does not change the behavior of nanoparticles. Here, it was also observed that the inclusion of IVM in the oil core practically did not influence the activator effect of the nanoparticles. Indeed, until 1000 cm^2/ml, the CH50 consumption of IVM-LNC compared to blank LNC is lower than 10 %, which may be considered not significant. At higher LNC concentrations (surface area >1000 cm^2/ml), this difference is practically negligible.

To confirm the CH50 test data, parallel in vitro studies on THP-1 cells were performed, using Nile Red-loaded (fluorescent) nanocarriers. The results are reported in Fig. 3 and were expressed as the percentage of fluorescence after FACS analysis. Regarding a control represented by cells alone, none of the LNC formulations showed any significant difference in terms of macrophage uptake. The fluorescence level recorded was only 5 %, meaning that LNC loaded with different concentrations of IVM were not internalized after contact with THP-1 cells (Fig. 4).

Experiments were also conducted at 37 °C (Fig. 3). In this case, the internalization of all LNC was minimal, with only small differences among different nanoparticles. The slight increase of observed fluorescence could be due to interactions of the systems with the cell membrane as reported previously (Basilea et al. 2012).

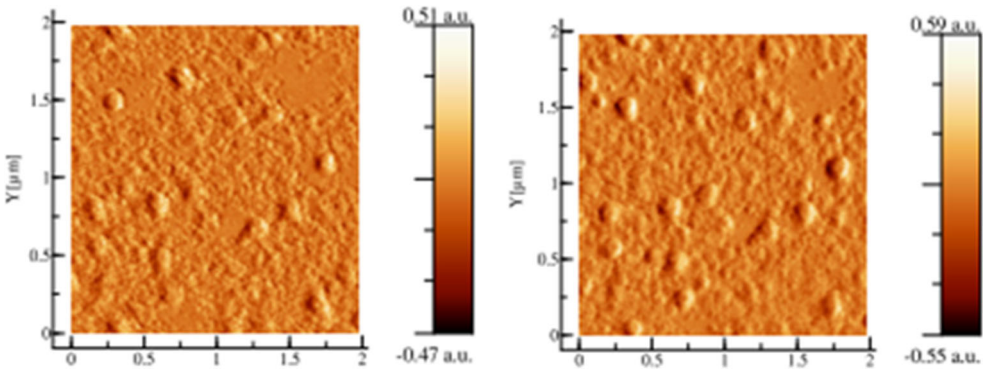

Fig. 1 AFM photographs of IVM-LNC deposited and dried on mica plate and observed by the contact mode. *Left*, IVM-LNC 2 µm TOPO BKW 2 µm × 2 µm. *Right*, IVM-LNC 2 µm TOPO FRW 2 µm × 2 µm

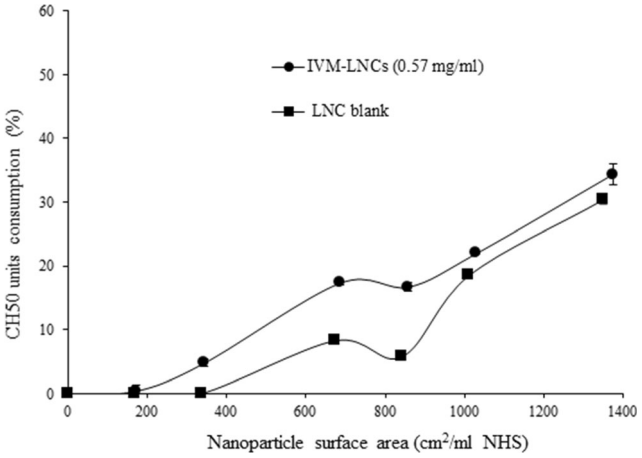

Fig. 2 Influence of IVM load LNC on complement consumption variation (0.57 mg/ml final IVM concentration)

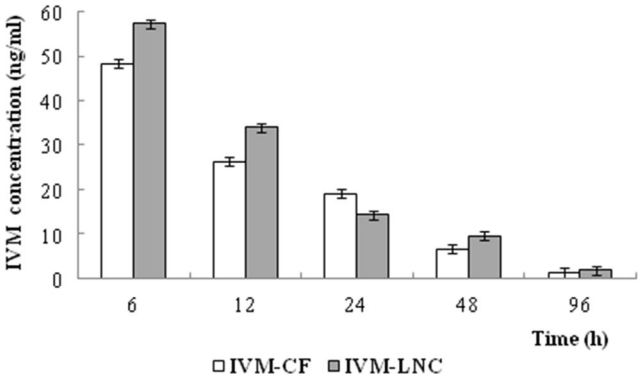

Fig. 4 Comparative mean (±SD) plasma concentration profiles ($n = 3$) for ivermectin (IVM), obtained after the subcutaneous administration of IVM (0.2 mg/kg), in a commercial formulation (IVM-CF) and load into LNC (IVM-LNC) to rats

Pharmacokinetics and biodistribution study

The concentration of the active compound was within the expected range for all formulations. Moreover, IVM was detected at the first sampling time, 6 h post-treatment in the plasma of both groups. However, C_{max} (57.3 ± 5.77 ng/ml) values reflected a faster process of drug absorption compared to Ivomec®, which had a lower value of C_{max} (48.4 ± 4.17 ng/ml) as shown in Table 3. The plasma concentrations decreased progressively thereafter during the elimination phase as characterized by a similar half-life of elimination values for both treated groups. In this study, statistically different AUC (1367 ± 63 ng day/ml) and a tendency to higher mean residence time (MRT) (20.3 ± 1.3 h) of IVM load into LNC evidenced longer drug persistence and greater absorption from the site of injection compared to the CF (1194 ± 37 ng day/ml and 18.8 ± 3.3 h, respectively).

No clinical signs of local or systemic drug intolerance were detected during the period of observation after the administration of IVM in both groups of animals. The amount of drug reaching the target parasite is influenced by the drug concentration in the tissue where the parasite is located (Basilea et al. 2012). The broad IVM distribution matches with the high concentration of this molecule found in the GI tract mainly in the wall of small intestine, lungs, and liver (Fig. 5 and Table 4). Besides, taking into account the ratio *AUC tissue* (IVM-LNC)/*AUC plasma* (IVM-LNC) for the three tissues of interest, the values are between 4.5 and 6.5, which clearly demonstrated the accumulation of this molecule in each tissue. As observed in plasma, IVM concentrations tended to be higher in target tissues after the administration of LNC. Mean IVM concentrations in lungs were 53.2 ng/g (IVM-LNC) and 39.5 (CF) after 48 h post-treatment. In the liver, the IVM concentrations measured at 96 h after the administration of IVM-LNC were two fold higher ($P < 0.05$) compared to the CF. These results corroborated that IVM-LNC formulation is as efficient as the commercial one to reach a high and clinical relevant concentration of the drug in the principal tissues, where the parasites are located.

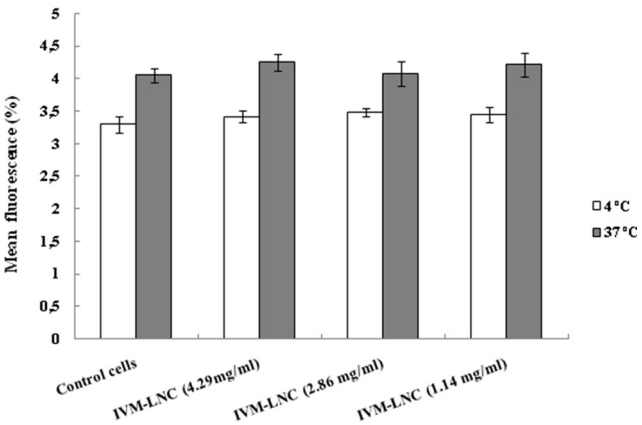

Fig. 3 Macrophage uptake of different formulations of LNC after 90 min incubation with THP-1 cells at 4 and at 37 °C

Table 3 Mean pharmacokinetic parameters for IVM obtained after the administration to rats (200 μg/kg) load into lipid nanocapsules (IVM-LNC) and commercial formulation (IVM-CF)

Parameters	IVM-LNC	IVM-CF
C_{max} (ng/ml)	57.3 ± 5.7[a]	48.4 ± 4.2[a]
AUC $_{total}$ (ng h/ml)	1367 ± 63[a]	1194 ± 37[c]
$T_{1/2\ el}$ (h)	20.3 ± 1.3	18.8 ± 3.3
MRT (h)	30.0 ± 2.2	27.6 ± 4.9

Data values are expressed as mean ± SD ($n = 3$). Values lacking a common letter are significantly different at $P < 0.05$

C_{max} Peak plasma concentration, *AUC* total area under the concentration vs time curve extrapolated to infinite, $T_{1/2el}$ elimination half time, *MRT* mean residence time

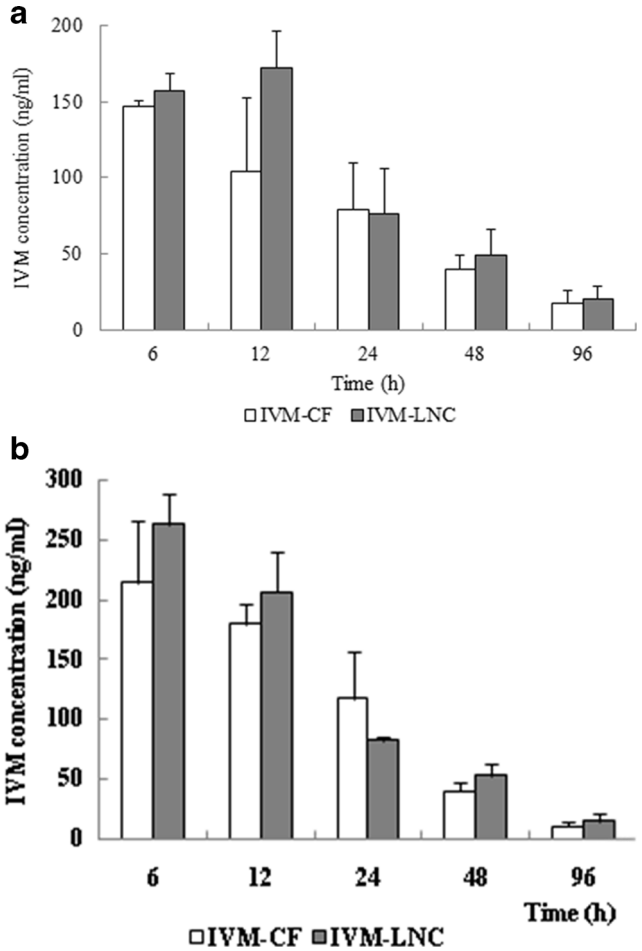

Fig. 5 Comparative mean (±SD) ($n = 3$) of ivermectin (IVM) concentrations in **a** small intestine wall; **b** lungs; and **c** liver; measured after their subcutaneous (SC) administration (0.2 mg/kg) to rats

Discussion

A primary goal of modern antiparasitic chemotherapy is to specifically target drugs to the respective pathogen while minimizing side effects to the host by finding and adapting innovative and "intelligent" formulations to enhance the anthelmintic efficacy (Bassissi et al. 2006). Pharmaceutical technology has been applied to develop different drug formulations and delivery systems to optimize the pharmacological potency of IVM and other macrocyclic lactones currently available (Hennessy 1997). The most promising approach for improving formulation lies in innovative delivery systems using carriers with defined physicochemical properties. In the present work, the LNC have been prepared according to an original method and they are stable for at least 2 months in suspension ready for injection, which should reduce the cost and convenience for treatment considerably.

The photon correlation spectroscopy measurements demonstrated that all the batches, either blank or loaded with IVM, were formed by weakly negative nanoparticles with a mean size between 54 and 64 nm, in good accordance with the previously reported results (Heurtault et al. 2002). The system was constituted by an oily liquid core corresponding to free triglycerides, which was surrounded by a tensioactive rigid shell made from a mixture of surfactants (i.e., Lipoid® and Solutol®) (Heurtault et al. 2002; Huynh et al. 2009). In this way, the lipid environment inside the LNC would solubilize drugs with low aqueous solubility such as IVM. Since PIM proposes the encapsulation of lipid oil in which the drug is previously dissolved, the high IVM encapsulation efficiency (≥90 %) was expected and has been already reported for other aqueous insoluble drugs (Garcion et al. 2006).

Colloidal drug carriers are rapidly removed from systemic circulation after intravenous injection due to their recognition as foreign bodies by the mononuclear phagocytic system (MPS), especially by Kupffer cells in the liver and macrophages in the spleen and bone marrow (Moghimi et al. 2001). This recognition is enhanced by the opsonization of the complement system by plasma proteins (Passirani and Benoit 2005). The consumption of CH50 units was measured at a fixed level of human serum in the presence of an increasing surface area of particles. In our study, according to the ratio host weight/doses, the administered amount of LNC would be usually low enough so that the corresponding particle surface will be lower than 10 cm^2/ml, where the CH50 unit consumption would

Table 4 Pharmacokinetic parameters (mean ± SD) for ivermectin (IVM) obtained after the subcutaneous administration (0.2 mg/kg) in a commercial formulation (IVM-CF) or loaded into lipid nanocapsules (IVM-LNC)

	Liver		Small intestine wall		Lungs	
	IVM-FC	IVM-LNC	IVM-FC	IVM-LNC	IVM-FC	IVM-LNC
C_{max}(ng/ml)	163.2 ± 58.4	195.0 ± 19.9	149.5 ± 9.2	180.48 ± 10.2[a]	214.1 ± 51.2	262.7 ± 25.7
$T_{1/2\ el}$(h)	21.0 ± 1.8	24.1 ± 1.3[a]	31.8 ± 8.9	28.81 ± 3.4	19.9 ± 1.6	22.3 ± 2.9
MRT (h)	31.6 ± 3.8	35.9 ± 2.2	47.1 ± 13.8	43.72 ± 5.7	29.4 ± 1.9	33.3 ± 4.1
AUC (ng h/ml)	4821 ± 732	6025 ± 841[a]	5980 ± 1063	6961 ± 681	6967 ± 998	7664 ± 460

Cmax peak plasma concentration, *AUC* area under the concentration versus time curve extrapolated to infinity, *T1/2el* elimination half-life.

[a] Differences with IVM-FC are statistically different at $P < 0.05$

be practically negligible. This observation allows us to infer that IVM-LNCs are not recognized by the CS with an extended circulation time, which was also corroborated through macrophage uptake assays. Thus, as reported previously, (Mosqueira et al. 2001a) the high density of PEG at the LNC surface, PEG flexibility linked to the curvature radius, and PEG length seems to play an important role in macrophage uptake.

When developing a combined formulation, the pharmacokinetic characteristics of one active ingredient could be affected by the other ingredients, the vehicle or the excipients. For example, among the different subcutaneous drug formulations given to cattle, the lowest (33 %) bioavailability was observed using a propylene glycol/glycerol-formal vehicle (60:40 *v*/*v*) compared with 41 % with an aqueous glycerol-formal vehicle (50:50 *v*/*v*) and 55 % with an aqueous vehicle (Lo et al. 1985).

The pharmacokinetics of IVM has been studied in several animal species due to its wide use and proven efficacy. Since the antiparasitic activity of these molecules depends on drug concentrations and exposure time of the parasite, the evaluation of the comparative pharmacokinetic profiles may help to estimate drug efficacy (Lanusse et al. 1997). It is known that the bioavailability of IVM is affected by the route of administration and its formulation; the greatest bioavailability is achieved by subcutaneous injection, especially in large animals. These types of formulations could be evaluated in the future to be used in pets and in humans. In this context, it is necessary to assess new injectable formulations of IVM that avoid the irritant vehicles included in the traditional preparations and improve the drug concentration profiles. Therefore, the pharmacokinetic study in rats was carried out here as a proof of concept to investigate the potential use of this novel strategy in anthelmintic therapy.

While a commercial injectable solution containing 1 % of IVM in glycerol formal and propylene glycol (40:60) was used in group A, a lipid-based formulation of IVM containing a mix of partial triglyceride surfactants and water in adequate proportions was administered to rats in group B. Whether the PEG chains from the surfactants in the LNC formulation could have some influence on the stealth properties of the carrier (Moghimi et al. 2001; Mosqueira et al. 2001b) is not possible to elucidate with the results of the present study. Nevertheless, the differences observed in $T_{1/2el}$ explain the significantly higher values of AUC for the group treated with LNC, in comparison with those of the CF group. The longer MRT for LNC could also be related to its longer distribution half-life and the longer half-life of elimination, but this last point should be confirmed.

Traditionally, the pharmacokinetic studies have been focused on drug concentration profiles obtained from the bloodstream. However, many compounds exert their effects on well-defined organs or no-vascular target tissues, which could be accessed after its distribution from the central blood compartment. Moreover, the helminthic parasites live on the surface of the mucosa of the GI tract, bronchopulmonary, or intracellular bile ducts (Lifschitz et al. 2000). Thus, the drug must be released from its formulation, absorbed, and transported by the circulation to the parasite locations. For this reason, the three tissues selected for the biodistribution studies were lungs, small intestine wall, and liver.

Ivermectin has a high volume of distribution due to its highly lipophilic property, so it tends to accumulate in fat tissue which acts as a drug depot. The drug may be readily distributed to other tissues because high drug concentrations were detected in the liver, kidney, and muscle of cattle (Chiu et al. 1990); in lungs, skin, and earwax of pigs (Scott and McKellar 1992); and in skin and hair of goats. In these tissues, the drug persisted for 17 days after treatment (Lespine et al. 2005). In the present study, a new type of nanocarrier-encapsulating IVM was assessed to study their influence on its biodistribution to the principal tissues of parasitic localization. The concentrations of IVM obtained in the current work after the administration of the LNC formulation tended to be higher in target tissues. Thus, the use of this strategy could represent a promising tool to improve drug efficacy by favoring its incorporation into the parasite membrane or allowing a better access to the target tissues. Nevertheless, in vitro and in vivo investigations should be carried out in order to demonstrate the ability of LNC to improve IVM efficacy.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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