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RESEARCH PAPER

Tissue Tolerance of Diclofenac Sodium Encapsulated in Liposomes After Intramuscular Administration

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ABSTRACT

In this work the effect of the encapsulation of diclofenac sodium within liposomes on the reduction of the myotoxicity after intramuscular administration in rats was studied. Diclofenac sodium was encapsulated in small unilamellar liposomes obtained from phosphatidylcholine, cholesterol, and α-tocopherol (40:10:0.04 mM), and administered by intramuscular injection in the quadriceps femoral muscle of male Wistar rats. After a single dose of 0.2 mg diclofenac formulations the local tissue damage was assessed by plasma creatine kinase (CPK) activity and histological analysis. It was demonstrated that formulations containing free diclofenac produced a higher increase in CPK activity, while those encapsulated in liposomes exhibited CPK activity similar to the control groups. Histopathological analysis of local muscle tissue performed on the third and seventh days following the injection showed intense cellular damage when free drug solution was used, while encapsulation in liposome protected the tissue against the local tissue inflammation.

Key Words: *Creatine kinase; Diclofenac sodium; Liposomes; Myotoxicity*

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INTRODUCTION

Liposomes have been extensively investigated as drug delivery systems due to their ability to increase the therapeutic index and reduce drug side effects (1–4). Liposome systems are considered highly suitable formulations given their effects of promoting sustained release (5,6), reducing drug toxicity derived from systemic absorption (7–10), and drug targeting (11–13).

Non-steroidal anti-inflammatory drugs (NSAIDs) may cause a large variety of side effects. When NSAIDs are encapsulated in liposomes, a better pharmacological activity can be achieved after either local or systemic administration (14).

Diclofenac sodium (DS) is one of the most widely used NSAIDs, and is the most suitable agent for the treatment of rheumatic conditions (15). However, NSAIDs show a high incidence of side effects. The incidence of side effects ranges from 12% to 25% and includes predominantly gastrointestinal disorders, although more severe side effects are not unusual (16).

Intramuscular (IM) injections of NSAIDs, including diclofenac sodium, can also cause adverse reactions (17). These side effects may vary from a small cutaneous lesion to large areas of tissue necrosis (18–21).

Evaluation of local toxicity following an IM injection represents an important criterion when developing parenteral formulations (22). Toxicity of a formulation depends on several parameters, such as pH, osmotic pressure, vehicle, adjutants used, and intrinsic toxicity of the drug.

Liposomes obtained from phospholipids are highly biocompatible and able to decrease the interaction between encapsulated drugs and the surrounding tissue, reducing the degree of muscle tissue damage after intramuscular injections (14,20,23,24).

Since an increase in creatine kinase (CPK) activity is usually related to muscle damage at the location of injections (25), the enzymatic determination method can be used to evaluate the local drug toxicity (26). In addition, histological changes at muscle tissue can also be related to the development of myotoxicity. These evaluations are performed by injecting the drug intramuscularly into laboratory animals and subsequently analyzing plasma samples for CPK, or the muscle tissue where the product was injected (27).

Since the liposomes prepared from phospholipids are highly biocompatible with skeletal muscle, this structure can be used as a depot form of drug, reducing the toxicity for local drug administration.

In this work we describe how to obtain small unilamellar liposomes containing diclofenac sodium. The effect of protection of diclofenac-load liposome against muscle tissue damage caused by drug was assessed through the measurements of plasma CPK activity and the histological analysis of muscle tissues of rats, extracted from injection sites.

MATERIALS AND METHODS

Drugs and Reagents

Diclofenac sodium was a gift from CIBA-Novartis, Brazil. Soya phosphatidylcholine (Epikuron 200) was supplied by Lucas Meyer, Hamburg, Germany. Cholesterol was purchased from Avanti Lipids (USA); α-tocopherol (Sigma Chemical Co., St. Louis, MO); Sephadex G-25 medium (Pharmacia Biotech AB, Uppsala, Sweden); Tris–hydroximethyl-aminomethane (Merck SA, Brazil); diclofenac sodium commercial injection solution (Voltarem®, CIBA-Novartis, Brazil); creatine kinase quantitative colorimetric kit (Merck SA, Brazil). All other reagents were of analytical grade.

Preparation of Liposomes

Liposomes containing phosphatidylcholine (PC), cholesterol, and α-tocopherol (molar ratio 40:10:0.04) were prepared in Tris–HCl buffer 10 mM, pH 7.2. Phosphatidylcholine, cholesterol, and α-tocopherol were dissolved in chloroform and placed in a round-bottomed glass tube. α-Tocopherol was included in the formulation as a lipid antioxidant. Diclofenac sodium (30 mM) was incorporated into the liposomes, dissolved in ethanol, and added to the chloroform solution. The organic solvent was removed under a N_2 stream in order to obtain a lipid film and then dried under vacuum for 2 hr. The lipid film with and without drug was hydrated with Tris–HCl buffer during 30 min and dispersed by vortex stirring. Small unilamellar vesicles (SUVs) were obtained by sonication of the lipid dispersion. Non-encapsulated diclofenac was separated from load-liposomes by size exclusion chromatography using a Sephadex G-25 medium column.

Quantitative Analysis of Diclofenac Sodium

Diclofenac sodium was assayed quantitatively by high-performance liquid chromatography (HPLC). Chromatographic analysis was carried out in a Shimadzu SPD-AV UV–Vis chromatography system, with LC9-A pump, SLC-6B system controller, C-R6A chromatopac integrator, CTO-6A column oven, and DGU-2A helium degassing unit. The Shim-pack CLC-SIL column (4.6 mm i.d.× 25 cm stainless column) packed with adsorption silica support (5 μm particle diameter and 100 Å pore diameter) was used. The mobile phase *n*-hexane/*n*-propanol (1:4) was previously filtered through a 0.22-μm polycarbonate membrane and degassed. The temperature of the column was set at 30°C and the detection was performed at 272 nm. The flow rate of the mobile phase was 1.0 mL/min.

Determination of Diameter by Light Scattering

Liposome diameters were determined with and without diclofenac sodium. All samples were filtered with a 0.22-μm polycarbonate membrane in order to ensure dust-free samples and diluted to 1:3 and 1:6 using 10 mM Tris-HCl buffer pH 7.2. The liposome size distribution was determined by dynamic laser light scattering using a Brookhaven apparatus with BI-9000 particle sizing software from Brookhaven. The samples were analyzed at 90°. As light source was applied a 35 mW Spectra Physics He–Ne laser, model 127, $\lambda_0 = 632.8$ nm.

Tissue Tolerance

Tissue tolerance experiments were performed using Wistar male rats, weighing approximately 200 g. Animals were randomly divided into groups of six rats, and maintained during 48 hr in an ambient atmosphere with controlled temperature at 22°C, light cycle of 12 hr, and with free access to water and standard food. Each group was treated as in Table 1.

All tested formulations were sterilized by filtration using a cellulose acetate membrane 0.22 μm before injections. Intramuscular injections were administered in each quadriceps femoris muscle, using a hypodermic needle ($8 \times 0.3\ mm^2$). The drug was administered as aqueous solution or liposomes dispersion. Empty liposomes and Tris–HCl buffer 10 mM, pH 7.2 were used as controls.

Plasma Creatine Kinase Activity

The creatine kinase activity was determined using a quantitative colorimetric procedure from Merck SA, Brazil. The basal level of enzymatic activity was measured for animals from group A, without treatment. Animals from groups B to F were submitted to the same treatment protocol. Approximately 0.5 mL of blood was withdrawn from each animal, from the tail vein under light anesthesia, at 0, 1, 2, 3, 6, and 24 hr after injection. The blood was transferred to tubes containing heparin and centrifuged for 5 min at 3000***g*** to obtain plasma. The creatine kinase activity was measured and the area under the curve (AUC) was determined using a trapezoidal framework without subtracting the basal CPK activity from experimental data.

Histological Evaluation

Seventy-two hours after injection, three animals from each group were anesthetized and the femoral muscle was removed by biopsy. Another three animals from each group were submitted to the same biopsy procedure on the seventh day following injection. Isolated muscle tissues were maintained in 10% formaldehyde. The muscle of the injection site was included in silicon molds. Ultra-fine (60–70 nm)

Table 1

Animal Groups Used in the Experiments

Group A	Animals without treatment
Group B	Animals treated with 1 mL of 10 mM Tris–HCl buffer, pH 7.2
Group C	Animals treated with 1 mL of empty liposomes
Group D	Animals treated with 1 mL of 0.2 mg/mL DS buffered solution
Group E	Animals treated with 1 mL of 0.2 mg/mL DS-load liposomes
Group F	Animals treated with 1 mL of 0.2 mg/mL commercial DS solution (Voltarem)

sections were cut and stained with hematoxilin-eosin-safran for histological analysis.

RESULTS AND DISCUSSION

The results of the liposome diameters with and without diclofenac sodium are presented in Tables 2 and 3. A small polydispersability coefficient was obtained, indicating that both liposome populations exhibited a homogeneous size distribution. The mean of the empty liposome diameters was 56.9 nm, while those encapsulating DS had a mean diameter of 39.5 nm, indicating that probably a small fraction of the drug was incorporated into the bilayer structure. Since the liposomes will be used as a drug depot form in the muscle, the smaller possible structure was chosen to prevent mechanical tissue damage in the locality of administration.

Table 2

Determination of the Diameter of Empty Liposomes by Light Scattering

Diameter (nm)	Diffusion Coefficient (cm^2/sec)	Polydispersability
57.0	5.94×10^{-8}	0.29±1.3%
56.6	5.76×10^{-8}	0.32±1.2%
58.2	5.78×10^{-8}	0.30±2.0%
56.1	5.70×10^{-8}	0.33±1.0%
56.4	5.74×10^{-8}	0.31±1.2%
56.5	5.75×10^{-8}	0.32±1.3%
Mean 57.8	5.78×10^{-8}	0.31±1.3%

Table 3

Determination of the Diameter of DS-Load Liposomes by Light Scattering

Diameter (nm)	Diffusion Coefficient (cm^2/sec)	Polydispersability
40.0	7.84×10^{-8}	0.30±2.2%
40.1	7.91×10^{-8}	0.30±2.3%
38.7	8.41×10^{-8}	0.28±1.9%
39.7	7.71×10^{-8}	0.30±1.9%
39.1	8.16×10^{-8}	0.29±1.9%
Mean 39.5	8.00×10^{-8}	0.29±2.0%

Liposomes have been used as drug carriers for about 30 years, and several researchers have demonstrated their ability to improve the therapeutic index of several drugs (28–32). One important advantage of liposomes is the complete biocompatibility of their structural components with the biological environment. This compatibility results from the similarity between liposome structure and biological membranes.

Although not frequently, NSAIDs are also administered intramuscularly. The IM route represents some advantages when compared to the oral route; particularly by avoiding first-pass metabolism and promoting a faster initiation of the therapeutic effect. However, the literature has shown that the administration of DS intramuscularly may cause muscle cell damage that varies from local pain to severe necrosis (20).

Basal plasma CPK activity determined for animals without treatment (group A) showed the mean value of 955 IU/L (±274). The mean plasma CPK activities determined for the formulations studied in this work (Table 1) are presented in Fig. 1. The means of AUCs (±SD) of the data in Fig. 1 are illustrated in Fig. 2, and the corresponding statistical analysis presented in Table 4.

All groups of treated animals exhibited an increase in the CPK activity, with a maximum value at about the first hour, and after 24 hr the levels of CPK activity decreased to values similar to the control (Fig. 1). The maximum CPK activity at about 1 hr was found previously with free diclofenac and diclofenac-load nanocapsules of poly(D,L-lactide) acid polymer (33,34). O aumento em kinase de creatine em ratos previamente foi descrito e atribuiu a mecanismo não elucidado (35). On the other hand, the release of CPK can be related not only to necrotic muscle fibers, but also to cells undergoing slight and reversible damage (26). Olling et al. (36) affirm that tissue damage can result from either the presence of the drug or the solvents of the formulation.

In our experiments, the injection of aqueous dispersion of empty liposomes did not provoke significant CPK release over buffer solution (Fig. 2). However, after the administration of an aqueous buffered DS solution a statistically significant increase in the CPK activity was achieved. A smaller increase in CPK activity was obtained with the injection of DS-load liposomes, but not statistically significant from controls of buffer solution and

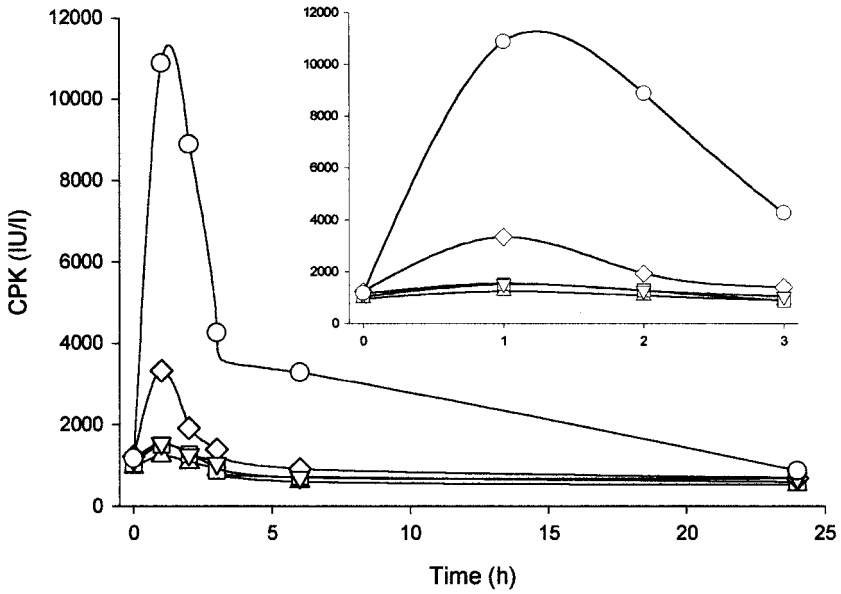

Figure 1. Serum CPK activity (IU/L) in rats after intramuscular injection of diclofenac sodium. Blood samples were taken at time intervals of 0, 1, 2, 3, 6, and 24 hr after injection: ○, commercial DS solution; ◇, buffered DS solution; ▽, DS-load liposomes; □, empty liposomes; △, Tris–HCl buffer.

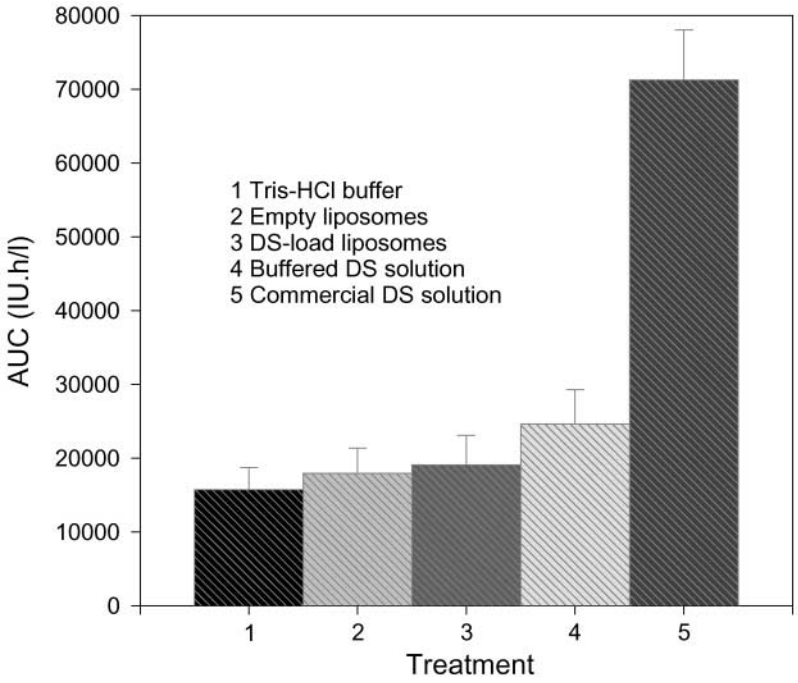

Figure 2. Mean (±SD) of area under curve (AUC 0–24 hr) of plasma creatine kinase activity (IU hr/L) in rats ($n = 6$) after intramuscular injection in quadriceps femoris muscle of 0.2 mg of diclofenac sodium formulations and controls of Tris–HCl buffer and empty liposomes. See figure insert.

Table 4

Statistical Analysis of the AUC Results from Fig. 2 by Student's Test

	Buffer	Empty Liposome	DS/Buffer	DS/Liposome
C/DS[a]	< 0.05	< 0.05	< 0.05	< 0.05
DS/liposome[b]	NS	NS	< 0.05	—
DS/buffer[c]	< 0.05	< 0.05	—	—
Empty liposome	NS	—	—	—

[a]C/DS, commerical DS solution.
[b]DS/liposome, DS-load liposome.
[c]DS/buffer, buffered DS solution.

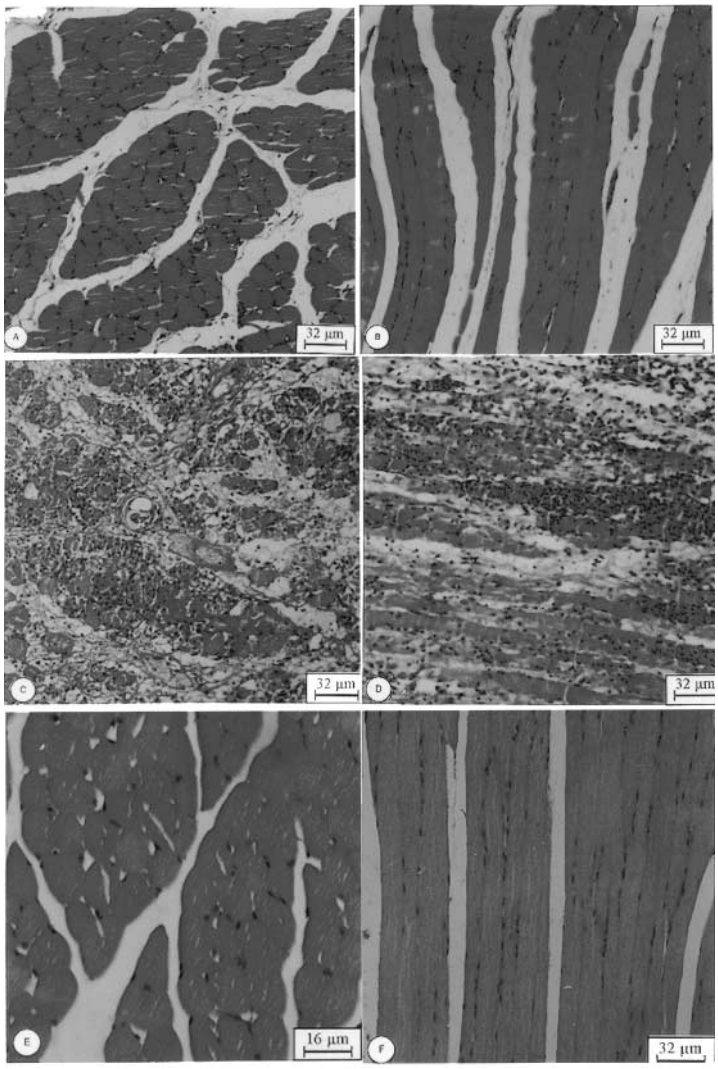

Figure 3. Photomicrographs of histological analysis showing transversal and longitudinal sections of rat femoris muscle tissue. (A,B) Muscle tissue from animals without treatment (control). (C,D) Muscle tissue from animals treated with commercial diclofenac sodium injection. (E,F) Muscle tissue from animals treated with diclofenac sodium liposomes.

empty liposomes. The injection of the commercial diclofenac caused the highest increase in CPK activity that is statistically significant from all other animal treatments (Fig. 2 and Table 4). In fact, the IM administration of diclofenac commercial solution (Voltarem) was described in the literature and provokes a great increase of CPK activity (37). Guterres et al. (34) evaluated the effect of poly(D,L-lactide) diclofenac nanocapsules on the protection against muscular damage in rats and found that the level of CPK activity is dependent on the chemical nature of the central oil core.

Histopathological evaluation of rat muscle tissue showed that the injection of free diclofenac sodium solution (Fig. 3C,D) caused severe tissue damage, represented by hyaline degeneration, myocitolysis, inflammatory cells infiltrate, mainly lymphocytes, and macrophages (Fig. 3C,D). It was observed that on the third day after injection, the local inflammatory response was more intense, whereas on the seventh day the inflammation was milder. This phenomenon indicates clearly the tendency for recovery and regeneration of the local tissue, including new capilarization and fibrosis. However, with animals from group F the damage was so severe that an intense inflammatory reaction could be observed on the seventh day. The analysis of the transverse and longitudinal sections of rat femoris muscle showed that for animals treated with DS-load liposomes no lesions could be verified (Fig. 3E,F), since the tissue histology was similar to the control (Fig. 3A,B). However, middle lesions were observed with the injection of an aqueous buffered solution of diclofenac. Similar protection from muscular damage was verified using diclofenac encapsulated in nanocapsules, however, even empty nanocapsules were responsible for some extent of damage (34), which was not observed with the DS-load liposomes in this work.

Enzymatic (Figs. 1 and 2) and histological (Fig. 3) analysis can be compared, leading to more consistent analysis of results. Groups D and F, exhibiting more extensive tissue damage, also showed higher levels of plasma CPK. Similarly, comparing tissue samples extracted from animals without treatment (group A) with those from groups B, C, and E, it can be verified that there are no significant histological differences or statistical divergences among CPK levels.

According to Sutton et al. (38), muscle cell damage can be related to high local drug concentration and to prolonged contact time of the drug with the local tissue. These authors suggested that a slow drug release from formulation would minimize muscle damage by promoting the dilution of drug in the interstitial fluid, lymphatic clearance, and cell repair. The liposomes used in this study are able to reduce the rate release of diclofenac to biological medium. So, it is interesting to speculate that liposomes can act as a drug reservoir, lowering the drug concentration in the local tissue, and protecting the muscle cells from the toxic effect of drug.

These results open new perspectives for further investigations toward the use of liposomes as carriers for anti-inflammatory drugs, particularly for parenteral administration.

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