

Interaction of sodium diclofenac with freeze-dried soya phosphatidylcholine and unilamellar liposomes.

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Phospholipids are widely used as structural amphiphilic compounds in liposome formulations. In this study, we have analyzed the interaction the sodium diclofenac (SD) with soya phosphatidylcholine (PC) and soya phosphatidylcholine from lyophilized small unilamellar liposomes (SUV). The changes in the properties of the co-lyophilized drug/PC from SUV liposomes, lyophilized PC from SUV liposomes, and lyophilized soya phosphatidylcholine, were studied by Differential Scanning Calorimetry (DSC). The DSC data showed that the previous organization of phospholipids molecules to form liposome affects intensely the thermal behavior of PC when compared to non-liposomal PC. SD modified the thermal properties of PC from liposomes. It was verified that SD affects intensely the located group peaks in the regions of 120-140 °C and in the higher temperature region of 240-260 °C. The results of this work demonstrated that the presence of the drug modified the DSC behavior for both liposomal and non-liposomal PC and that these modifications can be easily monitored by DSC analysis.

Uniterms

- Liposome
- Diclofenac
- Phosphatidylcholine
- Freeze-drying
- Differential scanning calorimetry

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INTRODUCTION

Diclofenac, (2-[(2,6-dichlorophenyl)amino]benzeneacetic acid) is a non-steroidal anti-inflammatory drug with good analgesic properties; however it may cause side effects including gastrointestinal disorders when administered by oral route and cutaneous lesion by intramuscular injection (Giovannetti *et al.*, 1993).

Phospholipids are frequently used as structural amphiphilic compounds in colloidal drug delivery systems such as liposomes and microemulsions (Canto *et al.*, 1999; Lima, Oliveira, 2002; Komatzu *et al.*, 2001). The

interaction of non steroidal anti-inflammatory drugs with phospholipids (e.g. phosphatidylcholine (PC)) is of importance in the pharmaceutical field due to modification of bioavailability improving the drug efficacy (Lichtenberger *et al.*, 1996a, b) and to decrease side effects (Lichtenberger *et al.*, 1995; Schutze, Muller-Goymann, 1998).

Liposomes are colloidal supramolecular aggregates frequently used as drug carriers for a wide range of drugs including analgesics and anti-inflammatories (Canto *et al.*, 1999; Lima, Oliveira, 2002; Lasic, 1997; Venema, Weringa, 1987; Bermúdez *et al.*, 1999; Lima *et al.*,

2001). However, in spite of the properties highly biocompatible of the phosphatidylcholine that can protect the local tissue from the side effects of the anti-inflammatory drug (Lima, Oliveira, 2002), PC is not sufficiently stable in aqueous medium and decomposes by hydrolysis and oxidation (New, 1997). Thus, the dehydration plays an important role in the stability of the structural phospholipids of liposomes. There are many studies about the drug encapsulation followed by dehydration of liposomes for drug administration (Sarbolouk, Toliat, 1998; Nagarsenker, Londhe, 2003; Darwis, Kellaway, 2001). Coprecipitation, coevaporation and colyophilization have been used for dehydration of drug dosage forms, leading to amorphous product with modification of physicochemical and biological properties of the drug (Mura *et al.*, 2002; Briges, Taylor, 2001).

DSC has been used as a useful tool in drug analysis to access information about drug-drug and drug-excipient interactions (van Windenn *et al.*, 1998; Adeyeye, Price, 1991), molecular complexation (Souza *et al.*, 2002; Medeiros *et al.*, 2002; Bettinetti *et al.*, 1992; Castelli *et al.*, 1992) and materials stability and compatibility (Medeiros *et al.*, 2002; Coffin, McGinnity, 1992; Koenigsnauer *et al.*, 1992; Torricelli *et al.*, 1991) in pharmaceutical formulations. The modification of the physicochemical properties of dehydrated drug/lipid systems also can be easily accessed by DSC analysis (Bermúdez *et al.*, 1999; Kinigt, 1981; Biltonen, Lichtenberg, 1993; Castelli, *et al.*, 2001; van Winden *et al.*, 1997; Sanches-Migallon *et al.*, 1996; Rodante *et al.*, 2002).

In this work empty SUV structured with PC and SUV containing sodium salt of diclofenac were obtained. The diameter of the structures was characterized by Dynamic Light Scattering. The amorphous product derived from lyophilization was analyzed by DSC in order to verify the differences in the properties of PC, PC previously structured in the liposomes and the effect of both acid diclofenac and sodium diclofenac on these properties.

MATERIAL AND METHODS

Material

Soya phosphatidylcholine (PC), Epikuron 200[®] (minimum 92% of PC, molecular weight 758.07), was purchased from Degussa Texturants Systems Deutschland GmbH & Co. (Hamburg, Germany). Analytical grade chloroform, hydrochloric acid min. 37%, and ethyl alcohol (Merck Darmstadt, F.R. Germany), sodium diclofenac (SD) (Biogalenica, Brazil), Tris (hydroxymethyl) aminomethane (Sigma Chemical Co, St Louis, USA) were used as received. All other reagents were analytical grade.

Methods

Liposome Preparation

Liposomes containing 40 mM of soya phosphatidylcholine (PC) were prepared in 10 mM Tris-HCl buffer, pH 7.2 by sonication as previously described (Canto *et al.*, 1999; Lima, Oliveira, 2002; Lima *et al.*, 2001). A solution containing 160 mM (121.3 mg/mL) of PC was prepared in chloroform. 1 mL of the solution was placed in a sonication tube and the organic solvent was eliminated under N₂ stream in order to obtain a lipid film in the inner of tube. The film was kept under vacuum for 2 h to remove the solvent traces.

For films containing sodium diclofenac a solution of 31.6 mM (40 mg/mL) was prepared in ethanol. 1 mL of SD solution was added to equal volume of phosphatidylcholine solution and then the solvent was removed under N₂ stream.

The dry lipid films with and without drug were hydrated at 25 °C room controlled temperature for 45 min with 4 mL of Tris-HCl buffer or SD buffered solution in order to obtain the concentrations and final PC/SD ratios described in Table I. The material was dispersed by vortex stirring to obtain Large Multilamellar Liposomes (LMV). Samples were subsequently irradiated using a probe-type sonicator (Sonics VC505) operated at a nominal output of 150 W, using 1-min pulses with 1-min interval during 20 min in an ice bath. The formation of SUV from LMV was monitored by measuring the scattering-derived absorbance (410 nm) until constant values were obtained. Table I summarizes the preparations used in the different experiments of this work.

Non-liposomal mixtures preparation

A solution containing 160 mM (121.3 mg/mL) of PC was prepared in chloroform. A solution containing 31.6 mM (40 mg/mL) of SD was prepared in ethanol. Equal volumes of SD and PC solutions were mixed in order to obtain the same molar ratio PC/SD of the liposomal preparations (Table I). Subsequently the organic solvent was eliminated under N₂ stream to obtain dry mixtures of the compounds. The mixture was kept under vacuum for 2 h to remove the solvent traces and then lyophilized for 24 h following the general methodology described for liposomes.

Liposomes Lyophilization

For freeze-drying 4 mL of the liposomes dispersion were put in 10 mL vials and quickly prefrozen in liquid nitrogen. The vials were placed together in a tray dryer and the water content was sublimated by 24 h using an Edwards Mod. Modulyo apparatus. During all process the

temperature and pressure were maintained at $-40\text{ }^{\circ}\text{C}$ and 0.13 mBar , respectively. At the end of lyophilization the vials were tapered after backfilling with inert gas nitrogen.

Dynamic Light Scattering

The diameters were measured for empty liposomes and diclofenac-load liposomes (see Table I). The dispersions were filtered with a $0.22\text{ }\mu\text{m}$ polycarbonate membrane in order to ensure dust-free samples, and diluted to 1:3 and 1:6 using 10 mM Tris-HCl buffer. The size distribution was determined directly on the aqueous dispersion at $25\text{ }^{\circ}\text{C}$ by Dynamic Laser Light Scattering using a Brookhaven apparatus with BI-9000 digital correlator and particle sizing software from Brookhaven. The samples were analyzed at 90° and as light source was applied a 35 mW Spectra Physics He-Ne laser, model 127, $\lambda = 632.8\text{ nm}$.

Differential Scanning Calorimetry (DSC)

The calorimetric analysis was performed in order to determine the differences in the properties of PC and PC previously structured in the liposome bilayer and the effect of the sodium diclofenac on these properties. For this, empty liposomes and liposomes containing drug concentrations according Table I were prepared. Sodium diclofenac, non-liposomal PC and mixtures of PC/SD at molar ratio of 5.06 were also analyzed. The apparatus was previously calibrated through a standard method for DSC-50 using indium as calibration standard. The samples were packed in aluminum cells and the used mass was 2 mg . The DSC curves were recorded using a Shimadzu DSC-50 calorimeter oven with a temperature range of $25\text{-}500\text{ }^{\circ}\text{C}$, under dry nitrogen atmosphere at constant flow of 50 mL/min and a heating rate of $10\text{ }^{\circ}\text{C/min}$. Empty aluminum cell with a lid was used as reference.

Optical Microscopy

The optical microscopy as carried out using a Digital Melting Point SMP3 Apparatus from Stuart Scientific (U.K.). The samples were placed in capillary tubes, the temperature adjusted in the range of ambient to $300\text{ }^{\circ}\text{C}$, and heating ramp rate programmed to $2\text{ }^{\circ}\text{C per min}$. Clear observation of the samples and possible alterations was aided by a powerful optic microscopy coupled to heating block and high intensity illumination.

RESULTS AND DISCUSSION

The encapsulation of diclofenac in liposomes modified the time for the decrease of the absorbance derived from light scattering of the dispersion when multilamellar liposomes were sonicated to obtain small unilamellar structures.

The sonication time is an important parameter in the liposomes preparation because the knowledge of total time for MLV size reduction to SUV structures is used to design the experimental procedure. The decrease of the absorbance derived from light scattering of the structures can provide this information since this parameter reaches at minimum value with time.

In the data of Figure 1 is shown that the scattering-derived absorbance (410 nm) for the downsize of the multilamellar liposomes in order to obtain unilamellar structures decreases rapidly in the presence of SD reaching at minimal constant values at least of 10 min . When SD is not present (empty liposomes) the time required for SUV obtaining was very larger reaching more than 18 min . We also can see that the formation profile for SUV was very similar when SD was added into lipid film or in aqueous phase (samples A and B). Moreover there were not significant differences in the obtained profiles when SD was

TABLE I - Liposome formulations used in the DSC analysis

Sample	SD in film hydration	SD in lipid film (mM)	Total diclofenac (mM)	Molar ratio PC/ Total SD	Experimental pH
Empty SUV	4 ml of Tris-HCl buffer	—	—	—	7.21
A	4 ml of Tris-HCl buffer	7.9	7.9	5.06	7.25
B	4 ml of buffered 7.9 mM SD solution	—	7.9	5.06	7.30
C	4 ml of buffered 15.8 mM SD solution	7.9	23.7	1.69	7.35

Phosphatidylcholine 40 mM , Tris-HCl buffer 10 mM , pH 7.2 . SD=sodium diclofenac

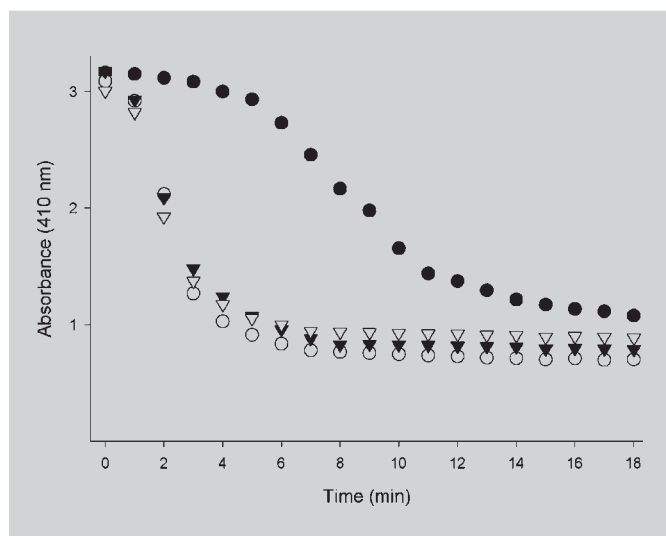


FIGURE 1 - Effect of sonication time on the absorbance derived from light scattering by MLV to obtain SUV. Key: ● empty liposomes containing phosphatidylcholine 40 mM¹; ▽ 7.9 mM of SD added to aqueous phase; ▼ 7.9 mM of SD added to lipid film; ○ 23.7 mM of SD added to aqueous and lipid phases. The data represents the mean of triplicate experiments.

TABLE II - Light scattering analysis of diclofenac encapsulated liposomes

Samples	Diameter (nm) Mean ± SD
Empty liposomes	57.8 ± 0.386
Sample A	41.8 ± 0.479
Sample B	39.4 ± 0.129
Sample C	31.8 ± 0.118

Phosphatidylcholine 40 mM; Tris-HCl buffer, 10 mM, pH 7.2. See Table I for samples details

added in both, aqueous and lipid phases and the concentration were increased three times. These phenomena suggest that the ionic drug can be easily incorporated into bilayer due predominantly to the hydrophobic effect.

The encapsulation of sodium diclofenac in liposomes decreased the diameter of the liposomes from 57.8 nm to 41.8 nm by the addition of SD directly into lipid phase (sample A). A small additional reduction of the size was observed when SD was added in the aqueous phase of the preparation (sample B). However, the largest decrease in the liposome size was verified when SD was added simultaneously in both aqueous and lipid phases and the total drug concentration were increased three times (sample C, Tables I and II). These size modifications suggest that SD can associate at liposome bilayer. This phenomenon can

be understood since surface-active drugs as non-steroidal anti-inflammatory compounds are reported to self-associate and bind membranes causing partial disruption and solubilization, in a detergent-like way (Kriwet, Müller-Goymann, 1994; Rades, Müller-Goymann, 1997; Schultze, Müller-Goymann, 1998; Schreier *et al.*, 2000; Lopes *et al.*, 2004; Souza *et al.*, 2004).

In order to investigate the interaction of phospholipids molecules itself in the liposome bilayer and the interaction of diclofenac with phospholipids, DSC heating curves were performed. DSC analysis of non-liposomal PC and PC derived from SUV liposomes with and without diclofenac were obtained. This procedure allows assessing the possible differences in the DSC profile of non-liposomal PC, PC from liposomes and the effect of SD in the phospholipid thermal behavior.

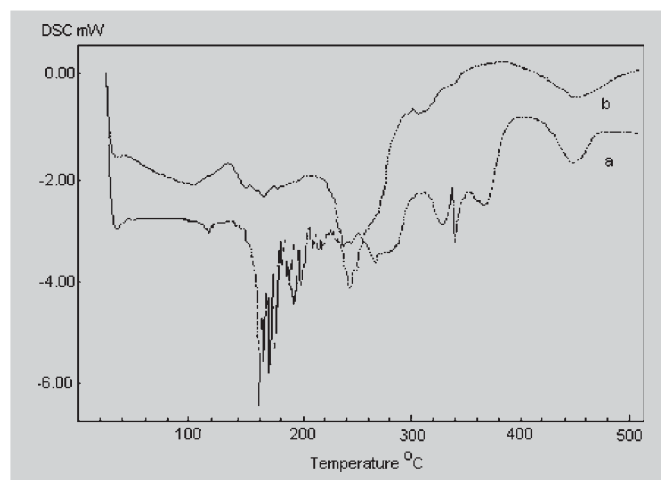


FIGURE 2 - DSC heating curves of phosphatidylcholine. Key: (a) freeze-dried non-liposomal soya phosphatidylcholine; (b) freeze-dried empty liposomes of soya phosphatidylcholine.

The results of Figure 2 suggest that the DSC curve of PC can be dependent on the previous organization of PC molecules to form liposome bilayer. In fact, Figure 2 shows that the DSC heating curves of non-liposomal PC (Figure 2, curve a) was very different from PC liposomes derived (Figure 2, curve b). The DSC curves for non-liposomal PC showed a major group of peaks in the region 159-193, 267, 330, 340, 381 and 449 °C, whereas for PC from freeze-dried liposomes the peaks appeared only at 243 and 448 °C. Great difference in the DSC profile can be observed because the decomposition of non-liposomal begins at about 140 °C, while for the liposomal PC the main temperature range for decomposition appears after the 220 °C. In fact, the DSC analysis shows that for PC from dehydrated liposomes different structures from non-liposomal PC can exist in the

medium. It is known that the presence of cryoprotectants is important to preserve the bilayer structure during the dehydration (Forrada *et al.*, 1995; Kim, Jeong, 1995; Zingel *et al.*, 1996; Joshi, Misra, 2001). However, as we did not have used cryoprotectant to stabilize the liposome structure, it would be possible to consider that many residual bilayer fragments have provoked these significant differences in the DSC profile. Nevertheless, in this temperature region the hydrophobic effect for organization of PC molecules in the bilayer residues can not be significant. Finally it is appropriate to highlight that while the DSC profile of the non-liposomal PC seems to be an amorphous material, the DSC profile of liposomal PC resembles a more organized material in which the decomposition was characterized by an intense peak at 243 °C (Figure 2).

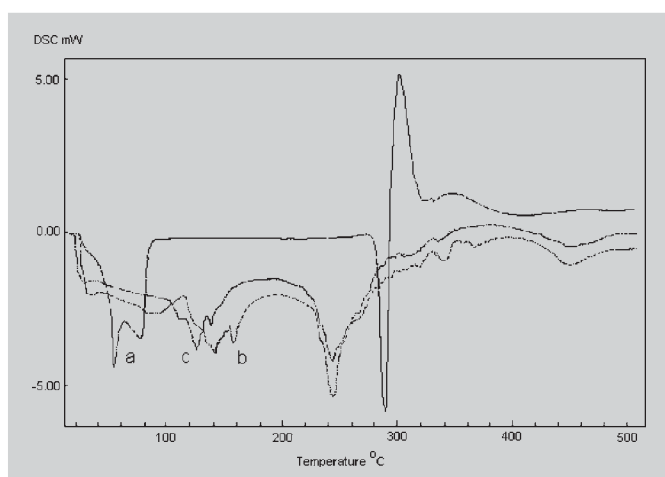


FIGURE 3 - Effect of sodium diclofenac on the DSC heating curves of phosphatidylcholine liposomes. Key: (a) sodium diclofenac, (b) freeze-dried empty liposomes, (c) freeze-dried liposomes containing sodium diclofenac added to lipid phase (molar ratio PC/SD = 5.06).

The DSC curve of pure SD showed two endothermic peaks at 52 and 78 °C and a group of peaks at 290, 300 and 345 °C produced by the drug decomposition (Figure 3, curve a) and is in agreement with the data of literature (Giordano, *et al.*, 2003). The peaks at 141 °C for empty liposomes (Figure 3, curve b) can be related to phase transition from amorphous solid to liquid product, appeared shifted to lower temperature (125 °C) when SD was added to the lipid phase during the liposome preparation (Figure 3, curve c) and decreased significantly the peak intensity at 245 °C due to product decomposition (Figure 3, curve c). This DSC behavior can be explained since the sodium diclofenac can interact with phosphatidylcholine during the preparation of the liposomes decreasing both the drug decomposition temperature and the peak intensity related to drug decomposition at 245 °C.

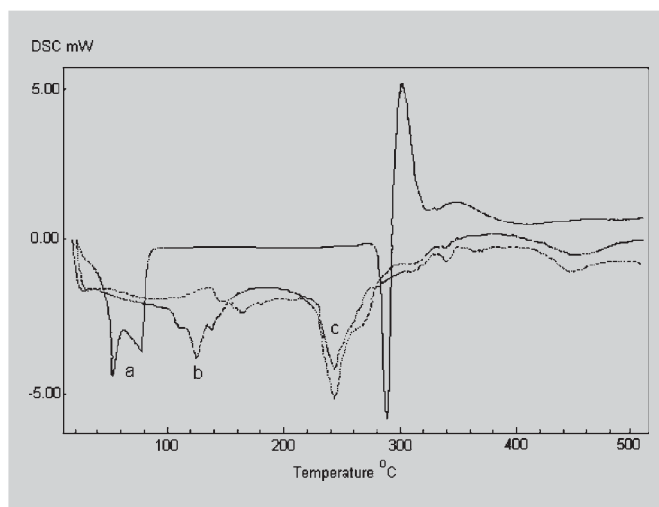


FIGURE 4 - Effect of sodium diclofenac on the DSC heating curves of phosphatidylcholine liposomes. Key: (a) sodium diclofenac, (b) freeze-dried empty liposomes, (c) freeze-dried liposomes containing sodium diclofenac added to aqueous phase (Molar ratio PC/SD = 5.06).

The profile of phosphatidylcholine from liposomes containing sodium diclofenac in the aqueous phase (Figure 4, curve c) was different from that obtained for phosphatidylcholine from empty liposomes (Figure 4, curve b). In the presence of SD the DSC curve was characterized by an endothermic peak at 125 °C (Figure 4, curve c) which does not exist for PC from empty liposomes. This data suggests that SD interacts with lipid phase even when the drug is added to aqueous phase of the liposomes that is in agreement with the low water solubility of the sodium diclofenac.

On the other hand, the liposomes were obtained using Tris-HCl buffer, which was freeze dried together with liposomes structures. It would be reasonable to consider the buffer as PC impurity, which could influence partially these differences in DSC curves. However, Figure 5 shows clearly that the peak in the region of 125 °C appears when the SD was mixed to non-liposomal phosphatidylcholine (Figure 5, curve b). This group of data demonstrates that the phenomenon seems to be directly related with the interaction between sodium diclofenac and phosphatidylcholine molecules and not with the possible presence of bilayer residues or Tris salt in the phospholipid mixture.

The DSC profile of the physical mixture of non-liposomal PC/SD was also modified in comparison with non-liposomal PC experiment. The data shows an intense peak at 120 due to phase transition of amorphous viscous to liquid transparent gel and another at 225 °C related to drug decomposition (Figure 5, curve b). This feature can be easily seen by optical microscopy, through a capillary di-

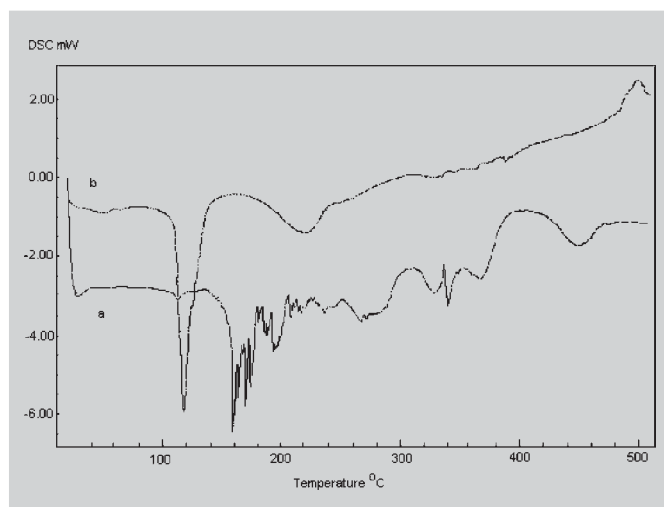


FIGURE 5 - Effect of sodium diclofenac on the DSC heating curves of non-liposomal phosphatidylcholine. Key: (a) non-liposomal phosphatidylcholine; (b) non-liposomal phosphatidylcholine/sodium diclofenac (Molar Ratio PC/SD = 5.6).

gital melting point apparatus. The curves of thermal behavior of non-liposomal PC mixed with SD showed peaks with the large intensity of the signal in the region of 120 °C, demonstrating that SD molecules interact with phosphatidylcholine even in the dehydrated state.

CONCLUSION

The obtained data demonstrates that previous organization of PC molecules to form liposomes affects significantly the DSC profile of the PC from freeze-dried liposomes when compared to non-liposomal PC. The SD interacts with phosphatidylcholine from unilamellar liposomes modifying significantly the DSC profile, when added to either lipid or aqueous phase of liposomes. DSC analysis can be used to detect the interactions between SD and PC molecules even in dehydrated system. These results indicate that in all cases the differences verified in the DSC profile can be easily monitored through DSC analysis.

RESUMO

Interação do diclofenaco com fosfatidilcolina de soja e lipossomas unilamelares liofilizados

Fosfolípidios são freqüentemente usados como compostos anfífilicos estruturais em formulações de lipossomas. Neste estudo foi analisada a interação do diclofenaco sódico (SD) com a fosfatidilcolina de soja liofilizada e a fosfatidilcolina de soja (PC) obtida de lipossomas unilamelares pequenos

liofilizados (SUV). As modificações nas propriedades da mistura fármaco/PC co-liofilizados a partir de SUV pré-formados, lipossomas de PC vazios e PC liofilizada foram estudadas por Calorimetria Diferencial de Varredura (DSC). Os resultados de DSC mostraram que a organização prévia das moléculas PC para formar lipossomas interfere significativamente no perfil de DSC da PC, quando comparada ao perfil de DSC da PC não-lipossômica. Verificou-se que o SD afeta intensamente o grupo de picos situados nas regiões de 120-140 °C e na região de mais alta temperatura (240-260 °C). Os resultados deste trabalho demonstraram que em todos os casos a presença do fármaco modificou o perfil de DSC da PC e que essas modificações podem ser facilmente monitoradas através da análise de DSC.

UNITERMOS: Lipossomas. Diclofenaco. Fosfatidilcolina. Liofilização. Calorimetria diferencial de varredura.

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