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Quantitative determination of hydrophobic compound entrapment in dipalmitoylphosphatidylcholine liposomes by differential scanning calorimetry

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Abstract

In this paper the feasibility of determing the percentage of drug entrapment (PDE) in dipalmitoylphosphatidylcholine (DPPC) liposomes by using differential scanning calorimetry (DSC) was assessed. Three different lipophilic compounds, namely testosterone, vitamin E acetate and retinoic acid, were encapsulated in multilamellar liposomes and their PDE values were determined by DSC and by conventional separative techniques, such as dialysis and centrifugation. PDE values of testosterone, vitamin E acetate and retinoic acid determined by DSC analysis, applying Van't Hoff equation, were 6.3 ± 0.1, 68.9 ± 0.7 and 87.8 ± 0.6, respectively. Dialysis was performed at different time intervals, 3, 6, 9 and 12 h, on DPPC liposomes entrapping the compounds tested. The duration of the dialysis process strongly affected the quantification of testosterone incorporated in DPPC liposomes while it slightly influenced the determination of vitamin E acetate and retinoic acid PDE values. Centrifugation of DPPC liposomes entrapping the compounds tested was carried out at different rpm (6000, 9000 and 12000 rpm). No significant difference was observed comparing PDE values obtained centrifugating at different rpm. Comparing PDE values obtained by dialysis (at 9 and 12 h) and centrifugation for each compound with the corresponding PDE values determined by DSC, linear relationships were observed. These results suggest that DSC analysis could be regarded as a suitable technique for the quantitative determination of the percentage of hydrophobic compound entrapped in DPPC liposomes.

Keywords: Liposomes; Differential scanning calorimetry; Dialysis; Centrifugation; Lipophilic compound; Percentage of entrapment

1. Introduction

During the last years, interest has been increasing in the use of liposomes in the pharmaceutical field. Many investigators have proposed that lipo-

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somes can be used as carriers for drug delivery to improve drug therapeutic effect while reducing unwanted side effects (Mezei and Gulasekharam, 1980; Axelsson, 1989).

For liposome to be effective, drugs should be entrapped within the liposomal vesicles and the percentage of drug entrapment (PDE), which can be affected by several factors (Nassander et al., 1990), may play an important role in determining liposome efficacy. PDE determination requires the separation of the nonencapsulated compound from the liposome dispersion: generally, this is the most critical step in the quantitative determination of the encapsulated drug. The most common separative techniques are dialysis, gel filtration and centrifugation (Nassander et al., 1990). However, each of these techniques shows some disadvantages since dialysis is rather time consuming, gel filtration produces a considerable sample dilution while centrifugation may induce vesicle aggregation or fusion due to mechanical forces during sedimentation.

Several researchers (Papahadjopoulos et al., 1975; O'Leary et al., 1986) have reported the use of differential scanning calorimetry (DSC) to evaluate the interaction of hydrophobic drugs with liposome bilayers. A lipid bilayer has a typical transition temperature due to phase transition from a gel state to a liquid crystal state and the introduction of a hydrophobic molecule from outside within the lipid bilayer causes a perturbation of the pseudo-crystal network producing a decrease in transition temperature (T_m) and enthalpy of transition (ΔH) (Mabrey and Sturtevant, 1976). Since the crystalline structure of a lipid bilayer in the gel state is similar to that of a solid compound, Van't Hoff equation, which is generally used to assess the degree of purity of crystalline substances, can be applied to determine the molar fraction of hydrophobic compound encapsulated in liposomes using DSC curve analysis (Estep et al., 1978).

In this study, we assessed the feasibility of determining the amount of hydrophobic compound entrapped in dipalmitoylphosphatidylcholine (DPPC) liposomes by using differential scanning calorimetry. DPPC was chosen as phospholipid for liposome preparation because it is one of the most widely used for evaluating drug interaction with lipid bilayers and its transition temperature can be easily measured since it shows narrow DSC peaks. In order to evaluate the effect of compound lipophilicity on PDE three different model compounds (testosterone, retinoic acid and vitamin E acetate), showing different lipophilic characteristics, were used. For each compound, the percentage of entrapment in DPPC liposomes was determined by dialysis and centrifugation and these results were compared to those obtained by DSC.

2. Materials and methods

2.1. Materials

Dipalmitoylphosphatidylcholine (DPPC) was purchased from Sigma Chemical Co. (St. Louis, MO). Testosterone and retinoic acid were obtained from Fluka (Basel, Switzerland). Vitamin E acetate was a gift from Basf (Germany). [1,2,6,7,-^{3}H] Testosterone (specific activity 80–105 Ci mmol^{-1}) was purchased from Amersham (U.K.).

Dialysis tubes, cut off 12 000 (average diameter 16 mm), were supplied by Sigma. Bidistilled water used for liposome preparation was tested for calcium ion absence by atomic absorption. All other reagents used were of analytical grade.

2.2. Liposome preparation

Multilamellar vesicles (MLV) were prepared following the 'film' method (Bangham et al., 1974). Ten milligrams of DPPC in mixture with different amounts of active compound (retinoic acid: 400 μg; vitamin E acetate: 1 mg; testosterone: 1 mg and 0.36 μCi of [1,2,6,7,-^{3}H] testosterone) were placed in glass tubes and dissolved in 1 ml chloroform. The solvent was evaporated in nitrogen flow and the resulting lipid film was kept overnight at 30°C under high vacuum. The dried film was then suspended in 1 ml of 0.9% sodium chloride solution at over the phase transition temperature of DPPC (42°) by vortexing twice for 2 min. For retinoic acid liposomes all the preparation steps, dialysis and centrifugation procedures

were carried out sheltered from the light to avoid photodegradation.

2.3. Morphology and size analysis

As previously reported (Panico et al., 1993), liposomes were examined under a photomicroscope (Zeiss III RS, Germany) for morphological evaluation. A rather homogeneous population of multilamellar vesicles without cluster or formation of crystals was observed.

Vesicle size was determined by photon correlation spectroscopy (PCS) light scattering analysis, as previously described (Panico et al., 1993). The apparatus consisted of an He-Ne Spectra Physic model 120 Laser (7 mW), a holding sample cell (PC8 Malvern) thermostated at 24°C by a Haake F3-R and equipped with a Microcontrol precise mechanical goniometer and an optical system (Melles-Griot f. 150); Hamamazu R1333 and RCA 8852 photomultipliers were used. All the data from PCS analysis were correlated by a Malvern 4700 C particle analyzer connected to an Olivetti 240 computer. The scattering angles were 20° and 40°. From the scattering behaviour of vesicles, the quality parameter or polydispersity index (PI) (Pusey et al., 1974) was determined. This parameter, which can range from 0 to 9, shows values approaching 0 for a monodisperse system and higher values for a polydisperse system.

2.4. Differential Scanning Calorimetry (DSC)

A Mettler TC10A processor equipped with a DSC 20 measuring cell was used for calorimetric analysis, after temperature and energy calibration using Indium and lauric, capric and myristic acids as standards. The plotting range full scale deflection was set to 1 mW. Enthalpy changes were evaluated using the integration program (Bezout method) of an IBM XT computer. Scanning rate was 2°C min^{-1}. Noise (RMS) was 0.006 mW; thermograms were detected both in heating and cooling modes, in the 30–80°C range, with a precision of ± 0.2°C.

2.5. Dialysis

Dialysis tubings of cellulose were conditioned as follows. Glycerin, included as a humectant, was removed by washing the tubing in running water for 4 h. Sulphur coumpounds were removed by treating the tubing with a 0.3% (w/v) solution of sodium sulphide at 80°C for 1 min and successively washing with hot water (60°C) for 2 min, acidifying with a 0.2% (v/v) solution of sulphuric acid and rinsing with hot water to remove the acid.

Tubings were stored in distilled water at 4.0 $\pm$ 1°C until used.

Two milliliters of each liposome suspension were sealed in a previously conditioned dialysis tubing and immersed in 400 ml of normal saline. In order to evaluate the effect of dialyzing time on PDE determination, dialyses were run for 3, 6, 9 and 12 h and the dializing solution was replaced at intervals. At the end of the experiment, samples of the dialized liposomal suspension were withdrawn and assayed for liposome integrity and active compound content. From morphology and size analyses, no significant change in liposome structure or size was observed after the dialysis process.

2.6. Centrifugation

Each liposomal suspension was centrifuged 3 times at 4°C for 20 min at different rpm: 6000, 9000 and 12 000, in a 50Ti type rotor of a Beckman L8-60M ultracentrifuge, in order to separate the incorporated active compound from the free form. Each time the supernatant was removed and the liposomal pellet resuspended in 1 ml of 0.9% sodium chloride solution. Samples of the resulting liposomal suspension were withdrawn and assayed for liposome integrity and active compound content. No significant changes of liposome morphology and size were found after the centrifugation procedures.

The recovery of active compound being tested from the resuspended liposomal pellet and from the supernatant obtained in the washing steps accounted for more than 95% of the amount used for liposome preparation.

2.7. Quantitative determination of active compounds

Active compound content in liposome samples after dialysis or centrifugation was determined as follows. Samples containing testosterone were mixed with 3 ml of liquid scintillation cocktail (Insta-Gel, Packard) and the radiolabeled compound was measured using a Beckmann LS 9800 series liquid scintillation counter (45–50% efficiency). Vitamin E acetate and retinoic acid were determined spectrophotometrically at 284 nm and 360 nm, respectively, by diluting the samples with a known amount of ethanol and determining the sample absorbance using a spectrophotometer UV Kontron mod. Uvikon 860. The spectrophotometric measurements were carried out in accordance with the method reported by Ma et al. (1991) who used empty liposomes dissolved in ethanol as reference standards in order to correct for the turbidity effects when active compound content in liposomal suspensions was determined. Standard absorbance curves of vitamin E acetate and retinoic acid in the suitable solvent were constructed daily. The concentration of vitamin E acetate and retinoic acid was determined from the standard curves in the linear region of the Lambert-Beer plot.

2.8. Calculation

The molar fraction of hydrophobic compound encapsulated in DPPC liposomes was determined by Van't Hoff equation (1) using DSC curve data.

$$X_2 = \frac{\Delta H_{fusion}\,(T_o - T_m)}{RT_o^2} \qquad (1)$$

Where X_2 is the molar fraction of the hydrophobic compound within the lipid bilayer, ΔH fusion is the heat of fusion of pure liposomes (cal mol^{-1}), R is the gas constant (1.987 cal mol^{-1} K^{-1}), T_o is the fusion temperature of the pure liposome and T_m is the fusion temperature of the liposomes encapsulating a hydrophobic compound. Due to the instrument difficulty of directly determining the exact decrease of the melting temperature of the liposome encapsulating the active compound (Palermo and Chiu, 1976), the fraction of melted sample (f) at different temperatures (T_s) in the melting range is determined. So, the DSC scan is sectioned in small areas of melting and the temperature of each is determined. f is then calculated as the ratio between each area of melting and the total area of the DSC curve. Since f is equal to:

$$f = \frac{T_o - T_m}{T_o - T_s} \qquad (2)$$

then:

$$T_s = T_o - (T_o - T_m)\,1/f \qquad (3)$$

As reported by other authors (Palermo and Chiu, 1976), plotting T_s values vs 1/f a straight line whose slope ($T_o - T_m$) represents the decrease of melting temperature of the liposomes containing the active compound is obtained. In this way, calculating H fusion from the integration of DSC curve and ($T_o - T_m$) from equation (3), we determined the molar fraction of active compound encapsulated in DPPC liposomes using equation (1) and then the corresponding PDE value.

PDE values are expressed as the percentage of active compound incorporated with respect to the starting amount for the preparation of the liposomes.

3. Results and discussion

As shown from morphological evaluations, all the DPPC liposomes prepared in this study were large multilamellar vesicles (MLV) and their average size, determined by PCS light scattering analysis, was about 700 nm with polidispersivity indexes ranging from 0.7 to 1.3 (Table 1).

The percentage of encapsulation of testosterone ($X_a = 0.2$) in DPPC liposomes determined by DSC was 6.3 $\pm$ 0.1% (Table 1). Measuring from higher molar fractions ($X_a = 0.3, 0.4, 0.5$) did not produce further increase in testosterone encaspulation since no significant changes in T_m and ΔH were observed compared to $X_a = 0.2$ (data not shown). This low percentage of encapsulation could seem in contrast with the hydrophobic character of this molecule. However, this result agrees well with the data reported by Shaw et al. (1976)

Table 1
DSC data (molar fraction, X_a; main transition enthalpy changes, ΔH; mean size; polidispersivity index, P.I.) and percentage of encapsulation (PDE) of testosterone, vitamin E acetate and retinoic acid in DPPC liposomes determined by DSC. $n = 3$ for each determination

Compound	X_a	ΔH (Kcal mol^{-1})	Mean size (μm)	P.I.	PDE ± S.D.
Testosterone	0.20	7.2	707	1.1	6.3 ± 0.1
Vitamin E acetate	0.13	8.6	690	0.7	68.9 ± 0.7
Retinoic acid	0.10	7.8	670	1.3	87.8 ± 0.6

who pointed out poor encapsulation of steroids in liposomes: the authors attributed the low percentage of entrapment to the geometric structure of steroid molecules which prevent them to strongly interact with lipid bilayers.

From DSC measurements on vitamin E acetate ($X_a = 0.13$) and retinoic acid ($X_a = 0.10$) DPPC liposomes, the percentage of drug entrapment was 68.9 ± 0.7% and 87.8 ± 0.6%, respectively (Table 1). As observed for testosterone DPPC liposomes, higher molar fractions of vitamin E acetate or retinoic acid ($X_a = 0.2, 0.3, 0.4$) did not provide higher percentage of drug encapsulation (data not shown). Vitamin E acetate PDE values determined by DSC are in good agreement with those reported in a previous paper (Bonina et al., 1994). Gomez-Fernandez et al. (1988), investigating vitamin E acetate interaction with DPPC liposomes by DSC, reported that this compound decreased the phase transition of DPPC and should interact with the hydrophobic tails of the lipid bilayer. Regarding retinoic acid, this drug could interact with DPPC aliphatic tails inducing a packing of the lipid chains and retinoic acid molecules could be located perpendicularly to the phospholipid chain within the bilayer (Castelli and Gurrieri, 1988). The high percentage of retinoic acid entrapment in DPPC liposomes observed in this study is in good agreement with that reported by Nastruzzi et al. (1990) who found an entrapment efficiency over 95%.

In order to compare PDE values determined by DSC with those obtained by conventional separative techniques, testosterone, vitamin E acetate and retinoic acid percentage of entrapment in DPPC liposomes was assessed by dialysis and centrifugation.

As may be noted in Table 2, using dialysis the quantification of testosterone incorporated in DPPC liposomes was strongly affected by the duration of the dialysis process and a plateau was achieved only after 9 h. On the contrary, the percentage of vitamin E acetate or retinoic acid in DPPC liposomes was slightly influenced by the dialysis duration (Table 2). As expected, PDE values of vitamin E acetate and retinoic acid determined after 3 h were higher than those obtained at the following time points, but a plateau

Table 2
Testosterone, vitamin E acetate and retinoic acid percentage of encapsulation in DPPC liposomes determined by dialysis at different dializing times. Values are expressed as the percentage of drug encapsulation with respect to the starting amount for the preparation of the liposomes. $n = 3$ for each determination

Compound	Dializing time (h)			
	3	6	9	12
Testosterone	62.3 ± 1.4	18.7 ± 1.8	6.9 ± 1.3	6.8 ± 0.9
Vitamin E acetate	73.7 ± 1.9	68.8 ± 1.8	67.6 ± 1.3	67.6 ± 1.1
Retinoic acid	89.1 ± 2.2	83.8 ± 3.7	82.4 ± 3.8	83.1 ± 2.4

Table 3
Testosterone, vitamin E acetate and retinoic acid percentage of encapsulation in DPPC liposomes determined by centrifugation at different rpm. Values are expressed as the percentage of drug encapsulation (± S.D.) with respect to the starting amount for the preparation of the liposomes. $n = 3$ for each determination

Compound	Centrifugation (rpm)		
	6000	9000	12 000
Testosterone	8.0 ± 0.9	7.8 ± 0.8	7.5 ± 0.9
Vitamin E acetate	65.7 ± 1.4	63.2 ± 1.1	61.4 ± 1.3
Retinoic acid	99.3 ± 0.8	99.5 ± 0.7	99.6 ± 0.9

was observed at 6 h since no significant difference was noted among PDE values determined at 6,9 and 12 h ($P > 0.05$ for all the comparisons). Since all the drug tested were lipophilic, the effect of dializing time on the determination of the percentage of testosterone encapsulation could be ascribed to a different interaction of this compound with DPPC liposomes. However, further studies are needed to better elucidate the different behaviour of the compounds tested during the dialysis process.

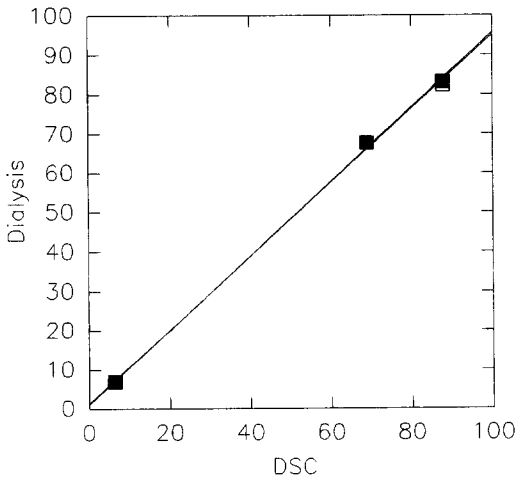

Fig. 1. Relationship between PDE values determined by DSC and dialysis at different dializing intervals. (■) 9 h ($r = 0.999$); (□) 12 h ($r = 0.999$). Since dialysis PDE values at 9 and 12 h are very close, lines and symbols are overlapping.

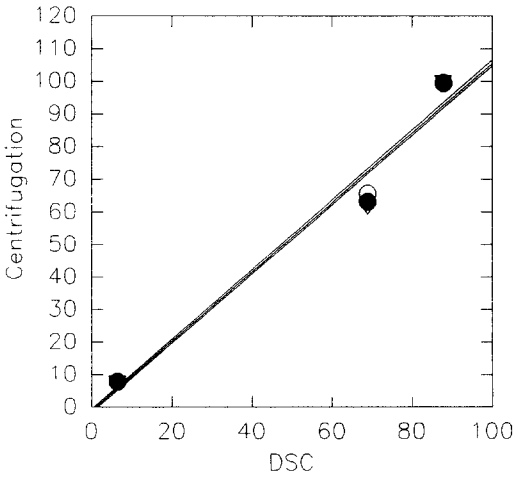

Fig. 2. Relationship between PDE values determined by DSC and centrifugation at different rpm. (●) 6000 rpm ($r = 0.988$); (○) 9000 rpm ($r = 0.983$); (▽) 12 000 rpm ($r = 0.979$). Since PDE values determined by centrifugation at different rpm are very close, lines and symbols are almost superimposed.

The amounts of testosterone, vitamin E acetate and retinoic acid encaspulated in DPPC liposomes, determined by centrifugation, are reported in Table 3. Centrifugating at different rpm (6000, 9000 and 12 000) did not significantly affect the percentage of entrapment of the drugs tested. These results suggest that for DPPC liposomes entrapping testosterone, vitamin E acetate or retinoic acid centrifugation did not induce significant alterations of vesicles structure or changes of drug interaction with the lipid bilayer. Comparing PDE values obtained by dialysis at 9 and 12 h for each compound with the corresponding PDE values determined by DSC linear relationships were observed ($r = 0.999$ for both comparisons) (Fig. 1). Since the percentage of testosterone encapsulation determined by dialysis achieved a plateau only after 9 h, only the results at 9 and 12 h were considered to evaluate the correlation with DSC data. Similar linear relationships (Fig. 2) were obtained comparing the centrifugation results at different rpm for each compound with the corresponding DSC data (PDE at 6000 rpm vs DSC PDE, $r = 0.988$; PDE at 9000 rpm vs DSC PDE, $r = 0.983$; PDE at 12 000 rpm vs DSC PDE, $r = 0.979$). The results of this study suggest that DSC

analysis could be regarded as a suitable technique for the quantitative determination of the percentage of hydrophobic compound entrapped in DPPC liposomes since a good correlation between PDE values determined by DSC and those obtained using conventional separative techniques (dialysis and centrifugation) was found. Since DSC analysis does not require the separation of the nonencapsulated compound from the liposome dispersion, it could provide a less time consuming, easier and more accurate techinque for the quantitative determination of hydrophobic drug encapsulation in liposomes. However, further studies are needed to investigate if DSC analysis could be successfully applied to determine the percentage of hydrophobic drug entrapment in other liposomal systems containing different phospholipids and active compounds.

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