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INFLUENCE OF ONE QUINOLONE ON THE FORMATION AND THE PHYSICAL STABILITY OF LIPOSOMES

by

Ignasi Carrera[1], Concepció Martorell[2],
Joan Freixas[2], and Jordi Hernández-Borrell[1].*
1. Unitat de Físicoquimica, Facultat de Farmacia, U.B. 08028 Barcelona,Spain.
2. ITEVE Laboratories S.A. , 43202 Reus, Spain.

Abstract

The influence of one quinolone on the formation and physical stability of phosphatidylcholine (PC) liposomes is described. Based on Photon Correlation Spectroscopy (PCS) measurements, namely mean diameter and polydispersity, the maximum proportion of quinolone which is compatible with liposome morphology and homogeneous population was established in 30 % mole of drug in a PC matrix. This lead to a pharmaceutical formulation which includes the additive D-α-tocoferol as antioxidant, glycerol as a co-solvent and phosphatidylcholine-cholesterol (2:1, mole/mole) as the formal lipid matrix. Drug encapsualtions between 30-40 % were found. The feasibility of the method to scale-up this liposome formulation is discussed in terms of PCS, efficiency of encapsulation and preliminary assays *in vivo*.

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* To whom correspondence should be addressed.

Introduction

The quinolones are a group of antibacterial agents which have a widespread action against Gram negative microorganisme, especially Salmonella, Pseudomonas, Mycobacteria and so on (1). Structurally, all are related to nalidixic acid, the oldest predecessor. Substances of this class constitute 4 groups of low soluble heterocyclic carbon acids like the well known compounds norfloxacin, ciprofloxacin, enoxacin, ofloxacin, cinoxacin and perfloxacin, which can be identified by the carboxyl group in the 4-position. Today, the fluoroquinolones, produced by the introduction of the piperazine group and fluorine into the nucleus, have become the most promising group, with much greater efficacy than classical quinolones or other antibiotics.

Coinciding with the chemical modifications, a renewed interest in quinolone research has appeared, especially in an attemp to understand their mechansim of action (all are gyrase inhibitors), which is related to the binding to DNA (2). A hydrophobic route of diffusion through membranes, which seems likely as a first step in the pharmacological behaviour, has been related to the partition coefficient of the drug (3) and can may be useful in the passive methods of encapsulation in liposomes (4). In this area, some methods take the advantage of covalent binding to phospholipids (called pharmacosomes) (5, 6). Indeed, as can be expected from the poor solubility of quinolones in water, a double process of encapsulation (in aqueous inner volume and in lipid bilayer) may be expected.

The industrial interest in the production of quinolone-containing liposomes is considerable since a more powerful effect than that of other antibiotics or the free drug by itself has been observed when preliminary experiments *in vivo* were performed on pigs and sheep (unpublished data). Thus liposome-associated quinolone led to unusual rates of survival and these findings encouraged us to develop a new method to scale up liposomes for veterinary purposes. Previously, we studied the effect of the quinolone on the morphology of the liposomes of pure lecithin and their physical stability tested at temperaturtes between 20 and 50°C. Now a complete pharmaceutical formulation designed to encapsulate quinolones in liposomes is proposed and the role of each constituent is discussed. Morphological characteristics and efficiency of encapsulation of these liposomes are also studied.

Materials and Methods

Materials

The quinolone ITV 8912 ® 1-cyclopropil-1,4-dihydro-4-oxo-6-fluor-7-(N-3-methyl-piperazinil)-3-quinolincarboxilic acid was synthesized by ITEVE S.A Labs., (Reus, Spain). Crude Phosphatidylcholine approx. 96 % pure (Natterman Phospholipid GmbH, Germany) was dissolved in petroleum ether and twice precipitated with acetone. The precipitate was stored dry at -20°C. Product was judged pure for industrial purposes. Cholesterol 99% (CHOL) (Sigma Chemical Co. St. Louis, MO), was re-crystallized twice from 95% ethanol, liophylized for 12 h and stored at -20°C. D-α-tocoferol was also obtained from Sigma. Glycerol was redistilled from glass apparatus before use. Lactic acid was purified by fractional distillation and fractional crystallization from ethyl ether:isopropyl ether (1:1,v/v). Other common chemicals were of pharmaceutical grade and used as received. Deionized water was distilled from sodium permanganate in an all-glass apparatus and further purified by reverse osmosis through a Milli-Q system (Millipore, U.S.A.).

Methods

Liposome preparation and quinolone quantitation

Liposomes were prepared as follows (7). Firstly, quinolone was dissolved in lactic acid suitably heated to 30-40° C. The resulting solution was transferred to a monomixer and CHOL, D-α-tocoferol and PC, previously dissolved in ethanol, were immediately added to the drug solution. This operation was carried out at 50°C and short periods of sonication were occasionally applied. Mixture was stabilized for 1 h at room temperature and heated under reduced pressure to 75-80°C in order to remove alcohol. In the following step, glycerol and buffer solution were added at convenient temperature (50-55°C). For PCS measurements, neither CHOL nor additives was present in the preparation. Separation of liposomes from free quinolone at large scale was carried out by using a Bioran column.The percentage of encapsulation determined at laboratory scale by using a Franz cell (8).Thus, 2 mL of liposome preparation was placed in the upper glass holder of the cell and dinamically dialysed against 200 ml acceptor medium of buffer at 25 °C. The half-time for drug removal by dialysis was 12 h. Quinolone was determined by high-performance liquid chromatography (HPLC) methods elsewhere described (9). The chromatographyc equipment consisted of a Model 110 B pump with a spectrophotometric detector Model 167 (Beckman

Associates). The analytical column (300 x 39 mm) was filled with μ-Bondapak C-18 (10 μm) packing material. Quinolone was chromatographed with a mobile phase of methanol:water:H_3PO_4 (500:500:1, v/v/v) containing 25 mM of H_2KPO_4 and 1 mM of sodium heptanesulphonate. A flow rate of 1.5 mL/min was used. Detection was done at 270 nm. The data were analyzed using the System Gold software package.

Photon Correlation Spectroscopy

For particle-size analysis in solution, photon correlation spectroscopy (PCS), also known as quasielastic light scattering (QELS), was used to calculate normalized autocorrelation functions. Liposome diameter was obtained from the Stokes-Einstein relationship. Light scattering measurements were performed in an Autosizer IIc photon correlation spectrometer (Malvern Instruments, U.K.) using a helium-neon laser as a source of incident light (λ =632.8 nm), operating at 5 mW; other experimental conditions were: viscosity, $0.899 \cdot 10^{-3}$ Pa·s and refractive index, 1.330. Liposome solution conveniently diluted (usually x 100) was tranfered to a cuvette and placed inside temperature regulated cell-scattering enclosure.The data were collected at a scattering angle of 90° and the autocorrelation function was determined via a Malvern 7032-N, 72 channel multibit correlator. Assuming a log-normal size distribution of particles, the method of cumulant analysis (9) was available, while for broad monomodal distributions of the model, independent analysis which does not assume any particular form of the distribution was used (10).

TABLE 1

Characteristics of Quinolone-Containing Liposomes. (I: PC Liposomes; II: Complete Formula). Data are the average of 10 separate batches.

	Diameter mean± S.D. (nm)	Polydispersity	Encapsulation Efficiency (%)
I:	191 ± 40	0.34 ± 0.01	8 ± 1
II:	270 ± 60	0.35 ± 0.18	35 ± 5

Results and Discussion

We first studied the production of liposomes with the most simplified bilayer matrix: phophatidylcholine. The quinolone-containing liposomes, at a drug:PC molar ratio of 0.4:0.6 yielded a mean particle size ranging from 150 nm to 231.0 nm (polydispersity index 0.24 and 0.36) (I in Table 1). In order to determine whether the quinolone could influence liposome formation and physical characteristics of liposomes, the influence of the molar fraction of quinolone on liposomes size was monitored. As can be seen in Fig.1, liposomes undergo an increase in mean diameter which is proportional to the total amount of quinolone present, but no linear relation between size and quinolone concentration can be established. Nevertheless the polydispersity of samples with proportions of quinolone above 30 % was higher than 1, the upper limit of this parameter, and therefore it is possible that no liposomes were formed beyond than this mole fraction. Thus polydispersity indexes higher than 0.5 are not acceptable in a liposome form. On the other hand, these results also suggest a double process of

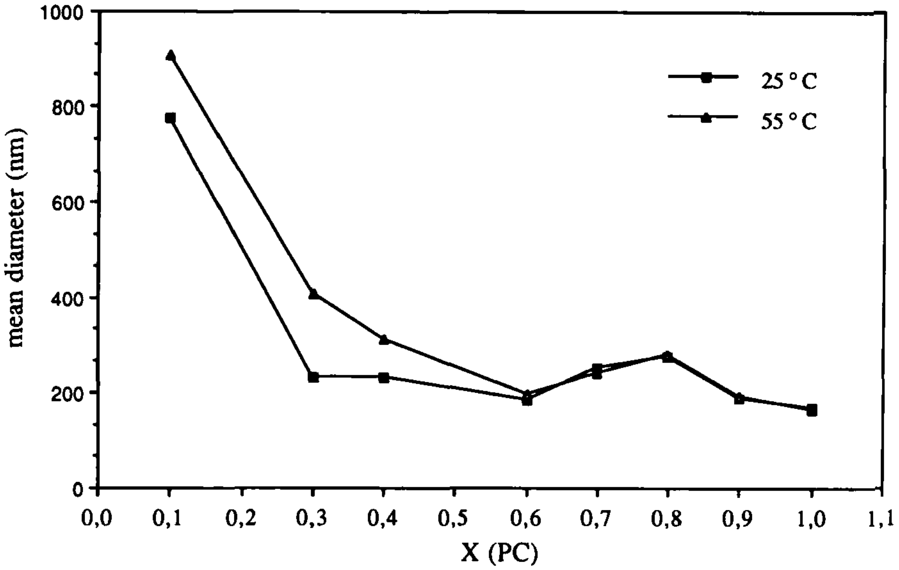

FIGURE 1

Dependence of liposome size on quinolone:PC mole fraction.

encapsulation that is a consequence of the partitioning phenomena between aqueous phase and lipid bilayer. This could improve encapsulation efficiencies, although values of 8 ± 1 % were obtained (Table 1). As will be discussed, these values increase with the presence of additives.

Given the thermal properties of phospholipids, the effect of temperature on liposome morphology was also studied. Liposomes are sensitive to temperature and size could be therefore affected (11) which may impair the physical stability of liposomes. In Figures 2a and 2b, sizes were monitored at 25,35,45 and 55°C. Only slight modifications were detected between 25 and 35°C, and even at 45°C. Samples with predominant PC, undergo a low increase (i.e.4% for 0.9:0.1, PC:Quinolone mole ratio at 45°C), compatible with a lipid phase

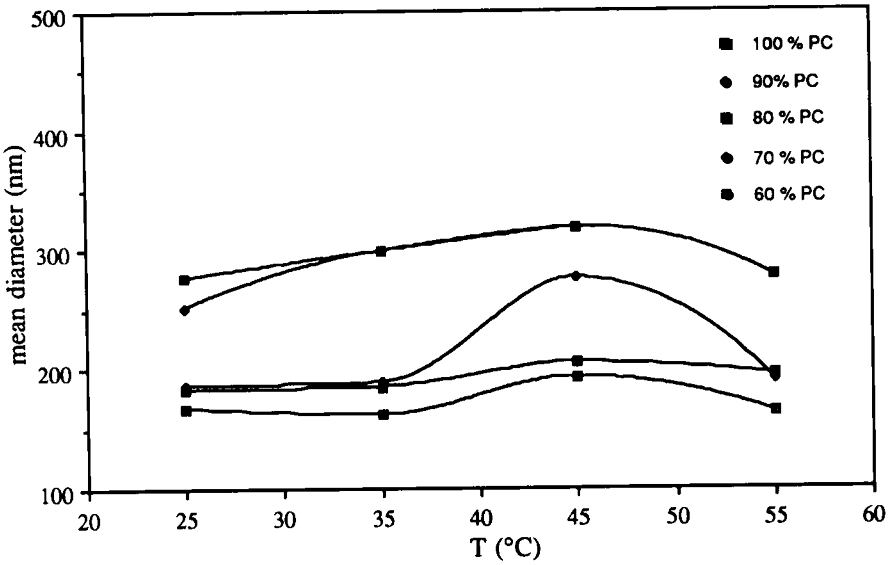

FIGURE 2a

Mean diameter-Temperature relations for liposomes with quinolone proportion until 40 %

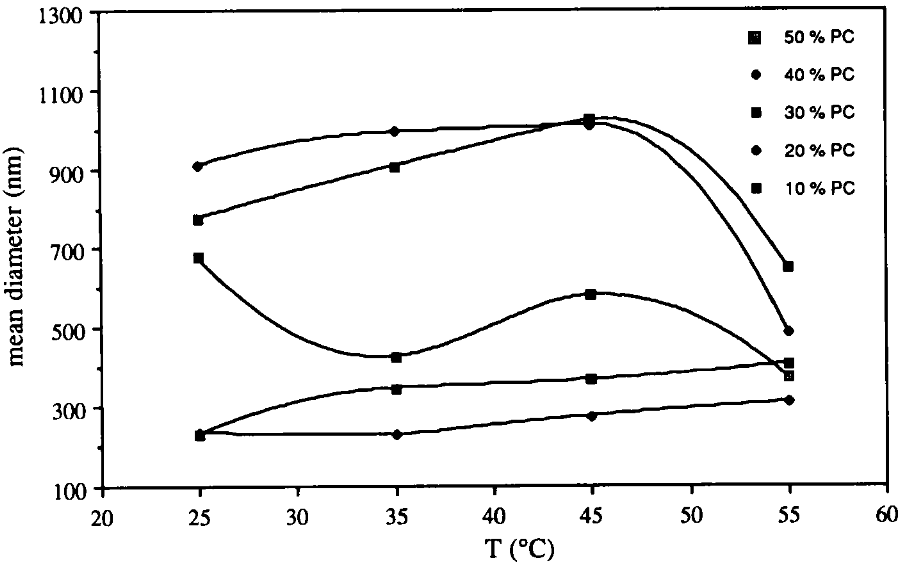

FIGURE 2b

Mean diameter-Temperature relations for liposomes with quinolone proportion until 90 %

transition. This was not apparent beyond 30% of quinolone presence. It is also apparent in Figures 2a and 2b that liposomes shrink at 55°C, which is more relevant when drug is in excess (percentages of quinolone higher than 70 %). These results may be in agreement with reported calorimetric studies of enrofloxacin, which were unable to establish the interaction between quinolone and PC (13) in apparent controversy with other studies in which affinity between both molecules appears to be established (3). Thus, monolayer studies (14) confirm the formation of quinolone domains in the lipid bilayer. However, two phenomena, interaction and exclusion, could occurs as a function of the drug/phospholipid ratio and thus explain the unusual behaviour at 55°C when drug concentration is higher than 70%.

Having established the feasibility of a quinolone-liposome system, we now study a complete formula, including additives, and the possibilities of scaling up this product with a guarantee of physical stability. The preparation proposed is shown in Table 2 and the role of each compound is summarized. Thus, quinolone, becomes soluble by moderate heating in acid solution owing to the formation of an acid salt, which explains the role of the lactic acid. The formula includes two lipids, phosphatidylcholine and cholesterol (at molar ratio 2:1) which constitute the bilayer matrix. The presence of cholesterol is desired because it reduces drug release. With this basic composition, liposomes will form spontaneously by evaporation of the organic solvent, ethanol, as can be expected from the method which is formally based on the organic extraction method followed by a phase change. The presence of remaining ethanol is obiously critical. These were evaluated to be less than 5 % of initial mass and are removed during the

TABLE 2

Composition Preparation

Ingredient	Amount (g / dm^3)	Role
quinolone	10	drug
lactic acid	10	increase solubility
ethanol	25 (initial)	organic solvent
CHOL	5	reduces drug release
PC	25	bilayer matrix
D-α-tocoferol	5	antioxidant
glycerol	60	co-solvent
Acetate buffer	q.s.	aqueous solvent
pH	4.7	

following steps (15). To avoid chemical degradation of phosphatidyl- choline, formula includes D-α-tocoferol, which protects against oxidation. Glycerol, the last component, was used as water-miscible co-solvent. As shown in Table 1, from encapsulation efficiency results, it may enhances quinolone-association with the lipid matrix. On the other hand, glycerol increases medium viscosity and could act as an additional factor reducing leakage. As can be seen in Table 1 comparable homogeneous populations of liposomes were obtained by using the complete formulae and with only the matrix of PC. The main factors limiting liposome comercialization may have been associated with large scale production and the stability of the particular liposome formulation. To solve the first homogenization with microfluidizer (16) and other devices based on filtration under pressure (17) have been propesed in order to obtain large volumes of liposomes of uniform size and lamellarity. In contrast to what was expected given the simplicity of the instrument, liposomes were obtained with relative simplicity as can be demonstrated by electron microscopy (18). On the other hand, besides the chemical stability of the drug, the liposomal formulation has to be stable in storage and neither aggregation nor fusion, which would lead to precipitation is desirable. While chemical degradation may be followed by conventional analytical methods, the changes in morphology undergone by vesicles can be followed by PCS which may be taken to be an easier method to evaluate the integrity of liposome preparations.

In the meantime, studies carried out with quinolone-containing liposomes stored for long time (8-12 months) at room temperature, evidenced only minor changes in size and polydispersity (less than 10 %) with a persistent pharmacological action on bacteria. On the other hand, liposomes undergo a 6 % leakage of quinolone after the same period of storage and experiments carried out *in vivo* by i.v. administration of quinolone liposomes to pig and sheep showed similar results to those obtained with solutions freshly prepared. These findings suggest that the pharmacological action depends more on the drug/lipid ratio than on the other characteristics of liposomes. These results are difficult to explain, but it seems that an improvement of the therapeutic index of quinolone could be expected when the drug is carried in or associated with liposomes. Finally, this leads to the conclusion that lipid-quinolone interaction needs further studys, before the beneficial action and the mechanism involved can be understood, whichever formulation is proposed. Research in this direction and the study of other phospholipids as a bilayer, which would avoid quinolone leakage, is now under devolopement in our laboratory.

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