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Formulation parameters of fluoroquinolone-loaded liposomes and in vitro antimicrobial activity ☆

Giovanni Puglisi [a,*], Massimo Fresta [b], Gioacchino Mazzone [a], Pio M. Furneri [c], Gianna Tempera [c]

[a] *Istituto di Chimica Farmaceutica e Tossicologica, Università di Catania, viale A. Doria 6, 95125 Catania, Italy*
[b] *Institut für Polymere, ETH-Zentrum, Universitätstrasse 6, CH-8092 Zürich, Switzerland*
[c] *Istituto di Microbiologia, Università di Catania, via Androne Catania, Italy*

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Abstract

To load pefloxacin and ofloxacin in liposomes, two preparation procedures were carried out, leading to the formation of multilamellar vesicles (MLVs) or reverse-phase evaporation vesicles (REVs). MLVs were able to entrap greater amounts of the two drugs than REVs, especially when the drugs were co-dissolved with the lipid mixture in the organic phase. The encapsulation efficiency was influenced by the presence of a negatively charged lipid in the liposome composition: the greater the content of charged lipidic compound, the larger is the amount of drug entrapped. Among the charged systems, a dipalmitoylphosphatidylcholine-cholesterol-dihexadecyl phosphate mixture (4:3:4 molar ratio) showed the highest trapping capacity. The fluidity of the bilayer could also influence the encapsulation efficiency. In fact, the increase in encapsulation capacity for the lecithin-cholesterol-dihexadecyl phosphate mixture (4:3:4 molar ratio) conformed to the following order: dipalmitoylphosphatidylcholine > dimyristoylphosphatidylcholine > egg phosphatidylcholine. Variation in pH values led to different encapsulation efficiency and release rate. In vitro experiments on the antimicrobial activity of the encapsulated fluroquinolones compared to the free drug demonstrated a reduction of at least 50% of the minimal inhibitory concentration.

Keywords: Ofloxacin; Pefloxacin; Liposome; Encapsulation capacity; Release rate; Antimicrobial activity

1. Introduction

Fluoroquinolones are broad-spectrum antimicrobial agents whose primary mechanism of action is the inhibition of DNA gyrase activity (Wolfson and Hooper, 1985; Hooper et al., 1986). Access to the target site is a major determinant of antibacterial activity, the outer membrane being the major permeability barrier in gram-negative bacteria.

Fluoroquinolones seemed to reach their intracellular target site (DNA gyrase) in *Escherichia coli* by means of a simple diffusion process through the outer and inner membranes (Bedard

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* Corresponding author.

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et al., 1987). Recent studies (Chapman and Georgopapadakou, 1988; Cohen et al., 1988) confirmed the lack of energy-dependent cell uptake of the antimicrobial drugs. In fact, it is generally considered that there are three routes by which antimicrobial agents can penetrate this structure. These are the porin pathway, the hydrophobic pathway (Mikaido and Vaara, 1985) and the self-promoted route used by cationic compounds (Hancock et al., 1981).

The porin pathway through microbial membranes has been shown to be a major route of entry, since any mutation affecting the outer membrane porins resulted in resistance to fluoroquinolones (Hancock et al., 1981; Hooper et al., 1986; Ayoma et al., 1987; Bedard et al., 1987). These mutations were sometimes associated with lower levels of drug uptake. However, before the drug penetration routes can become available, the aqueous layer of polysaccharide side chains of the lipopolysaccharide is encountered in the so-called smooth bacteria. In the case of some quinolones this layer represents a barrier to cell entry.

Colloidal dispersion systems may provide a means of decreasing the dissolution time and improving the bioavailability of drugs that are poorly water soluble (Francés et al., 1991), like fluoroquinolones. Association of pefloxacin and ofloxacin with polymeric carriers or lipidic supramolecular aggregates could improve the bioavailability of these drugs in topical treatment, i.e., ocular administration could be of therapeutic interest.

Liposomes are possible carriers for controlled drug delivery and targeting by the intravenous route. Enhancement of the therapeutic efficacy of antimicrobial agents (Desiderio and Campbell, 1983) and anticancer drugs (Beck et al., 1993) has been reported in the literature. The liposome lipid composition could be arranged in such a way as to increase or to reduce the uptake by the macrophages of the reticuloendothelial system (Gabizon and Papahadjopoulos, 1988).

The use of antibiotic delivery devices should achieve increased drug concentrations at the site of infection resulting from targeting of antibiotic to the infected tissues (Magallanes et al., 1993), improved intracellular antibiotic concentration due to targeting of the antimicrobial agent to the infected cells, and reduced toxicity of potentially toxic antibiotics provided by targeting of drugs to the infectious organisms (away from the host cell). Liposomes may thus represent an excellent device for improving the selective transport of antibiotic in these respects. Furthermore, liposomes, being constituted by natural compounds normally occurring in biological membranes, represent a biodegradable and highly biocompatible drug delivery system which can be administered safely.

The entrapment of the fluoroquinolones in lipid vesicles could be of therapeutic interest. In fact, liposomal devices might be capable of ensuring different pathways of interaction with microbial cells (Nässander et al., 1990), compared to the normal routes followed by fluoroquinolones in entering cells. This behaviour should be useful in the treatment of infections caused by quinolone-resistant bacteria or by microbes which are, normally, poorly sensitive to this class of drugs. In this paper, the interaction occurring between the two fluoroquinolones and liposomes, the formulation parameters and the in vitro response were studied.

2. Materials and methods

2.1. Chemicals

Dipalmitoyl-DL-α-phosphatidyl-L-serine (PS), cholesterol (CH) and egg phosphatidylcholine (EPC) were purchased from Sigma Chemical Co. (St. Louis, USA). 1,2-Dipalmitoyl-*sn*-glycerophosphocholine monohydrate (PC), 1,2-dimyristoyl-*sn*-glycerophosphocholine monohydrate (MC), 1,2-dipalmitoyl-*sn*-glycerophosphoethanolamine (PE), 1,2-dipalmitoyl-*sn*-glycerophosphatidic acid disodium salt (PA) and dihexadecyl hydrogen phosphate (DP) were obtained from Fluka Chemical Co. (Buchs, Switzerland). Before use, the lipid purity (greater than 99%) was assayed by two-dimensional thin-layer chromatography (TLC) on silica gel plates (E. Merck, Darmstadt, Germany). TLC was loaded with lipid solutions in chloro-

form/methanol (3:1 v/v) and eluted first with chloroform/methanol/5 N ammonium hydroxide (60:3:5 v/v) and then with chloroform/methanol/acetic acid/water (12:60:8:3 v/v). Phospholipid phosphorus content was assayed as inorganic phosphate, as described elsewhere (Bartlett, 1959).

For antimicrobial assay, standard bacterial strains indicated in the NCCLS method (National Committee for Clinical Laboratory Standards, 1990) were employed. The assayed bacterial strains were *E. coli* (ATCC 25922), *E. coli* (ATCC 35218), *Enterococcus faecalis* (ATCC 29212), *Staphylococcus aureus* (ATCC 29213) and *Pseudomonas aeruginosa* (ATCC 27853).

Pefloxacin (PFX) was a gift from Formenti Farmaceutici s.p.a. (Milano, Italy); ofloxacin (OFX) was kindly provided by Sigma-Tau s.p.a. (Pomezia, Italy). The purity of these two drugs was greater than 99.5% as assayed by HPLC analysis (Notarianni and Jones, 1988). Double-distilled water was used. All other materials and solvents were of analytical grade (Carlo Erba, Milano, Italy).

2.2. Liposome preparation

The desired amount of lipids was weighed in a round-bottomed flask and dissolved in chloroform in the presence of 30 g of glass beads (2–3 mm mean size) (Carlo Erba, Italy). By evaporating the organic solvent at 30°C, a thin film of dry lipid was deposited on the inner wall of the flask and along the glass bead surface. OFX or PFX (1.4×10^{-3} M final concentration) were added directly to the lipid organic solution (method A) or dissolved in the aqueous buffer (method B). Large multilamellar vesicles (MLVs) were prepared under an atmosphere of nitrogen by hydrating (vortex-mixing) the dry lipid film with isotonic pH 7.4 phosphate-buffered saline (PBS) (0.13 M NaCl, 3 mM KCl, 10 mM sodium phosphate), maintaining a temperature of 45°C during the whole process. A lipid concentration of 50 mg ml^{-1} was routinely employed. The frozen and thawed multilamellar vesicles (FATMLVs) were obtained by freezing the MLVs in liquid nitrogen and thawing the samples in a water bath at the same temperature as that employed for formation of MLVs. This procedure was carried out for eight cycles.

Reverse-phase evaporation vesicles (REVs) were prepared according to a method reported elsewhere (Szoka and Papahadjopoulos, 1978). Briefly, the lipid film, obtained as previously described, was dissolved in diethyl ether and isotonic buffer was then added such that the organic to aqueous phase ratio was 5:1 v/v. The buffer-lipid mixture was first shaken by vortexing, then sonicated for 30 min at 55°C in an ultrasonic bath resulting in a fine emulsion from which organic solvent was slowly removed at 35°C with a rotavapor under a nitrogen stream to produce the liposomes. The resulting viscous gel was resuspended in isotonic phosphate buffer. In this case, the antimicrobial drugs were also co-dissolved with the lipids (method A) or solubilized in aqueous buffer (method B). Following production all liposomes were maintained for 1 h at a temperature of 45°C to anneal the liposome structure.

2.3. OFX and PFX encapsulation efficiency

Free drug was removed from the liposomal suspension by centrifugation. FATMLVs prepared via both methods A and B were centrifuged for 45 min at 12000 rpm, whereas REVs were centrifuged at 20000 rpm for 45 min (Beckman model J2-21 equipped with a Beckman JA-20.1 fixed angle rotor). The drug content in the supernatant was determined by means of spectrophotometric analysis (Uvikon 860, Kontron) at 292 and 276 nm for OFX and PFX, respectively. The drug-loaded liposomes was determined by difference from the drug amount added during vesicle preparation. The encapsulation parameter was expressed as the encapsulation capacity (EC). The EC values were calculated from the ratio between the concentration of the drug entrapped (mmol/ml), and the product of the added drug (mmol/ml) and lipid concentration (mmol/ml) of the liposome suspension.

2.4. Fluoroquinolone release from liposomes

After separation of the free drug, the liposome pellet was made up to 5 ml with pH 7.4 isotonic

phosphate buffer, obtaining a final phospholipid concentration of 30 mg/ml, and submitted to dialysis. The dialysis donor compartment was a Spectra/Por 3 membrane (Mol. Wt. cut-off 3500; Spectrum, Medical Industries, Inc., Los Angeles, CA). The dialysis bag was immersed in the receiver compartment containing 150 ml of the buffer solution or culture medium. Experiments were performed at 37°C in a thermostated bath. At timed intervals, 1-ml samples were removed from the receiver compartment and the fluoroquinolone content was determined by spectrofluorimetric analysis (Hitachi F-2000). The excitation and emission wavelength were $\lambda_{ex} = 286$ nm, $\lambda_{em} = 452$ nm, and $\lambda_{ex} = 275$ nm, $\lambda_{em} = 436$ nm for OFX and PFX, respectively. Each sample removed was replaced by an equal volume of the dialysis liquid. Drug retention in the various liposome types was calculated by means of the following equation:

$$\% \text{ drug retention} = (C_0 - C_f)100/C_0$$

where C_o is the initial concentration of drug entrapped and C_f denotes the free fluoroquinolone concentration at various times.

2.5. Partition coefficient

The partition coefficient of PFX and OFX was determined with octanol/0.1 M buffer at different pH values. The buffer solutions were phosphate (pH 4, 6.05, 7.47 and 7.88) and pH 4.63 acetate. The organic solvent and the aqueous buffer were poured together into a glass flask under thorough stirring for 24 h at a thermostated temperature of 37°C, in order to presaturate each other fully. A 1 mg aliquot of PFX or OFX was added to 10 ml of buffer plus 10 ml of octanol previously presaturated. These mixtures were immersed in a thermostated water bath at 37°C and mixed by mechanical shaking for at least 3 h. At the end of the incubation period the mixtures were centrifuged at $3000 \times g$ for 10 min and then allowed to stand for 10 min for phase separation. Aliquots were removed from the organic and aqueous phases and then assayed by spectrofluorimetric analysis, reporting data on a calibration straight line. The partition coefficient (log P) was expressed as the logarithm of the ratio of the amount of the two antimicrobial drugs in the organic phase to that in the aqueous phase.

2.6. Differential scanning calorimetry (DSC)

DSC scans were recorded using a Mettler DSC 12E instrument. Calibration of the temperature scale and energy (ΔH) was carried out using indium as standards. The plotting range as full-scale deflection was set to 1 mW; the noise was < 20 μW. The thermometric and calorimetric sensitivity was 56 μV/°C and 3 μV/m, respectively. Each experimental measurement presented an accuracy of ± 0.4°C with a reproducibility and resolution of 0.1°C. The ΔH values were calculated from the peak area using Mettler system software (TA89E).

For DSC analysis, 40 μl of the liposomal suspension containing about 2.5 mg of phospholipids were sealed in an aluminium pan and submitted to calorimetric assay. The thermograms of the liposomal suspension were recorded during both heating and cooling over the range 20–60°C at a scanning rate of 0.5°C/min.

2.7. Size analysis

Dynamic light scattering was used to determine the vesicle size. The apparatus consisted of an argon ion laser (Spectra Physics model 166), equipped with a PC 8 sample holding system (Malvern, Worcs, UK), a microcontrol precise mechanical goniometer and a Melles-Griot f 150 optical system. Light coming from the laser source at 514.5 nm was focused on the vesicle sample in a quartz cuvette maintained at a constant temperature of 21 ± 0.01°C by a Haake F3-R thermostat (Berlin, Germany). The intensity of the scattered light was detected at 90° to the incident beam with an RCA (Fishers, NY, USA) model C 31034 photomultiplier cooled to -30°C.

The radius of the liposome in a sample was calculated from the correlating function (Aurora et al., 1985).

2.8. Freeze-fracture electron microscopy

Morphological characterisation was carried out by freezing the liposome colloidal suspension starting from room temperature, with the propane-jet technique (Müller et al., 1980). The cryofixed samples were fractured at −165°C in a Balzen BAF 300 at a pressure of 1×10^5 Pa. Platinum/carbon replicas were produced and examined in a Philips EM 301 electron microscope at 100 kV.

2.9. Lamellarity analysis

^{31}P-NMR was employed to provide an indication about the lamellarity of the various liposome samples. Briefly, Mn^{2+} was added to the vesicle colloidal suspension (2 ml, 50 μmol phospholipid per ml in a 10 mm diameter NMR tube) at levels (5 mM) sufficient to a broadening beyond detection of the ^{31}P-NMR signal from those phospholipids facing the external medium. The impermeability of the liposomes to Mn^{2+} was straightforward to demonstrate by following the time course of the signal intensity, which for the MC systems herein investigated was stable over a period of several days.

Proton-decoupled, ^{31}P-NMR spectra were recorded on a GN500 MHz spectrometer operating at 202.45 MHz. Trimethyl phosphate (Aldrich) (10% in D_2O) was used as a reference and set at 0.0 ppm. The number of scans was 9000 with a spectral width of 20 kHz and 8K data points. 90° pulses were used with a pulse delay of 250 ms and an acquisition time of 205 ms. An exponential multiplication corresponding to a 50 Hz line broadening was applied prior to Fourier transformation. Signal intensities related to peak areas were determined by means of computer integration.

From the integrated peak areas found before and after Mn^{2+} addition, the percentage relative loss of signal (RLOS), arising from the interaction between the paramagnetic ions and the outer phospholipid head groups, was calculated (Bergelson and Barsukov, 1977). The average number of bilayers $\langle N \rangle$ was calculated from the following expression (Schwartz and McConnell, 1978):

$$\langle N \rangle = 100/(2 \times \mathrm{RLOS}).$$

In fact, the principle of this method is comparable with the spin-label reduction technique previously reported (Schwartz and McConnell, 1978). When the phosphate head groups are randomly distributed between the lamellae, the RLOS value equals the fraction of the bilayer surface area that forms the outer surface of the liposomes. Therefore, the $\langle N \rangle$ value represents the surface average number of bilayers. This condition is verified only when the liposome mean size is much larger than the total thickness of the bilayers. Another important condition to be fulfilled is the independence of the bilayer number distribution per vesicle from the diameter of the liposomes, so that the effect on RLOS is proportional to the surface area of the vesicles.

2.10. Determination of bacterial susceptibility

The antimicrobial activity of the ofloxacin and pefloxacin liposome entrapped was determined in comparison to the free drug by means of MIC determinations on ATCC standard strains (National Committee for Clinical Laboratory Standards, 1990). The minimal inhibitory concentrations (MIC) was determined by a broth microdilution assay. Iso-sentitest broth (Oxoid) (100 μl) was inoculated into microtiter wells. The stock solution of each drug (in growth medium) (100 μl) was added to the first wells and serial 2-fold dilutions were performed with a multichannel pipette beginning in the second well, discarding the final 100 μl. A total of 11 concentrations of each drug were prepared. A suspension of organisms (1 μl) was added to each well containing the drug and the control well. Plates were sealed with transparent acetate and incubated at 37°C under atmospheric conditions up to 18 h. The final concentrations of each micro-organism was 1×10^5 CFU/ml. The MIC was defined as the lowest concentration of antibiotic inhibiting the visible growth after 18 h.

A positive control (growth) consisting of organisms in broth, a negative (sterility) control consist-

Fig. 1. Freeze-fracture electron micrograph of reverse-phase evaporation vesicles obtained in PBS following preparation procedure method A. The lipid mixture was PC/CH/DP (4:3:4 molar ratio) at a concentration of 50 mg/ml. Magnification, ×30000.

ing of uninoculated broth, and drug control consisting of broth containing the highest concentrations of drug were included for each bacterial tested. Each assay was repeated six times against each antimicrobial agent and six additional times on a different day with all drugs to ensure reproducibility of results.

3. Results and discussion

In order to encapsulate PFX and OFX, two different preparation procedures were carried out. The freeze-thaw procedure resulted in the formation of multilamellar vesicles, whereas, uni- or oligolamellar vesicles were obtained by means of the reverse-phase evaporation procedure (Fig. 1). The characterisation of the two liposome formulations was performed by light scattering and freeze-fracture electron microscopy, as concerns size analysis, and ^{31}P-NMR for lamellarity investigation.

At the end of the freeze-thaw procedure, no influence on the morphological characteristics (size and lamellarity) of the liposome systems was demonstrated, as compared to the initial MLV suspension (data not reported). FATMLVs showed greater mean size values than those obtained for REVs (Table 1). Also, the polydispersity index of the FATMVL systems presented a rather high value (approaching unity), compared to the REV formulations. This behaviour underlined the formation of a heterodispersed vesicle suspension. The phospholipid composition was able to influence either the mean size or the lamellarity of the lipid vesicles. In fact, on increasing the molar ratio of the charged phospholipid, a slight increase in size and a reduction in the number of lamellae per vesicle were observed (Table 1). These behaviours could be due to the interlamellar electrostatic repulsion forces that led to an enhancement of the captured water phase and a consequent reduction in lamellarity. No particular effect of lipid composition on the liposome characteristics was observed for the REV systems. REVs are poorly influenced by the presence of a charged phospholipid, owing to the presence of one or a few lamellae per lipid vesicle. This factor makes the repulsion forces irrele-

Table 1
Size, polydispersity index and lamellarity of the various liposome types at different lipid compositions

Phospholipid mixtures	FATMLVs [a]			REVs [b]		
	Size (nm) [c]	PI [d]	$\langle N \rangle$ [e]	Size (nm) [c]	PI [d]	$\langle N \rangle$ [e]
PC/CH/DP 4:3:4	1307	1.3	5.5	215	0.3	1.7
PC/CH/DP 4:3:2	1240	1.1	7.3	207	0.2	1.9
PC/CH 6:4	945	1.7	11.0	265	0.6	2.4

[a] MLVs submitted to eight freeze-thaw cycles. The thawing procedure was performed at 45°C.
[b] Liposome suspension obtained by the reverse-phase evaporation procedure.
[c] Each value is the average of six different experiments.
[d] Polydispersity index value.
[e] The lamellarity values $\langle N \rangle$ are calculated according to the equation reported in section.

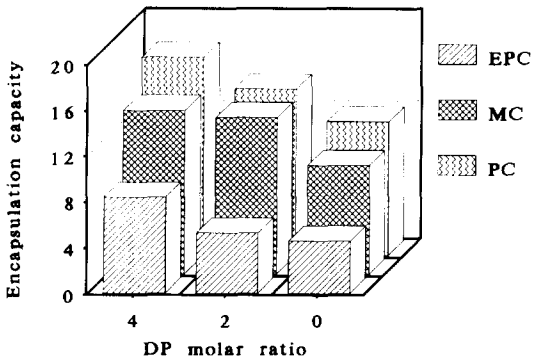

Fig. 2. Encapsulation capacity values of PFX-loaded liposomes obtained with FATMLV preparation procedure method A in the presence of PBS. The phospholipid mixtures were composed of different kinds of lecithins (PC, MC or EPC at a molar ratio of 4), CH at a molar ratio of 3 and DP at different molar ratio.

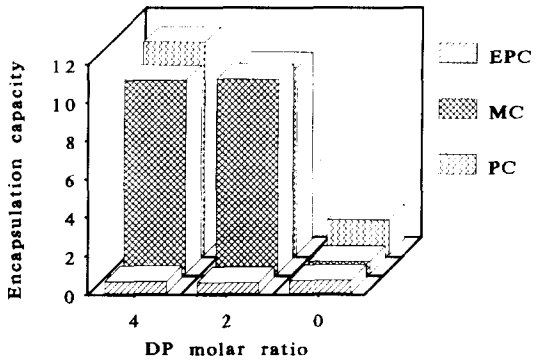

Fig. 3. Encapsulation capacity values of PFX-loaded liposomes obtained with FATMLV preparation procedure method B in the presence of PBS. The phospholipid mixtures were composed of different kinds of lecithins (PC, MC or EPC at a molar ratio of 4), CH at a molar ratio of 3 and DP at different molar ratio.

vant for liposome size and lamellarity. The presence of MC or EPC, instead of PC, as well as of a different charged phospholipid (PS, PA, PE) did not greatly influence the liposomal features (data not reported).

The encapsulation capacity of PFX-loaded FATMLVs as a function of liposome lipid composition is reported in Fig. 2. The FATMLVs, obtained in PBS according to method A, showed satisfactory encapsulation efficiency. It is noteworthy that the presence of a charged phospholipid, as well as DP, was able to influence the liposome encapsulation parameters. In particular, the amount of entrapped PFX is related to the molar ratio of DP present in the lipid vesicle formulation (Fig. 2). Furthermore, the encapsulation parameters depend on the fluidity of the phospholipid bilayer: the lower the fluidity, the greater is the encapsulation capacity (Fig. 2). Concerning the phospholipid mixture reported in Table 2 and Fig. 2, the membrane fluidity conformed to the following decreasing order: EPC > MC > PC.

FATMLV preparation method B led to lower drug encapsulation yields that those obtained with method A. Also, in this case, a dependence of the encapsulation parameters on the bilayer fluidity and molar ratio of liposome DP was demonstrated (Fig. 3). This dependence was more remarkable following FATMLV preparation method B. In fact, liposomes containing EPC displayed the lowest EC values, such as the lipid vesicles prepared without DP (Fig. 3). The higher encapsulation capacity values obtained with method A are probably due to a suitable interaction between the drug (codissolved with the lipid material) and the lipid bilayer, which led to partitioning of PFX between the aqueous and organic

Table 2
Encapsulation capacity values of PFX-loaded FATMLVs prepared using both method A and B in pH 4.65 isotonic acetate buffer

Phospholipid mixtures	EC values	
	Method A [a]	Method B [b]
PC/CH/DP 4:3:4	9.548	6.299
PC/CH/DP 4:3:2	10.501	5.088
PC/CH 6:4	10.267	5.467
MC/CH/DP 4:3:4	8.637	4.526
MC/CH/DP 4:3:2	9.645	3.967
MC/CH 6:4	10.517	3.462
EPC/CH/DP 4:3:4	12.565	2.327
EPC/CH/DP 4:3:2	9.650	1.066
EPC/CH 6:4	2.371	1.079

[a] The drug was codissolved with the lipid during the preparation of the film.
[b] PFX was solubilized in the aqueous phase.
Each value is the average of three different experiments.

phases. In contrast, in method B, since the drug was solubilized in the buffer, interaction between PFX and the lipid matrix of the bilayer was more difficult and drug binding with the liposomal membrane was reduced. As concerns the entrapment values, method B was much more affected than method A by the presence of charged phospholipids in the liposome composition. The presence of charged compounds in the bilayer is able to increase the interlamellar spaces in a multilamellar system, leading to an enhancement of the captured aqueous volume.

To encapsulate PFX, methods A and B were also carried out in the presence of a pH 4.6 isotonic acetate buffer in order to evaluate the effect of the pH on the encapsulation parameters. In both cases, PFX entrapment values were lower than those obtained in a pH 7.4 phosphate buffer (Table 2). The decrease in encapsulation efficiency was more evident for liposomes presenting negatively charged phospholipids in their composition. In fact, at this pH value the charged phospholipid head groups are partially protonated and, as a consequence, there occurs a reduction in the interbilayer repulsive forces, which leads to a decrease in the trapped aqueous volume. In addition, it should be considered that PFX at a pH value of 4.6 is also partially protonated, as demonstrated by the partition coefficient reported in Fig. 4. Therefore, there is a reduction in the affinity of PFX for the phospholipid bilayer, which contributes to the lowering of antimicrobial drug encapsulation.

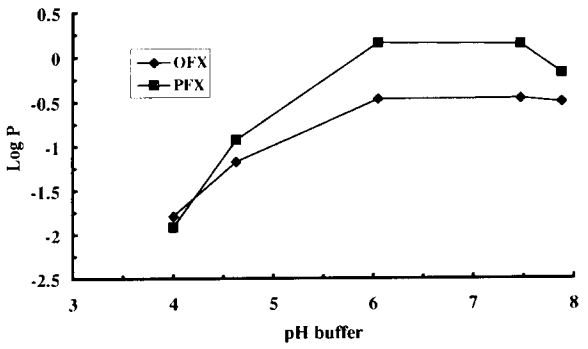

Fig. 4. Dependence of the fluoroquinolone partition coefficient on the pH value of the aqueous buffer.

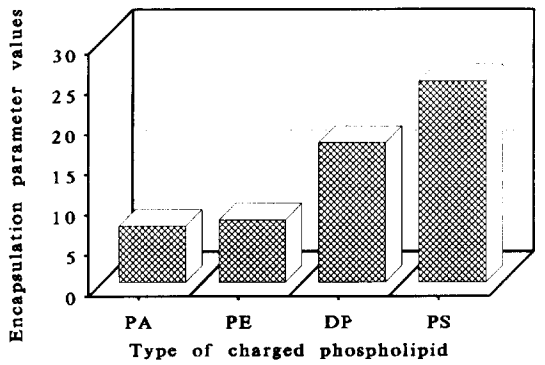

Fig. 5. Encapsulation capacity values of PFX-loaded FATMLVs prepared using method A in the presence of PBS. The phospholipid mixtures were composed of PC, CH and a charged phospholipid (4:3:4 molar ratio).

Beside the employment of DP to obtain lipid vesicles with a net negative charge, phospholipid mixtures containing PA, PE and PS were prepared. The mixture presenting PS showed the highest encapsulation values (Fig. 5), probably due to the ability of PS to form hydrogen bonds with the molecules entrapped in the liposome matrix (La Rosa et al., 1992a,b).

The OFX encapsulation parameters for FATMLV systems were lower than those of PFX (Table 3). In this case, no noticeable difference was observed between preparation methods A and B (Table 3). A slight reduction in drug entrapment was observed when pH 4.6 acetate buffer was used instead of pH 7.4 phosphate buffer (data not reported). This behaviour was due to the more hydrophilic nature of OFX, in agreement with the partition coefficient values (Fig. 4).

As shown in Fig. 6, for both fluoroquinolones there is a dependence of the encapsulation capac-

Table 3
Encapsulation capacity values of OFX-loaded FATMLVs prepared in PBS

Phospholipid mixtures	EC values	
	Method A	Method B
PC/CH/DP 4:3:4	8.797	9.907
PC/CH/DP 4:3:2	7.998	4.044
PC/CH 6:4	2.329	1.621

Each value is the average of three different experiments

ity on the pH value of the saline buffer employed during the preparation procedure. The highest levels of entrapment were achieved when the two antimicrobial drugs were in an uncharged form (pH value of 7.4, see Fig. 4). At pH values higher or lower than neutrality a reduction of drug entrapment was found.

The process of preparation of REV by either method A or B achieved lower drug entrapment values than those obtained with the FATMLV procedure (data not reported). Probably, the FATMLV preparation process was able to ensure a more appropriate interaction between the drugs and the phospholipid bilayers. For this reason, FATMLVs presenting the best encapsulation parameter values were used for further experiments.

The binding of these two drugs to the liposomes was thus the result of two different processes. One of these is ionic binding between negatively charged phosphate groups of the phospholipid and the positively charged piperazine ring at the C-7 position of the fluoroquinolone. The second form of binding is likely to be related to hydrophobic forces between the lipid bilayer and the drug. This hypothesis is in agreement with that reported elsewhere (Bedard and Bryan, 1989) and is confirmed by our DSC experiments. In fact, many antibiotics and chemotherapeutic agents show significant lipophilicity. These compounds are inherently capable of diffusing through the lipid bilayer region of any biological membrane. Fluoroquinolones contain more than

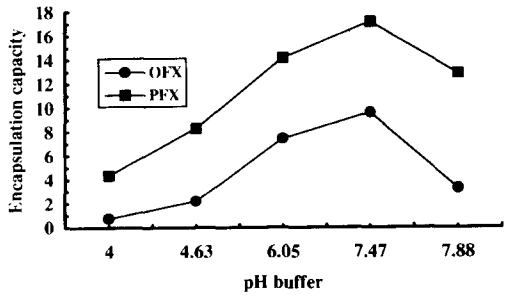

Fig. 6. Dependence of the PFX and OFX encapsulation capacity values on the pH value of the liposome preparation medium. Liposomes were prepared using FATMLV method A. The phospholipid mixture was PC/CH/DP (4:3:4 molar ratio) for both drugs.

Table 4
Thermodynamic parameters of the phospholipid mixture PC/DP (1:1 molar ratio) obtained in PBS in either the absence or presence of the drug

Drug	T_c (°C) [a]	ΔH (kcal/mol) [b]
Without drug	61.1	8.30
With PFX	57.8	5.76
With OFX	60.8	7.06

Each value is the average of three different experiments.
[a] Transition temperature values from gel to liquid crystalline phase.
[b] Enthalpy changes related to the phase transition from gel to liquid crystalline state.

one functional group that may become charged by protonation or deprotonation at physiological pH. The presence of these groups greatly affects both the rates of diffusion and the final equilibrium distribution of these compounds across the membrane bilayer.

DSC analysis is a powerful and non-perturbing thermodynamic technique for characterising the thermotropic behaviour of lipid bilayers and investigating drug-membrane interactions (La Rosa et al., 1992a,b; Fresta et al., 1993). There was no CH present in the phospholipid mixtures submitted to DSC analysis, since its presence would not allow any interaction to be demonstrated between drugs and the lipid membrane, owing to its depressant action on the phase transition from the gel to liquid crystalline phase (Puglisi et al., 1992). As reported in Table 4, the presence of PFX or OFX caused a variation in the thermodynamic parameters of the phase transition. The presence of OFX influenced only the enthalpic parameters, reducing the ΔH values of the gel to liquid crystalline phase transition. In contrast, the presence of PFX caused a lowering of both ΔH and temperature values of the phase transition. This phenomenon was due to the considerable perturbation of the phospholipid bilayer arrangement, as a result of the hydrophobic part of PFX incorporated inside the lipid core of the liposomal matrix. The lowering of the transition temperature is probably caused by an interaction between the negatively charged part of the molecule and the ionic head groups of the phospholipids, leading to increased repulsion among

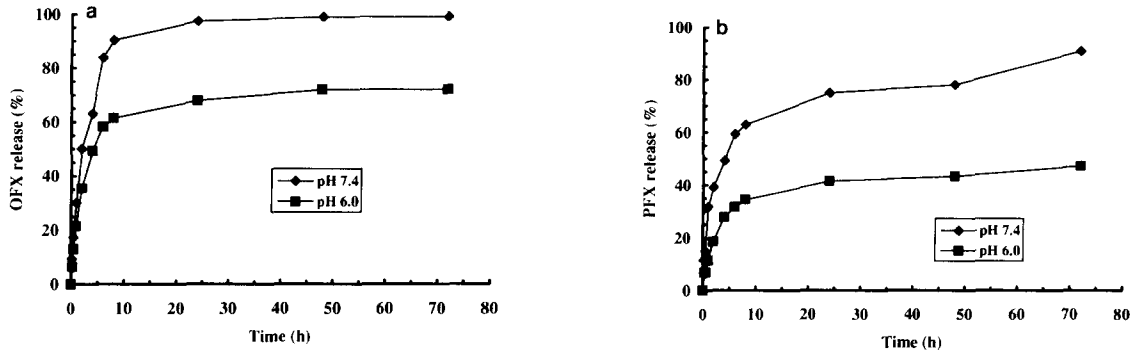

Fig. 7. Release profile of OFX (a) and PFX (b) from FATMLVs (MC/CH/DP 4:3:4 molar ratio) prepared using method A in the presence of buffer at different pH values. The release experiment was carried out at different pH values.

the charged phospholipids. Only a simple interaction with the hydrophobic zone of the bilayer was observed for OFX, whose influence was weaker than that of PFX, being less hydrophobic than PFX (see log $P_{(oct)}$; Fig. 4). Thus, OFX led to a reduction in the ΔH value related to the phase transition of about 15%, whereas PFX was able to lower the ΔH value about 30% with respect to the initial value.

The pH value of the environment also plays an important role in the release properties of the various liposome formulations. In fact, in PBS the drug release rate is more rapid than in pH 6.0 isotonic phosphate buffer (Fig. 7). This behaviour could be due to the greater hydrophobic character of the two drugs at pH 7.4, which permits easier permeation through the liposome membrane. At lower pH, with increasing drug hydrophilic character and protonation a stronger interaction between the drug and the phospholipid head group occurs and, as a consequence, slower release is achieved. The fluidity of the liposome bilayer scarcely exerted any influence on the release profile. The presence of PC instead of MC caused only a slight reduction in the release rate (data not reported).

The in vitro susceptibilities to OFX and PFX of the investigated strains are similar to those showed by NCCLS M7-A2 (M100S-3). The MIC values obtained with liposome formulations are shown for comparison. Both OFX and PFX-loaded liposomes showed MIC values which were at variance with those obtained with the free drugs. In particular, the MIC values were always lower except for *P. aeruginosa*. The MIC values are 1–2-fold less than those obtained with the free drugs. PFX was less active than OFX in either the encapsulated or free form (Tables 5 and 6). The in vitro activity of the two fluoroquinolones was thus enhanced as regards the MIC value by the liposome formulations. Our previous experiments demonstrated that free liposomed exert no activity against bacteria up to a concentration 100-times higher than that used throughout our experiments.

The liposome formulation could therefore be a

Table 5
MIC values of free and liposome-incorporated OFX against different bacterial strains

Bacterial strain	Free OFX	MC/CH/DP (4:3:4)	MC/CH/PS (4:3:4)
E. coli ATCC 25922	0.12	0.0525	0.0636
E. coli ATCC 35218	0.12	0.0525	0.0763
E. faecalis ATCC 29213	2	0.4201	1.0185
S. aureus ATCC 29213	1	0.8403	0.5093
P. aeruginosa ATCC 27853	1	1.6806	2.0371

Table 6
MIC values of free and liposome-incorporated PFX against different bacterial strains

Bacterial strain	Free PFX	MC/CH/DP (4:3:4)	MC/CH/PS (4:3:4)
E. coli ATCC 25922	0.12	0.0778	0.0774
E. coli ATCC 35218	0.12	0.0778	0.0929
E. faecalis ATCC 29213	2	1.2444	1.2383
S. aureus ATCC 29213	2	1.2444	0.6191
P. aeruginosa ATCC 27853	2	2.4889	2.1762

suitable device for antimicrobial agents, such as OFX and PFX. Liposomes were able to increase the antimicrobial activity of these two drugs against different bacterial strains. In addition, the liposomal delivery system represents a highly versatile drug carrier, suitable for both topical or systemic administration.

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References

Aurora, T.S., Li, W., Cummins, H.Z. and Haines, T.H., Preparation and characterization of monodisperse unilamellar phospholipid vesicles with selected diameters from 300 to 600 nm. *Biochim. Biophys. Acta*, 820 (1985) 250–258.

Ayoma, H., Sato, K., Kato, T., Hirai, K. and Mitsuhashi, S., Norfloxacin resistance in a clinical isolate of *Escherichia coli*. *Antimicrob. Agents Chemother.*, 31(1987) 1640–1641.

Bartlett, G.R., Phosphorus assay in column chromatography. *J. Biol. Chem.*, 234 (1959) 466–468.

Beck, P., Kreuter, J., Resska, R. and Fichtner, I., Influence of polybutylcyanoacrylate nanoparticles and liposomes on the efficacy and toxicity of the anticancer drug mitoxantrone in murine tumor models. *J. Microencapsul.*, 10 (1993) 101–114.

Bedard, J. and Bryan, L.E., Interaction of fluoroquinolone antimicrobial agents ciprofloxacin and enoxacin with liposomes. *Antimicrob. Agents Chemother.*, 33 (1989) 1379–1382.

Bedard, J., Wong, S. and Bryan, L.E., Accumulation of enoxacin by *Escherichia coli* and *Bacillus subtilis*. *Antimicrob. Agents Chemother.*, 31 (1987) 1348–1354.

Bergelson, L.D. and Barsukov, L.I., Topological asymmetry of phospholipids in membranes. *Science*, 197 (1977) 224–230.

Chapman, J.S. and Georgopapadakou, N.H., Routes of quinolone permeation in *Escherichia coli*. *Antimicrob. Agents Chemother.*, 32 (1988) 438–442.

Cohen, S.P., Hooper, D.C., Wolfson, J.S., Souza, K.S., McMurry, L.M. and Levy, S.B., Endogenous active efflux of norfloxacin in susceptible *Escherichia coli*. *Antimicrob. Agents Chemother.*, 32 (1988) 1187–1191.

Desiderio, J.V. and Campbell, S.G., Intraphagocitic killing of *Salmonella typhimurium* by liposome-encapsulated cephalothin. *J. Infect. Dis.*, 148 (1983) 563–570.

Francés, C., Veiga, M.D., Español, O.M. and Cadòrniga, R., Preparation, characterization and dissolution of ciprofloxacin/PEG 6000 binary systems. *Int. J. Pharm.*, 77 (1991) 193–198.

Fresta, M., Villari, A., Puglisi, G. and Cavallaro, G., 5-Fluorouracil: various kinds of loaded liposomes: encapsulation efficiency, storage stability and fusogenic properties. *Int. J. Pharm.*, 99 (1993) 145–156

Gabizon, A. and Papahadjopoulos, D., Liposome formulations with prolonged circulation time in blood and enhanced uptake by tumours. *Proc. Natl. Acad. Sci. USA*, 85 (1988) 6949–6953.

Hancock, R.E.W., Raffle, V.J. and Nicas, T.I., Involvement of the outer membrane in gentamicin and streptomycin uptake and killing in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.*, 19 (1981) 777–785.

Hooper, D.C., Wolfson, J.S., Souza, K.S., Tung, C., McHugh, G.L. and Swartz, M.N., Genetic and biochemical characterization of norfloxacin resistance in *Escherichia coli*. *Antimicrob. Agents Chemother.*, 29 (1986) 639–644.

La Rosa, C., Grasso, D., Fresta, M., Ventura, C. and Puglisi, G., Phospholipid vesicles as a drug delivery system: I. Interaction of cytidine 5′-diphosphate choline with charged and zwitterionic phospholipids. *Thermochim. Acta*, 195 (1992a) 139–148.

La Rosa, C., Grasso, D., Fresta, M., Ventura C. and Puglisi, G., Phospholipid vesicles as a drug delivery system: II. A

study on kinetic fusion between vesicles containing CDP-choline and dipalmitoylphosphatidylcholine vesicles. *Thermochim. Acta*, 198 (1992b) 181–190.

Magallanes, M., Dijkstra, J. and Fierer, J., Liposome-incorporated ciprofloxacin in treatment of murine salmonellosis. *Antimicrob. Agents Chemother.*, 37 (1993) 2293–2297.

Mikaido, H. and Vaara, M., Molecular basis of bacterial outer membrane permeability. *Microbial Rev.*, 49 (1985) 1–32.

Müller, M., Meister, N. and Moor, H., Freezing in a propane jet and its application in freeze fracturing. *Mikroskopie*, 36 (1980) 129–140.

Nässander, U.K., Strom, G., Peeters, P.A.M. and Crommelin, D.J.A., Liposomes. In Chasin, M. and Langer, R. (Eds), *Biodegradable Polymers as Drug Delivery Systems*, Dekker, New York, 1990, pp. 261–338.

National Committee for Clinical Laboratory Standards, Methods for dilution antimicrobial susceptibility for bacteria that grow aerobically, 2nd Edn, Approved Standard M7-A2, NCCLS, Villanova, PA, 1990.

Notarianni, L.J. and Jones, R.W., Method for the determination of ofloxacin, a quinolone carboxylic acid antibiotic, by high-performance liquid chromatography. *J. Chromatogr. Biomed. Appl.*, 431 (1988) 461–464.

Puglisi, G., Fresta, M., La Rosa, C., Ventura, C.A., Panico, A.M. and Mazzone, G., Liposome as a potential drug carrier for citicoline (CDP-choline) and effect of formulation conditions on encapsulation efficiency. *Pharmazie*, 47 (1992) 211–215.

Schwartz, M.A. and McConnell, H.M., Surface areas of lipid membranes. *Biochemistry*, 17 (1978) 837–840.

Szoka, F. and Papahadjopoulos, D., Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation. *Proc. Natl. Acad. Sci. USA*, 75 (1978) 4194–4198.

Wolfson, J.S. and Hooper D.C., The fluoroquinolones: structures, mechanism of action and resistance and spectra of activity in vitro. *Antimicrob. Agents Chemother.*, 28 (1985) 580–586.