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Influence of physicochemical properties of fluoroquinolones on encapsulation efficiency in liposomes

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Abstract

Three fluoroquinolones, ciprofloxacin, norfloxacin and a newly synthesized compound (CNV-8912), were encapsulated in liposomes of DPPC at pH 7.40 and encapsulation efficiency was determined by two methods: spectrophotometry of isopropanol-lysed vesicles and HPLC. These methods are compared with a view to the reproducibility and sensitivity of each technique. Physicochemical properties such as octanol-buffer coefficients and pKs were determined in order to interpret the encapsulation efficiency values in terms of affinity for lipid environments and microspeciation at pH 7.40.

Keywords: Fluoroquinolones; Liposomes; Octanol:Buffer partition coefficients; Ionization constants

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1. Introduction

Although some studies have shown the therapeutic efficacy of fluoroquinolones encapsulated in liposomes (Majumdar et al., 1992; Di Ninno et al., 1993), few details are avalaible from these papers about the methods used to obtain liposomes or the efficiency of the encapsulation process. To our knowledge, only papers from this laboratory (Pons et al., 1993; Pons et al., 1995; Mestres et al., 1994) provide information about the encapsulation of fluoroquinolones in liposomes. Several lines of evidences suggest (Carrera et al., 1993; Hernández-Borrell et al., 1993) that fluoroquinolones can diffuse passively across lipid bilayers and, therefore, some caution should be exercised in the preparation and storage of fluoroquinolone liposomes.

Frequently, physicochemical properties of drugs may be overlooked before testing liposomes preparations. This can not the case for fluoroquinolones because they have been extensively studied and much information is available

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about their properties, e.g. aqueous solubility (Ross and Riley, 1990), acid ionization constants (Ross and Riley, 1992), octanol-buffer partition (Ross et al., 1992), complexation with metal ions (Ross et al., 1992; Ross et al., 1993) and spectroscopical characteristics (Montero et al., 1994).

A point of crucial importance derives from the zwitterionic amphoteric nature of fluoroquinolones (Takács-Novák et al., 1990). Indeed, these molecules are ionized over the whole pH range and this property may influence their distribution and affinity for lipid environments, particulary bilayers. Consequently, we expect to find differences in the efficiency of the encapsulation in liposomes as a function of the pH.

In this paper, three fluoroquinolones, ciprofloxacin, norfloxacin and a newly synthesized compound (CNV-8912), have been studied. Physicochemical properties such as pKs and partition coefficients in octanol/buffer, which are generally accepted as an indication of the affinity of drugs for lipid environments, have been investigated in order to correlate those properties with their encapsulation in liposomes. On the other hand, the relative concentrations of the molecular forms present in solution at a given pH can be calculated from ionization constants obtained by potentiometry (Ross and Riley, 1992). With this approach, it is possible to interpret the mechanism of encapsulation in liposomes and obtain information about the factors that may influence the stability of these preparations.

Here we discuss two diferent methods for the quantitation of the amount of fluoroquinolones encapsulated. The first consists in the measurement of the absorbance of the drug after addition of an organic solvent (e.g. isopropanol), which destroys the liposome integrity and releases the drug. The second method consists in a regular HPLC assay. Both methods are currently used in many laboratories but, as discussed below, there are differences in the results obtained. These are mainly due to interferences and the limit of detection that should be considered for better reproducibility and comparison between laboratories.

2. Materials and methods

2.1. Materials

Ciprofloxacin, Norfloxacin and L-α-dipalmitoylphosphatidylcholine (DPPC) specified as 99% pure were purchased from Sigma Chemical Co. (St.Louis, MO); 1-cyclopropyl-1,4-dihydro-4-oxo-6-fluor-7-(N-3-methyl-piperazinyl)-3-quinolincarboxylic acid (named here CNV-8912) was obtained from CENAVISA Labs. (Reus, Spain). Phospholipid purity was assessed by thin layer chromatography (developing solvent $CHCl_3$-CH_3OH-H_2O-HCO_2H, 80:25:3:1, H_2SO_4 spray and chart staining). 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.). All other common chemicals were HPLC grade. Gel for chromatography was obtained from Pharmacia (Upsala, Sweden).

2.2. Buffer solutions

The buffer solutions used were: 0.15 M Acetate at pH 4.96, 0.15 M Tris hydroxymethyl-amino-methane at pH 7.46, and 0.15 M borate pH 9.10; $\mu = 0.15$ M with NaCl.

2.3. Partition coefficient determinations

The partition coefficient was determined between octan-1-ol and buffer using the following method. Briefly, 100 μl of a stock solution of fluoroquinolone (20 $\mu g\ ml^{-1}$) was diluted with 1.9 ml of buffer and mixed with 2 ml of octan-1-ol (the organic and aqueous phases were mutually saturated). The two phases were vortexed for 1 min and agitated for 3 h in a shaking water bath at 25 ± 0.1°C (control experiments showed that equilibrium was achieved in approximately 3 h). After equilibration, the octan-1-ol phase was removed with a Pasteur pipette and both phases were assayed spectrophotometrically to determine concentration. The partition coefficient was calculated as the ratio between molar concentrations in octan-1-ol and aqueous phase.

2.4. Determination of protonation macroconstants

For the potentiometric measurements, a Crison micropH 2001 pH-meter (precision of display 0.01, 1 mV) was used. pKa values were determined according to conventional potentiometric techniques. Aliquots (50 ml) of 0.5 mM fluoroquinolone and 2 mM HCl or NaOH solutions were titrated with 50 mM NaOH or HCl, respectively. The temperature was kept at 25.0 ± 0.1°C and the electrode was calibrated with standard buffer solutions (Crison) in the 4–7.02 range.

2.5. Vesicle preparation and determination of encapsulation efficiency

The interior of a conical 10 ml tube was coated with a thin lipid film by evaporation to dryness under nitrogen stream of a standard chloroform:methanol (V:V) solution containing DPPC and the fluoroquinolone at the desired ratio. After liophilization, multilamellar vesicles (MLVs) were obtained by hydration of the lipid film in the appropriate buffer. Heating was applied between vortexing periodes to give a stable milky suspension. Vesicles were then extruded 10 times through 100 nm polycarbonate membranes (Nuclepore, U.S.A.) following a method described elesewhere (Hope et al., 1985).

The fluoroquinolone content associated with liposomes was determined after the separation of free quinolone from liposome-entrapped quinolone following the same minicolumn centrifugation method described elsewhere (Pons et al., 1995). After chromatographic separation, an aliquot of the effluent was suitably diluted and the amount of fluoroquinolone encapsulated was determined by measuring the absorbance of isopropanol-lysed vesicles at maximum absorbance wavelength. Another aliquot was assayed in parallel by HPLC (Carrera et al., 1993). Phospholipid concentrations were determined as described elsewhere (Keough and Kariel, 1987).

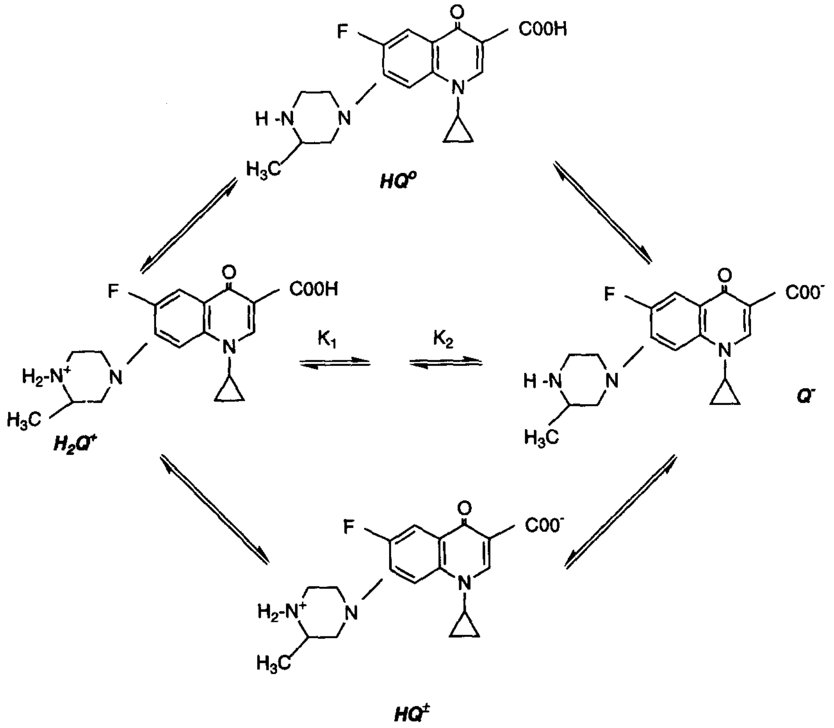
2.6. Spectrophotometry and particle size analysis

Spectrophotometric determinations were performed at room temperature on a Hewlett-Packard 8451 diode array spectrophotometer (Hewlett-Packard Co., San Diego, CA). Absorption quarz cells with a path length of 1 cm were used throughout. For the preparation of calibration curves for spectrophotometry, a standard solution containing 0.2 mg ml^{-1} of fluoroquinolone was prepared with the buffer in a 10 ml volumetric flask. A series of appropriate dilutions in the same buffer were prepared to check adherence to Beer's law at the maximum absorption wavelength of each compound.

Photon correlation spectroscopy (PCS), also known as quasielastic light scattering (QELS), was used to determine particle size and polydispersity of samples (Chen et al., 1976). 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. Samples suitably diluted (usually 0.30 mM DPPC) were transfered to a cuvette and placed inside a 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 interfaced with an Olivetti PC calculator for data analysis. The measurements of the correlation function were anayzed and the diffusion coefficient was obtained (Koppel, 1972). The hydrodynamic radius was then obtained from the Stokes-Einstein relation.

2.7. HPLC determinations

HPLC assays were performed in a Waters 600 multisolvent delivery system (Waters-Millipore, Bedfor, MA) connected to a fluorescence detector set at suitable excitation-emission wavelengths. A Spherisorb OD52 column (5 μM, 15 x 0.46 cm) was used. The mobile phase was acetonitrile-salt solution (13:87, v/v). The salt solution contained KH_2PO_4 (33mM), Na_2HP_4 2 H_2O (33 mM) and



Scheme 1. Scheme of CNV-8912 dissociation.

$(n\text{-}C_4H_9)$ 4 N^+ HSO_4^- (4.38 mM). The mobile phase was adjusted to pH 3.0 using phosphoric acid (85%) inmediately prior to use and defined at a flow rate of 1 mL/min at room temperature. Injection volume was 25 μl using an autosampler. Standards of fluoroquinolones were prepared in water for injection. Standard solutions contained drug in the concentration range 5-100 μg/ml. A calibration curve was obtained by plotting the peak height area against the drug concentration. Adherence to linearity and reproducibility were established.

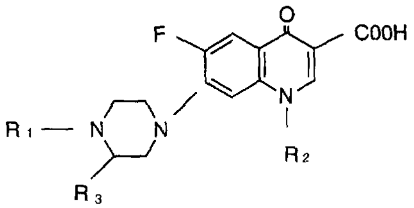
2.8. Data analysis

Linear regression equations were calculated with the StatWorks™ Package on a Macintosh LCIII personal computer.

3. Results and discussion

Fig. 1 Shows the structures of the three fluoroquinolones studied. Ciprofloxacin and norfloxacin molecules differ only in the presence of a cyclopropyl ring and an ethyl substituent attached to the N-1 position of the quinoline moiety. On the other hand, CNV-8912 resembles ciprofloxacin with a methyl substituent at the C-3 position of the piperazinil group. On the basis of molecular structures and previous studies (Montero et al., 1994), the spectrophotometric and fluorimetric characterisitics of these compounds we assumed to have similar features.

Fluoroquinolones show multiple protonation equilibrium in solution, as expected from the two proton binding sites (see Scheme 1) on the carboxylate of the nucleus and on the amine of the 7-substituent. Four different forms, normally called microspecies, are present in solution: H_2Q^+, Q^-, the zwitterionic $HQ^\pm$ species and the uncharged species HQ°. Although microspecies concentration can be estimated by a combination of a spectrophotometric method and potentiome-



	R_1	R_2	R_3	λ_{max}	λ_{exc} (nm)	λ_{em}
Ciprofloxacin	H	cC_3H_5	H	278	276	448
Norfloxacin	H	C_2H_5	H	278	277	446
CNV-8912	H	cC_3H_5	CH_3	278	278	448

Fig. 1. Structures of the fluoroquinolones studied. Included are the maxima wavelengths at UV and fluorescence.

try (Takácks-Novák et al., 1990), the protonation macroconstants (Ka_1 and Ka_2) can be used to determine the neutral-zwitterion ratio (Ross et al., 1992). This is crucial information for liposome encapsulation, since it is believed that only the neutral species (and probably the zwitterion) can permeate freely through the bilayer (Furet et al., 1992). This suggests a mechanism for leaking by simple diffusion after encapsulation in liposomes, which may depend on the proportion of each microspecies present. The pKa values and the ratio between the zwitterionic and the neutral species of each fluoroquinolone studied are shown in Table 1 along with the octanol-buffer coefficients. These results agree with values reported in the literature (Ross and Riley, 1992). The concentration of the neutral form is higher for CNV-8912 and lower for norfloxacin and ciprofloxacin in all media studied (Table 1). However, in all cases, the concentration of the zwitterionic form predominates over the neutral microspecies. Since the total concentration of the zwitterionic and neutral forms are maximal near neutrality, it is concluded that the highest octanol-buffer coefficients shown by CNV-8912 may be correlated with the predominance of those microspecies, which are those that may diffuse to the octanol phase (Takácks-Novák et al., 1992).

The lipophilic character of a drug, revealed by octanol-water coefficients, is normally accepted as a measurement of the affinity phospholipids used as a matrix in liposome preparations. Then, hypothetically, neutral conditions in which octanol-buffer coefficients were higher may be indicative of a tendency of fluoroquinolones to incorporate into liposomes at pH 7.40. Since fluoroquinolones are amphoteric, they could be partially integrated into the bilayer structure, as demonstrated by monolayer epifluorescence spectroscopy (Montero et al., 1994), as well as in the internal aqueous volume of the liposomes. This is a fairly common situation for non-polar drugs but it is beyond the purposes of this paper to elucidate the location of the drug molecules and their exact mechanism of interaction with phospholipids.

Two different methods were used to determine encapsulation efficiencies. The first consisted in the addition of acidified isopropanol, which solubilizes the liposomes. This led to the formation of a milky solution which clarified quickly and spontaneously. The disrupting effect causes increases of up to 990 nm mean diameter, and maximum polydispersity coefficients of the resulting particles (Table 2). This method (Majumdar et al., 1992) is effective only with 0.068 N HCl in 90% methanol and vigorous vortexing of the samples, although we found that the disrupting effect of the iso-

Table 1
Apparent (D) and thermodinamic (Kd) partition coefficient octanol-buffer, pKs (mean ± S.D., $n = 3$) and zwitterionic/neutral species ratio

	Ciprofloxacin	Norfloxacin	CNV-8912
D Acetate (pH 4.96)	0.0069 ± 0.0086	0.0547 ± 0.0007	0.0117 ± 0.0002
D Borate (pH 9.00)	0.0307 ± 0.0052	0.0300 ± 0.0028	0.0450 ± 0.0038
D Tris (pH 7.46)	0.0740 ± 0.0540	0.0677 ± 0.0036	0.1855 ± 0.0104
Kd	0.106 ± 0.030	0.095 ± 0.019	0.307 ± 0.105
pKa_1	6.09 ± 0.11	6.13 ± 0.10	6.54 ± 0.11
pKa_2	8.74 ± 0.55	8.51 ± 0.14	8.50 ± 0.03
$[HQ\pm]/[HQ^\circ]$	446.59	239.80	91.14

propanol is more effective and shows better reproducibility.

The three fluoroquinolones and DPPC are soluble in isopropanol at the working concentrations. Indeed, the application of Beer's law when liposomes are dissolved in the organic solvent could be appropiate because no interference of DPPC on fluoroquinolone absorption spectrum was observed. Although a UV multicomponent analysis was possible, we determined the phospholipid concentratrion independently by a classic phosphorus assay (Keough and Kariel, 1987). On the other hand, after the disruption step with isopropanol, by measuring the absorbance at λmax the concentration of the drug was obtained from the respective standard curve (Table 3). Encapsulation efficiencies obtained by this method are shown in Table 4 along with results obtained by HPLC. These results are obtained from aliquots of the same sample, therefore discrepancies between values obtained by spectrophotometry and chromatography should be attributed exclusively to non analytical signals. Given the characteristics of the material obtained after addition of isopropanol, the higher values obtained by spectrophotometry can be attributed to the light-scattering background caused by large particles present in solution, as demonstrated by PCS measurements (Table 2). The use of spectrophotometry could lead to erroneous determination of high encapsulation efficiencies. Nevertheless, the quantitation of fluoroquinolone concentrations encapsulated in liposomes determined spectrophotometrically could be in some way corrected (Majumdar et al., 1992). Conversely, the error associated with the encapsulation efficiencies calculated may be substantial. Such a criticism should be taken seriously because in many papers the technical details followed to determine encapsulation efficiency are avoided, especially in those papers focused on biological applications of liposomes, rather than microencapsulation technology.

In view of these results, we conclude that HPLC should be selected over spectrophotometry to quantify drug concentration when it is available. Otherwise light-scattering corrections have

Table 2
Quasi-elastic light scattering measurements of liposomes of before and after lysis with isopropanol

	Diameter mean ± S.D.		Polydispersity	
	Before lysis		After lysis	
Ciprofloxacin	148.2 ± 23.1	0.051	990.0 ± 1228.4	0.98
Norfloxacin	123.1 ± 19.1	0.056	890.0 ± 1290.1	1.00
CNV-8912	117.0 ± 24.1	0.060	947.0 ± 1422.4	1.00

Table 3
λ max. absorption, dinamic range ($\triangle$), molar absortibity (ϵ) in isopropanol acidified, number of samples (n) and correlation coefficient (r)

Drug	λ nm	$\triangle$ (drug) (μM)	ϵ ($M^{-1}cm^{1}$)	n	r
Ciprofloxacin	282	0-44	37 000	10	0.997
Norfloxacin	282	0-50	38 000	12	0.998
CNV-8912	282	0-38	35 000	15	0.999

Table 4

Encapsulation efficiency (μmol fluoroquinolone/μmol DPPC) in liposomes: (I) 0.7:0.3, (II) 0.5:0.5, (III) 0.3:0.7 DPPC/drug molar initial ratio at pH 7.40. Values are the average of three independent determinations

Drug	(I)		(II)		(III)	
	UV-Vis	HPLC	UV-Vis	HPLC	UV-Vis	HPLC
Ciprofloxacin	0.0055	0.0030	0.0039	0.0012	0.0028	0.0013
Norfloxacin	0.0041	0.0006	0.0035	0.0013	0.0023	0.0006
CNV-8912	0.0018	0.0063	0.0016	0.0054	0.0020	0.0038

to be performed to minimize background signals using an adequate reference. Moreover, because no derivatization was needed, HPLC become the simplest method to determine encapsulated fluoroquinolones in liposomes.

The present study shows that using a higher concentration of phospholipid enhances encapsulation efficiencies (see I in Table 4). On the other hand, we can conclude that when the methyl substituent is attached to the C-3 position of the piperazinil of ciprofloxacin the main consequence is an enhancement of the encapsulation efficiency of fluoroquinolones in liposomes. These findings can be correlated with the highest octanol-buffer coefficient shown by CNV-8912, which is also in concordance with the lowest $HQ\pm/HQ^{\circ}$ ratio calculated. These results reinforce the hypothesis that at neutral pH the bilayer-drug interaction is mainly hydrophobic (Bedard and Bryan, 1989) and, therefore, the drug with greatest lipophilicity shows the maximum encapsulation. Similarly to other nonpolar drugs, fluoroquinolones could be sequestered in the bilayer structure (Mestres et al., 1994) and, therefore, we can assume that the mechanism of encapsulation is not dependent only upon the internal aqueous volume of the vesicles. Hence, we can conclude that the extent of the encapsulation of fluoroquinolones is a function of pH. Although encapsulation efficiencies obtained approaches the expected efficiencies for amphoteric drugs in oligolamellar vesicles at the lipid concentrations used for encapsulation, the stability of those liposomes encapsulating fluoroquinolones should be monitored continuously to detect drug leakage before their eventual use. In the light of their amphoteric nature, neutral and possibly the zwitterionic species could diffuse passively through the lipid bilayer to some extent until equilibrium is reached. Further stuties on kinetical stability and encapsulation at other pHs are currently under development in our laboratory.

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