ELSEVIER

International Journal of Pharmaceutics 171 (1998) 75–86

international journal of pharmaceutics

6-Fluoroquinolone–liposome interactions: fluorescence quenching study using iodide

J.L. Vázquez [a], M.T. Montero [a], J. Trias [b], J. Hernàndez-Borrell [a,*]

[a] *Department de Fisicoquímica, Facultat de Farmacia, U.B., 08028-Barcelona, Spain*
[b] *Department of Biology, Versicor, 270 East Grand Ave., San Francisco, CA 94080, USA*

Received 8 December 1997; received in revised form 26 March 1998; accepted 24 April 1998

Abstract

Two models (octanol:buffer and lipid:buffer) were used to estimate the interaction of ciprofloxacin and two alkyl derivatives with neutral membranes. To investigate the existence of a mechanism of permeation through the lipid bilayers quenching methods of fluorescence were applied to ciprofloxacin liposomes in order to discriminate relative membrane location of ciprofloxacin. Different mechanisms of quenching appear depending on conditions (pH 4.80, 7.40 and 9.10) which were interpreted under the basis of 6-fluoroquinolone microspeciation and the ability of only the neutral microspecies to permeate across apolar environments. No major effects of temperature (below and above transition temperature, T_m) on quenching mechanism were observed. The high accessibility for iodide shown by ciprofloxacin and its methyl and ethyl derivatives together with the low drug/phospholipid ratio indicate a fast release of the drug from liposomes. Previously the quenching effect of iodide on ciprofloxacin and its inability to access the apolar hydrophobic regions of the bilayers were studied.

Keywords: Ciprofloxacin; Fluorescence quenching; Permeation; Quasi-elastic light scattering; Transition temperature

1. Introduction

Fluoroquinolones are bactericidal drugs broadly used to treat a large variety of infections. They inhibit bacterial gyrase and topoisomerase IV and are most active against Gram-negative bacteria. Newer fluoroquinolones, such as ciprofloxacin, have increased in vitro activity against Gram-positive bacteria and slow growing mycobacteria but cannot be used for treatment of some respiratory tract infections because of an unfavourable pharmacokinetics profile. Ciprofloxacin has also show significative activity against *Mycobacterium avium–M. intracellulare* complex (MAC) (Klopman et al., 1993). A better delivery system of quinolone could increase the concentration of the drug in the lower respiratory tract and yield a more effective drug formulation.

* Corresponding author.

0378-5173/98/$19.00
PII: S0378-5173(98)00154-9

The affinity of the target, gyrase and topoisomerase, for the drug and the ability of quinolones to cross the membranes and avoid efflux systems are the most important factors for the overall susceptibility of bacteria to quinolones. Thus, interaction of quinolones with membranes is key to understanding the resistance and susceptibility of a strain of a drug. In addition to the permeability barrier due to the bacterial membranes, intracellular pathogens have an additional protection layer because drugs have to reach the inside of the cell before reaching the pathogen.

Liposomes have been used to deliver small molecules into a cell. Liposomes carrying fluorescence probes have been delivered to intracellular compartments in macrophages (Oh and Straubinger, 1997). Ciprofloxacin can be encapsulated in liposomes and used to clear intracellular MAC infection in macrophages derived from human peripheral blood monocytes with better efficacy than the free drug (Majumdar et al., 1992; Montero et al., 1996).

The interaction between the lipid layer and ciprofloxacin is not fully understood. Using monolayers and DSC techniques Mestres et al. (1994) have shown that there is a slight interaction between fluoroquinolones and neutral phospholipids but enriched drug domains can be segregated from the lipid membrane (Nag et al., 1996). The distribution of ciprofloxacin in liposomes has not been investigated and a study would help understand the interaction of this compound with biological membranes, passive permeation to the cytoplasm and for liposome formulations (Georgopapadakou, 1995; Pons et al., 1995).

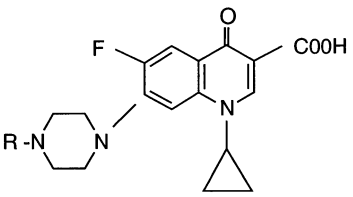
Fluoroquinolones are intrinsic fluorescent amphoteric molecules and phospholipid thermotropic in nature therefore the influence of pH and T are key factors in understanding the fluoroquinolone–phospholipid interaction. In this paper, we studied the interaction of ciprofloxacin and ciprofloxacin derivatives (see structures on Table 1) with liposomes using fluorescence quenching.

2. Experimental procedures

2.1. Materials

Ciprofloxacin was provided by Cenavisa Labs. (Reus, E). *N*-methyl and *N*-ethylciprofloxacin were synthesized following a procedure described earlier (Montero et al., 1997). The purity of the compounds was determined with HPLC and IR. L-α-Dipalmitoylphosphatidylcholine (DPPC), specified as 99% pure was obtained from Sigma (St. Louis, MO). 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). 1,6-Diphenyl-1,3,5-hexatriene (DPH) was obtained from Sigma. Deionized water was distilled from sodium permanganate in an all-glass apparatus and further purified by reverse osmosis through the Milli-Q system (Millipore). Buffer solutions: acetate buffer pH 4.80, 0.15 M; Tris hydroxymethyl-amino-methane pH 7.40, 0.15 M; borate buffer pH 9.10, 0.15 M; $I = 0.15$ M. All other common chemicals were ACS grade and all organic solvents were redistilled. Gels for chromatography were obtained from Pharmacia (Upsala, Sweden).

Table 1
Structure of ciprofloxacin and their alkyl derivatives, thermodynamic octanol/buffer (P) and bilayer/buffer (K_p) partition coefficient (mean ± S.D., $n = 3$)



R	P	K_p (°C)	
		25	50
–H	0.116 ± 0.029	—	—
$-CH_3$	0.553 ± 0.015	0.150 ± 0.030	0.182 ± 0.025
$-CH_2-CH_3$	1.133 ± 0.143	0.324 ± 0.025	0.511 ± 0.032

2.2. Solubility determination

Solubilities of ciprofloxacin and methylciprofloxacin were determined following a method described elsewhere (Ross and Riley, 1990) with minor modifications. Saturated solutions of each drug were prepared in buffers described above and stirred overnight at room temperature. Samples were filtered and concentration was measured spectrophotometrically.

2.3. Bilayer–buffer partition coefficient determinations

A method described elsewhere (Ma et al., 1991) was used, with a minor modification, to measure bilayer–buffer partition coefficients. Phospholipid films of DPPC were dried on the walls of glass tubes following evaporation of a chloroform/methanol (50:50, v/v) solvent. The dried film was dispersed using 1 ml of appropriate buffer solution to obtain different drug:phospholipid mol ratios. Multilamellar vesicles (MLV) were obtained after a few cycles of freezing and thawing alternated with vigorous vortexing. MLVs were incubated below or above DPPC transition temperature (T_m) and partition coefficients were estimated after centrifugation (12500 rpm) in a Eppendorf DuPont Sorvall RM C14 bench centrifuge. The concentration of the drug in the supernatant was determined spectrophotometrically by UV absorption. Partition coefficients were calculated using,

$$K_p = \frac{(C_o - C_s)}{C_o} \times \frac{W_{aq}}{W_b} \quad (1)$$

where C_o is the concentration of the drug in a control buffer containing a given amount of fluoroquinolone but no lipid, C_s the concentration of the supernatant depleted of fluoroquinolone by the presence of phospholipids and W_{aq} and W_b the weight of the aqueous and phospholipid phases in the sample respectively.

2.4. Obtention of liposomes

Chloroform/methanol (50:50, v/v) stock solutions of DPPC and ciprofloxacin were mixed to obtain an initial molar ratio of 0.70 drug:phospholipid. The interior of a conical tube was coated with a thin lipid film by the evaporation to dryness under nitrogen stream and a short period of lyophilization to ensure absence of organic solvent traces. MLVs were obtained by hydration in the appropriate buffer. Freezing and thawing cycles were applied between vortexing periods to get a milky stable suspension. The suspension was extruded ten times through 100 nm polycarbonate membranes (Nuclepore) to obtain large unilamellar vesicles following a method described elsewhere (Hope et al., 1985). Size and polydispersity of liposome preparations were systematically monitored by quasi-elastic light scattering using an Autosizer IIc photon correlation spectrophotometer (Malvern Instruments, UK). Fluoroquinolone content associated with liposomes was measured after the separation of free quinolone from liposome-entrapped quinolone using the minicolumn centrifugation method described elsewhere (Pons et al., 1993). An aliquot of the effluent was diluted after chromatographic separation, and the amount of fluoroquinolone encapsulated was measured in parallel by HPLC (Montero et al., 1996). Phospholipid concentrations were determined as described elsewhere (Keough and Kariel, 1987).

2.5. Column equilibration methods

A Sephadex G-50 column (1 × 40 cm) was previously equilibrated with Tris buffer pH 7.40 containing the same concentration of ciprofloxacin that was used in quenching experiments (see below). Liposomes of DPPC (1 ml) were incubated for 20 min with the same volume of solution of drug (pH 4.80, 7.40, 9.10) of the same concentration as that in the column. Samples were then loaded onto the column and the flow rate regulated by a peristaltic pump at 10 ml/h. After elution with column solution fractions were collected and absorbance measured at 272 nm.

2.6. Fluorescence quenching studies

Intrinsic ciprofloxacin fluorescence was used to measure the amount of free compound.

Table 2
Solubility (S) (mg/ml) and Drug:DPPC ratios (D/Ph, mol/mol) determined after chromatography of extruded liposomes at three different buffers (mean ± S.D., $n = 3$)

	pH					
	4.80		7.40		9.10	
R	S	D/Ph	S	D/Ph	S	D/Ph
–H	1.461	0.011 ± 0.001	0.679	0.055 ± 0.003	1.475	0.028 ± 0.002
$-CH_3$	1.351	0.014 ± 0.002	0.278	0.076 ± 0.004	1.269	0.031 ± 0.003
$-CH_2-CH_3$	1.020	0.012 ± 0.001	0.282	0.083 ± 0.004	1.123	0.034 ± 0.001

Ciprofloxacin was excited at 277 nm and fluorescence was monitored at 448 nm at a constant temperature. Quinolone bound to lipid bilayers was measured with fluorescence quenching. All fluorescence measurements were carried out using a SLM-Aminco 8100 spectrofluorometer provided with a jacketed cuvette holder. Isotonicity between liposomes and solutions was checked with a Fiske osmometer. The eventual scattering caused by different concentrations of KI added to liposomes sizes and polydispersities were monitored by quasi-elastic light scattering. In all cases, intrinsic sample fluorescence was corrected with an appropriate blank.

Liposomes encapsulating ciprofloxacin were incubated in a quartz cuvette at 25 and 50°C (below and above T_m of DPPC) in the presence of iodide (I^-) as a quencher agent. Sodium thiosulphate (0.1 mM) was added to the KI solution to prevent I_3^- formation. Data were analysed according to the Stern-Volmer equation for dynamic quenching (Lakowicz, 1983)

$$\frac{F_o}{F} = 1 + K_{sv}[Q] \quad (2)$$

where F_o and F are the fluorescence intensities in the absence and presence of the quencher, Q, respectively and K_{sv} the Stern-Volmer collisional constant.

To distinguish between two populations of fluorophores another treatment based on a modification of Stern-Volmer equation is commonly used (Lehrer, 1971)

$$\frac{F_o}{\Delta F} = \frac{1}{f_a} + \frac{1}{f_a K[Q]} \quad (3)$$

which introduces f_a as the fraction of the initial fluorescence which is accessible to the quencher ΔF being the difference between the fluorescence intensity in absence (F_o) and presence (F) of quencher. Linear regression equations were conducted with the Statworks™ Package on a Macintosh Performa 6200.

3. Results and discussion.

Ciprofloxin showed a moderate value of the thermodynamic coefficient of partition using the octanol–buffer model (Montero et al., 1997) (P in Table 1) and has shown an almost null affinity for DPPC bilayers (K_p in Table 1). These results are in concordance with differential scanning calorimetry studies where a very small effect of 6-fluoroquinolones on the transition width of the DPPC endotherm (Mestres et al., 1994; Nag et al., 1996) was observed. Therefore, in order to enhance phospholipid–drug interaction, ciprofloxacin was incorporated in liposomes preparing a dried film of drug and DPPC instead of resuspending the lipid film in a buffer solution containing the drug. Using this strategy ciprofloxacin undergoes a better and reproducible, although low, incorporation in the bilayer (D/Ph in Table 2) and quenching methods of fluorescence can be rationally applied. Another strategy designed to enhance drug lipophilicity was the introduction of alkyl groups in the N-4 position of the piperazinyl group of ciprofloxacin. As expected, the partition behaviour was found to be

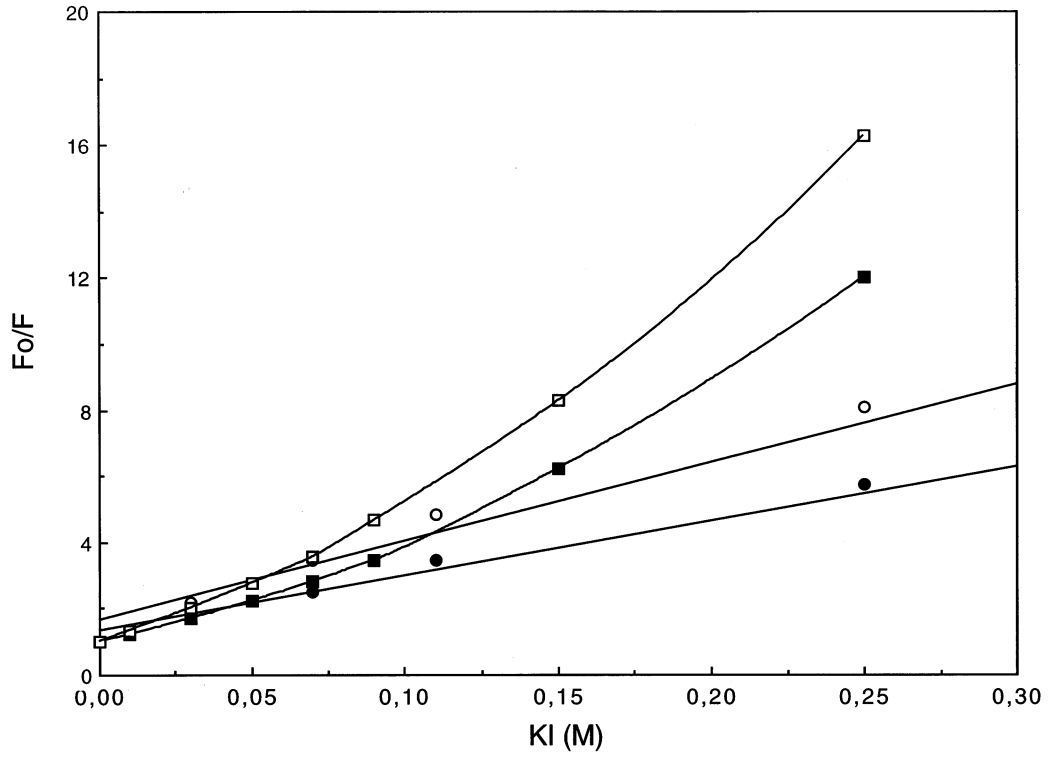


Fig. 1. Stern-Volmer plots for iodide quenching of ciprofloxacin free in acetate buffer, pH 4.70, (■, 25°C and □, 50°C) and encapsulated into liposomes (●, 25 and ○, 50°C).

several times higher for those compounds than for the parent compound (Table 1) as well as for the drug–lipid ratio (Table 2).

Our purpose in using iodide as a quencher was to establish the location of ciprofloxacin in liposomes of DPPC to better understand the mechanism of 6-fluoroquinolones permeation in bacteria. Previously the behaviour of the free drug in solution in front of the quencher was studied. The Stern-Volmer plots of free ciprofloxacin at 25 and 50°C in acetate, Tris, and borate buffers, together with the results obtained with ciprofloxacin encapsulated in liposomes are shown in Figs. 1–3. These results demonstrate that iodide is able to quench ciprofloxacin fluorescence but operates by a collisional and static mechanism at pH 4.80 and 7.40 and by only one of those mechanisms at pH 9.10. These can be verified by the upward curvature at acid and neutral pH and by the linear behaviour obtained in basic media. The coexistence of two mechanisms at pH 4.80 could be due to the absorption of the incident light by ground state complexes because of the propensity of fluoroquinolones to ion-pair at pH 4.80 (Ross et al., 1992). The low solubility at pH 7.40 (Table 2) may be due to the ciprofloxacin to aggregate a this pH which may also be the reason for the existence of two kinds of fluorophore (free and aggregated). It is understood that to discriminate between both mechanisms time resolved fluorescence methods should be applied but they were not available for the present study.

The features of the Stern-Volmer plots for ciprofloxacin liposomes are very different from those observed for the free drug in solution. Ciprofloxacin liposomes showed a linear behaviour of F_o/F data at pH 4.80 (Fig. 1) and 7.40 (Fig. 2), whereas a downward curvature was found at pH 9.10 (Fig. 3). K_{sv} values were calculated from the slope of the linear plots (Figs. 1

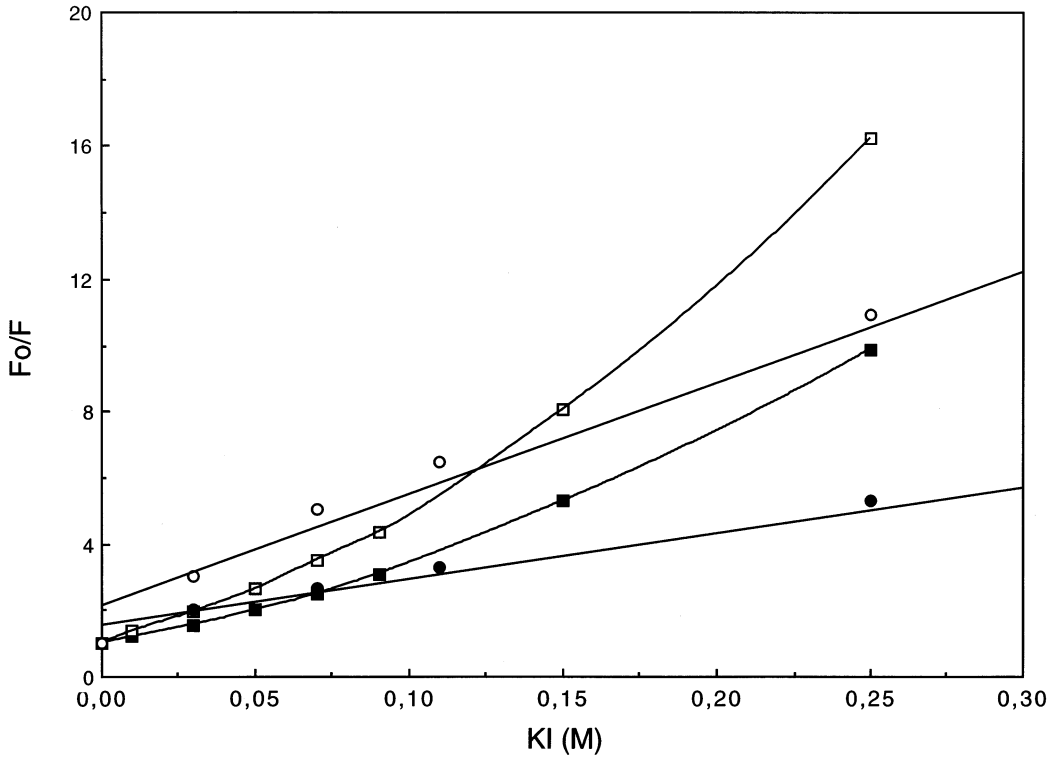


Fig. 2. Stern-Volmer plots for iodide quenching of ciprofloxacin free in Tris buffer, pH 7.40, (■, 25°C and □, 50°C) and encapsulated into liposomes (●, 25 and ○, 50°C).

and 2) indicating that the drug was more efficiently quenched at acidic pH (22 M^{-1}) than at neutral pH (17 M^{-1}). A collisional mechanism seemed to predominate in both cases. On the other hand at pH 9.10 it was only possible to obtain the K_{sv} value (13 M^{-1}) from the four first points of the curve and therefore we must be cautious when drawing conclusions. On first sight we interpret that only one population of fluorophores exists at neutral and acidic pHs, below and above T_m, while at the alkaline pH, the saturation character of the curves, may indicate the existence of an inaccessible fraction of fluorophore. But it should not be forgotten that both molecules, DPPC and ciprofloxacin, are zwitterions. Whether in the pH range used DPPC exists in the neutral zwitterionic state, ciprofloxacin shows different concentration of its four microspecies at each pH (Takáks-Novák et al., 1990). In brief, when the pH of the liposomal suspension was adjusted to 7.40 the zwitterion and neutral microspecies predominate and, at pH 4.80 and 9.10, the cation and anionic species will predominated respectively (Hernández-Borrell and Montero, 1997). Thus the saturation behaviour observed at pH 9.10 (Fig. 3) could be better understood as a consequence of a repulsion effect exerted by the anionic microspecies and the anionic quencher. Conversely, the quenching will be most efficient at pH 4.70, in concordance with the K_{sv} value, because it involves the interaction between the iodide and the cationic microspecies of ciprofloxacin.

Although the permeation through the bilayer is undergone only by the neutral microspecies, which predominates at pH 7.40, we are likely to assume that the drug will remain in this environment in an extent proportional to its concentra-

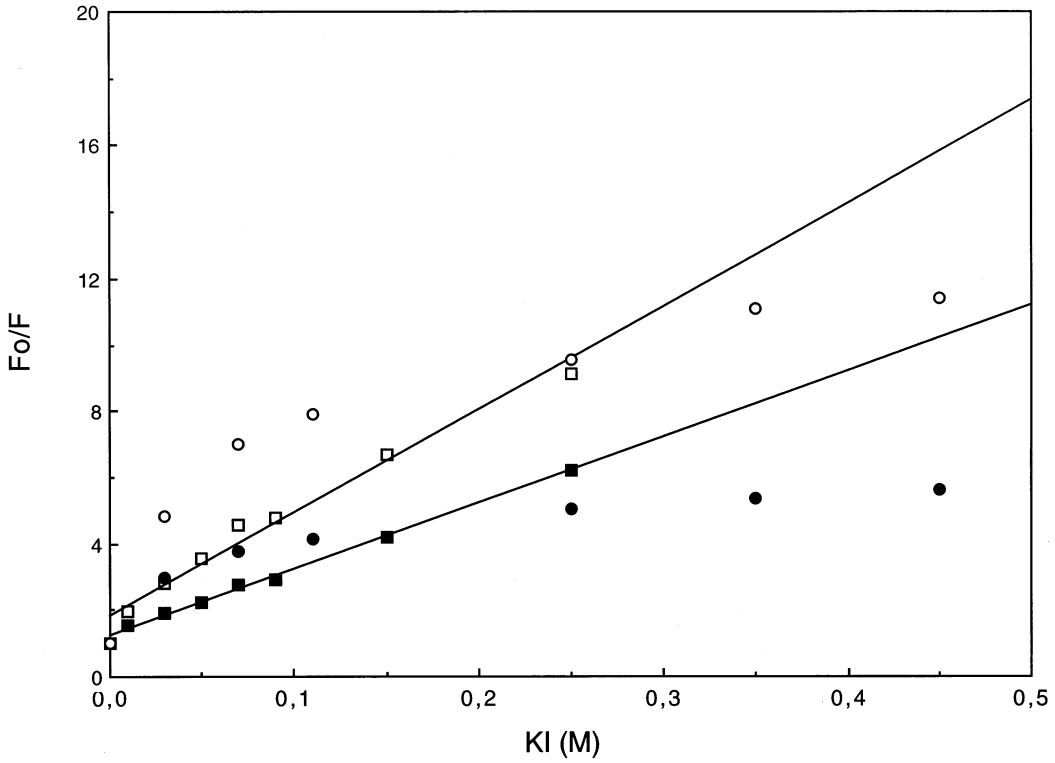


Fig. 3. Stern-Volmer plots for iodide quenching of ciprofloxacin free in borate buffer, pH 9.10, (■, 25°C and □, 50°C) and encapsulated into liposomes (●, 25 and ○, 50°C).

tion at each pH. Effectively, the quencher is clearly efficient in drug solutions and the effectiveness observed indicated the presence of a fraction of chromophore in spaces that are not accessible to iodide. These regions can be the hydrophobic core or the inner aqueous space of liposomes. In fact it was necessary to demonstrate that iodide did not reach those spaces in order to interpret ciprofloxacin location. Normally, it is assumed that iodide quenches fluorophores in hydrophilic environments but some arguments in favour of a possible ability of iodide to quench fluorophores embedded in the hydrophobic matrix have been recently reported (Moro et al., 1993). These authors came to such a conclusion because of the swelling of liposomes under the effect of iodide. Therefore we monitored changes in liposome structure, diameter and polydispersity, by using quasi elastic light-scattering which seems a more adequate technique than absorbance measurements. On this basis no major changes were observed (Fig. 4a) and therefore we discard passive diffusion of iodide in our experimental conditions even with long incubation periods (i.e. 4 min). Additionally, it was shown that iodide was unable to quench DPH fluorescence (Fig. 4b). Consequently, we conclude that iodide quenches ciprofloxacin present only in hydrophilic regions.

To estimate the extent of the accessible fraction quenched we used the Lehrer transformation (Eq. (3)). Although a poor correlation was obtained in some cases (Table 3) we took this representation as a convenient approximation. Namely, f_a values were slightly lower at pH 9.10, than at pH 4.80 and 7.40 (Table 3). The highest values were obtained for ciprofloxacin and ethyl-ciprofloxacin at pH 4.80. Generally the accessibility increased above T_m with the exception of the methyl and ethyl derivatives at pH 9.10. On the other hand the lower values were obtained at pH 7.40. Values

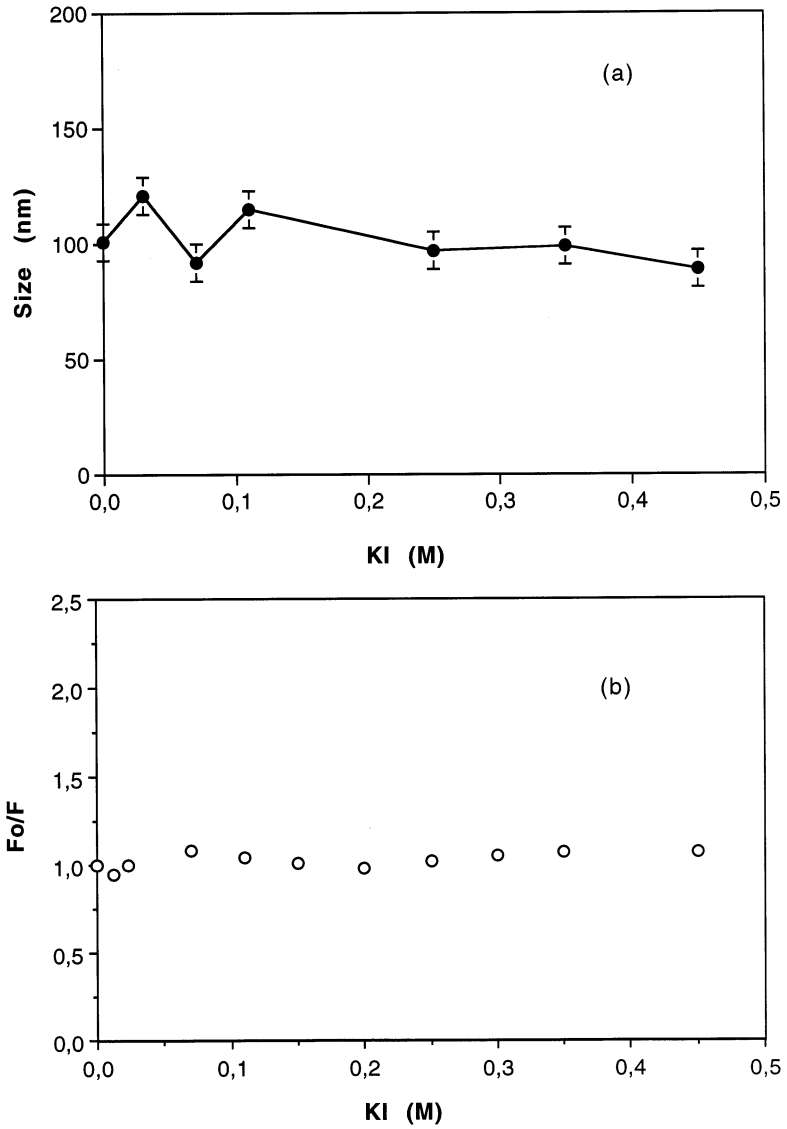


Fig. 4. Liposome size measured by light scattering upon addition of increased concentrations of KI (a); fluorescence quenching of DPH incorporated in liposomes iodide (b).

obtained can be explained taking into account that after the separation by chromatography (Pons et al., 1993) a concentration gradient interior-exterior will be established with a consequent release of the drug. This hypothesis lead us to perform column equilibration studies (Hummel and Dreyer, 1962) which were used to impose pH gradients across vesicles. Thus in response to negative ΔpH (acidic interior and neutral exterior pH) as well as in absence of ΔpH (both, internal and external neutral pH) the resulting elution profiles (Fig. 5a,b) exhibited a peak of ciprofloxacin which represents the associated drug to liposomes, and a smooth trough that can be interpreted as a result of depletion of ciprofloxacin by the vesicles. Interestingly no depletion of drug was observed with positive ΔpH (interior alkaline) (Fig. 5c). The observed deple-

Table 3
f_a, K values, and correlation coefficients (r) of Lehrer equation of the drugs studied

		pH								
		4.80			7.40			9.10		
R	T (°C)	f_a	K (M^{-1})	r	f_a	K (M^{-1})	r	f_a	K (M^{-1})	r
–H	25	0.95	25.24	0.999	0.87	29.37	0.986	0.82	129.64	0.990
	50	0.95	42.68	0.997	0.95	76.27	0.998	0.91	209.61	0.996
$-CH_3$	25	0.66	15.34	0.974	0.77	41.12	0.984	0.68	132.06	0.958
	50	0.69	33.04	0.970	0.88	79.90	0.986	0.50	119.36	0.993
$-CH_2-CH_3$	25	0.90	24.93	0.995	0.56	22.60	0.959	0.65	157.54	0.938
	50	0.95	30.79	0.996	0.69	35.06	0.963	0.58	165.22	0.955

tions occur as a result of the transmembrane distribution of the drug determined by the proton gradient. Thus when ΔpH is negative (Fig. 5a) the neutral microspecies cross the bilayer and becomes protonated on the arrival at the acidic interior. The bilayer is impermeable to the charged microspecies and therefore the drug remains trapped in the liposome. When $\Delta pH = 0$ (Fig. 5b) the neutral form will predominate on both sides of the membrane and a transmembrane equilibrium is established. The depletion observed for neutral liposomes was stronger than those observed for acid liposomes as a result of the higher relative concentration of the neutral microspecies at pH 7.40. The experiments carried out when ΔpH was positive have shown the inability of the drug to permeate the membranes under that condition. This can be interpreted as a consequence of the marginal incorporation of neutral microspecies at pH 9.10. These results suggest the ability of fluoroquinolones to diffuse through the lipid bilayer but at the same time they raise doubts on the encapsulation of these drugs in liposomes using passive methods (Gürsoy and Senyücel, 1997).

The Stern-Volmer plots for methyl-ciprofloxacin showed similar features that those obtained for the parent compound and when the Lehrer equation was used the f_a values (Table 3) showed a significant reduction in the accessibility of iodide for the fluorophore. As a consequence of the introduction of the alkyl substituents the molecules became partially integrated in the bilayer as predicted from their partitioning values. Part of the drug became integrated in hydrophobic regions leading to higher drug/DPPC ratios (Table 2) and lower accessibility for iodide than ciprofloxacin alone (Table 3). This is in agreement with f_a values (Table 3) which are lower for the alkyl derivatives than for the parent compound. In general, the drug become more deeply buried in the bilayer below T_m, with the increase in the alkyl-chain and at pH 7.40 where the neutral form of the 6-fluoroquinolones predominates.

Additional aspects arise from those experiments. For instance when we 'force' the interaction between the drug and the DPPC, by co-drying the compounds before the redispersion and after gel separation, the drug/DPPC ratio recovered is extremely low, $\approx 3-5\%$ in terms of ciprofloxacin (Table 2). This finding and the high accessibility for iodide support the idea that the drug has been mainly segregated from the liposome structure or remain adsorbed at the bilayer–bulk interface. On the other hand because 6-fluoroquinolones permeate passively through bilayers the introduction of alkyl chains has the effect of enhancing lipid solubility and of increasing the activity accordingly to their MICs (Montero et al., 1997). Moreover the enhanced membrane solubility of the alkyl-derivatives can be considered as a reservoir of ciprofloxacin which can be used to encapsulate these drugs in liposomes.

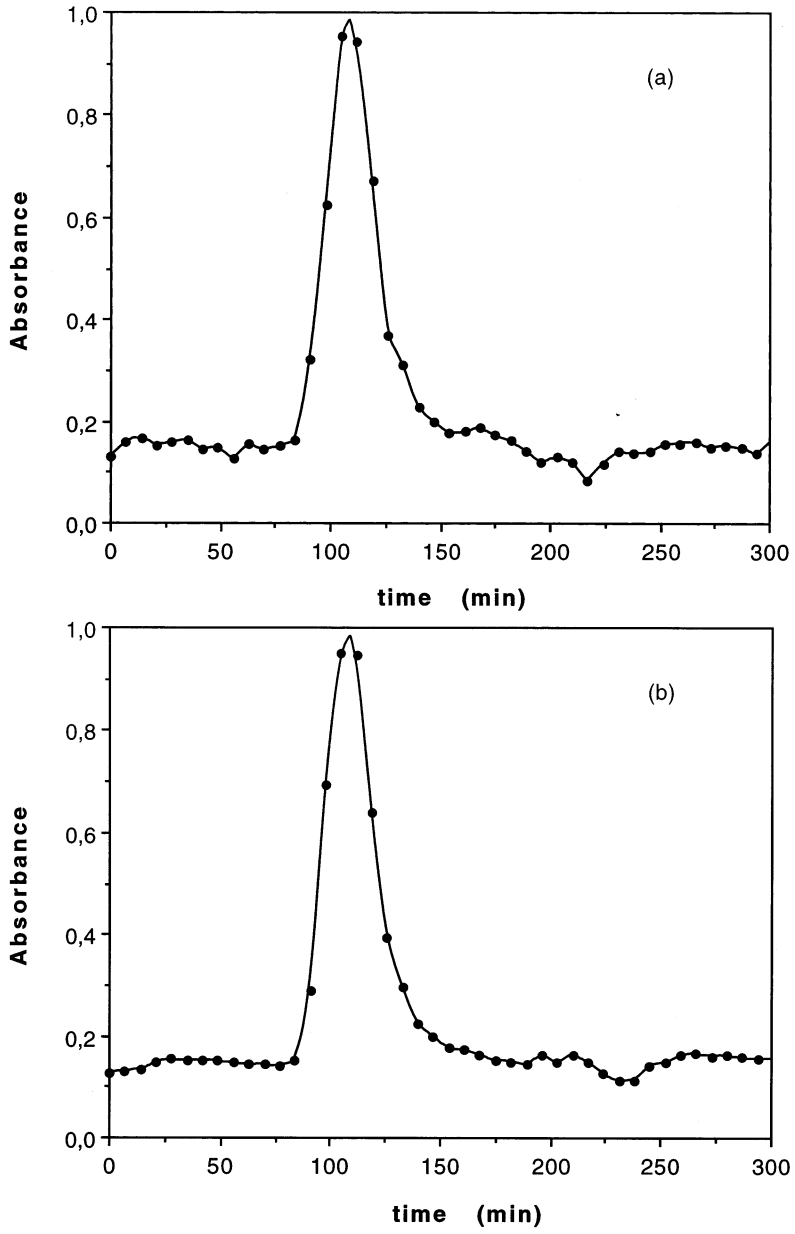


Fig. 5. Elution profiles of ciprofloxacin encapsulated in DPPC liposomes with acid (a), neutral (b), and basic (c) pH interior.

4. Concluding remarks

In conclusion, the interaction of 6-fluoroquinolones with phospholipid membranes depends, apart from the partitioning behaviour of the drugs, on their microspeciation. With regard to the mechanism of fluoroquinolone permeation through bacterial membranes it was earlier proposed (Furet et al., 1992) that an electrostatic interaction might be the first step in the mechanism. This can be correlated with high exposures to iodide indicating more interfacial locations and

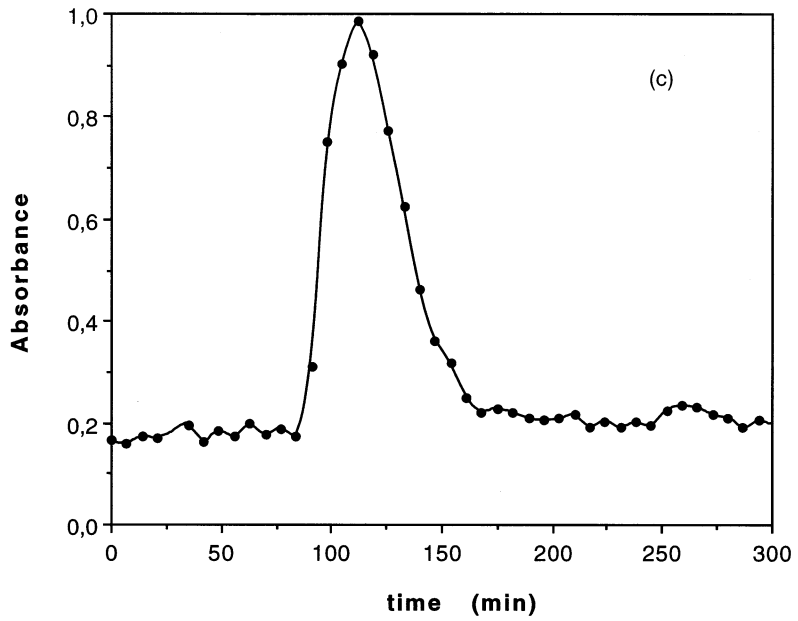


Fig. 5. (*Continued*)

the low hydrophobic interactions found using quenching methods in all range of pH values studied in this work.

Moreover, because the tendency of these compounds to aggregate in lipid environments (Nag et al., 1996), a second step of drug accumulation at the interface can be assumed. Therefore an accumulation following a gradient of concentration of the drug in the cytoplasm seems reliable. From our column equilibration results it has been demonstrated that ciprofloxacin can be loaded into liposomes with and acidic interior. Indeed, all those results point to the existence of a passive mechanism of fluoroquinolone permeation through bilayers. Finally, other variables such as membrane charge and composition can play a role in the global process. All these aspects are currently being investigated in our laboratory to better understand the passive mechanism of permeation of 6-fluoroquinolones through bacterial membranes.

Acknowledgements

This work has been supported by DGICYT, Spain (grant PB93-0809). José Luis Vázquez was recipient of a fellowship from the Health Science Division of University of Barcelona. Thanks to Cenavisa Labs (Reus, E) for the generous gift of ciprofloxacin. Thanks to the referees for valuable comments on the original manuscript and to Sandra Hurle for correcting the English.

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