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Determination of the partition coefficients of a homologous series of ciprofloxacin: influence of the *N*-4 piperazinyl alkylation on the antimicrobial activity

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Abstract

Partitioning of a fluoroquinolone antibiotic, ciprofloxacin, and its *N*-piperazinyl alkyl derivatives, between octanol or *Escherichia coli* lipid membrane extract and aqueous buffer pH 7.4, was studied. The experimental partition coefficients (P_{exp}) were corrected at this pH using an expression that includes the microconstant values of each compound. The relationship between the corrected partition coefficients expressed as log *P* (thermodynamic partition coefficient) and the diffusion through the lipid bilayers ('hydrophobic pathway') of entry has been considered here. In this work, we have explored the possibility of using our model to provide physicochemical evidences to support such a via. The correlation between log *P* values and antibacterial activities (expressed as minimal inhibitory concentration (MIC) values) of the homologous series of antibiotics against different bacteria were studied. A parabolic behaviour was observed which evidenced that the only increase in lipophilicity does not result in an enhanced antimicrobial activity for the homologous family studied.

Keywords: 6-Fluoroquinolones; Partition coefficients; Minimal inhibitory concentration; log *P*

1. Introduction

Fluoroquinolones, which are structurally related to nalidixic acid, have become the focus of attention in many research areas because of their broad-spectrum antimicrobial activity. During this decade, large numbers of fluoroquinolone derivatives have been synthesised and their effects have been tested against a variety of Gram-negative and Gram-positive bacteria. Furthermore, there is evidence that some compounds in this group, i.e. ciprofloxacin or sparfloxacin, may be efficient against multidrug resistant *Mycobac-*

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terium tuberculosis and particularly beneficial in improving the conditions of AIDS patients secondarily affected by *Mycobacterium* (Houston and Farming, 1994; Hooper and Wolfson 1995). Indeed, this unusual activity against acid-alcohol resistant bacteria, well known for their highly effective permeability barrier of complex architecture (Nikaido et al., 1993), suggests that a specific physicochemical feature enables these drugs to penetrate bacterial envelopes.

Three routes of fluoroquinolone entry through the outer or/and cytoplasmic membranes have been proposed (i) the porin pathway through hydrophilic channels (Dechéne et al., 1990), (ii) hydrophobic pathway through lipid bilayer domains (Nikaido and Thanassi, 1993), and (iii) a 'self-promoted' route similar to that used by cationic compounds (Hancock et al., 1981). Although the porin pathway is the most significant, the other two mechanisms might also allow the entry of antibiotics, especially quinolones due to their moderate hydrophobicity. Our knowledge of fluoroquinolone lipophilicity and its influence on the capacity to cross bacterial membranes is, therefore, a matter of interest.

Partition coefficients can often be correlated with biological effects, although some theoretical aspects should be taken into account before using them for predictions. This is also true for amphoteric compounds and their affinity for hydrophobic biophases, particularly when their ability to permeate microbe membranes is to be predicted. Fluoroquinolones, like other amphoteric drugs may exist in solution as a mixture of several species in equilibrium. As can be seen in Fig. 1, fluoroquinolones display two proton-binding sites and subsequently four microspecies can be found in solution, anion (Q^-), zwitterion ($HQ^\pm$), neutral (HQ°) and cation (H_2Q^+). Therefore, the experimental partition coefficients ($\log P_{exp}$) have to be corrected to pH to obtain the true partition coefficient ($\log P$) (Takács-Novák et al., 1995).

The octanol–water system as a measure of the distribution of a drug between aqueous and lipid environments provides reliable data for comparative purposes. Thus the low affinity of ciprofloxacin for neutral liposomes, (Maurer et al., 1998) or the extremely low encapsulation efficiencies observed in large unilamellar vesicles using passive methods, (Montero et al., 1997) are good examples. These findings are in agreement with the observation of both fast accumulation (Lasic et al., 1995; Maurer et al., 1998) and efflux (Vázquez et al., 1998) in liposomes when there is a favorable pH gradient. These arguments support a simple diffusion process (or hydrophobic pathway) energised by proton motive force.

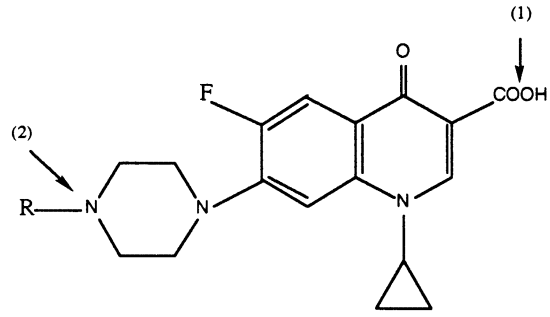
In this work, the partitioning behaviour of ciprofloxacin between octanol or lipid membrane extracts of biological origin and buffer solutions was studied. The introduction of *N*-alkyl substituents (from 1 to 5 C) in the *N*-4 position of the piperazinyl group provided a homologous series of compounds with increasing lipophilicity. These were also analysed by the same methods. This strategy enables us — (i) to test the correlation between $\log P$ and the chain length to ascertain the concordance with values predicted (Leo et al., 1971), (ii) to study the ability of the models to predict the affinity of fluoroquinolones for lipid environments, (iii) to investigate whether a correlation between $\log P$ and the antimicrobial activity [expressed as minimal inhibitory concentration (MIC)] of several bacterial species of clinical interest exists and (iv) to assess whether the lipid solubility potentiation is related with a possible hydrophobic pathway of entry.

2. Materials and methods

2.1. Chemicals

Ciprofloxacin was obtained from CENAVISA laboratories. (Reus, E). Total lipid membrane from *Escherichia coli* extract was obtained from Avanti Polar Lipid Co. (Alabaster, Al, USA) and was used without further purification. Deionised water was distilled from sodium permanganate in an all-glass apparatus and further purified by reverse osmosis through a Milli-Q system (Millipore, USA). Buffer solution, Hepes 50 mM, pH 7.40, 0.15 $I = 0.15$ m. Octanol was HPLC grade from Merck (Barcelona). All other common chemicals were ACS grade.

(a)



R	pK_{a1}	pK_{a2}	pK_{11}	pK_{12}	pK_{21}	pK_{22}
H	6.08±0.11	8.58±0.55	6.61	8.04	6.23	8.43
CH_3	6.10±0.15	7.86±0.38	6.50	7.46	6.31	7.64
CH_2CH_3	6.12±0.05	7.68±0.42	6.45	7.19	6.39	7.25
$(CH_2)_2CH_3$	6.20±0.17	7.55±0.12	6.82	7.19	6.71	7.30
$(CH_2)_3CH_3$	6.26±0.02	7.52±0.41	7.03	6.74	7.22	7.36
$(CH_2)_4CH_3$	7.04±0.05	7.55±0.14	7.50	7.08	7.23	7.36

Macro-$pK_{a,i}$ and micro-$pK_{i,j}$ of the 6-Fluoroquinolone homologous family

(b)

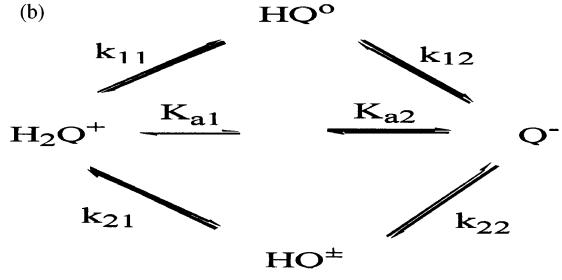


Fig. 1. (a) Structure of ciprofloxacin with protonation 'sites' and its derivatives. (b) Scheme for protonation of a quinolone. k_{a1} and k_{a2} are the two macroscopic acid dissociation constants. k_{11}, k_{12}, k_{21}, and k_{22} are the four microscopic acid dissociation constants. The four species are designated as follows; H_2Q^+, cation; Q^- anion; HQ° neutral, and $HQ^\pm$ zwitterion.

2.2. Synthesis of alkyl-derivatives

The alkyl-derivatives were synthesized according to the method elsewhere described (Koga et al., 1980). Under continuous stirring, ciprofloxacin (0.010 mol), triethylamine (0.015 mol) and alkyl bromide (0.012 mol) were dissolved in 40 ml of dimethylformamide and heated at reflux to 90–95°C for 2 h. Then the resulting solution was dried and the residue was resuspended in water. After filtration the product was recrystallized from ethanol. Compounds were judged pure (99.9%) after HPLC and IR determination.

2.3. Biologicals

The bacteria used were *Serratia marcescens* NIMA wild type (Williams and Qadri, 1980), *E. coli* K-12, *Pseudomonas aeruginosa* (CECT-108), *Staphylococcus aureus* (CECT-59). Strains were maintained on trypticase soy agar slants and cultured in Mueller–Hinton broth for susceptibility experiments. Media were purchased from Scharlau (Barcelona, Spain).

2.4. Determination of microconstants

Microconstants were determined by a combination of potentiometric and spectroscopic techniques initially designed to study amino acids (Edsall and Wyman, 1958) and adapted successfully to study the microspeciation of several drugs such as cephalosporins (Streng et al., 1976), doxorubicin (Sturgeon and Schulman, 1977) and fluoroquinolones (Takács-Novák et al., 1994). The values are shown in Fig. 1a. Details of the experimental procedures and discussion on calculations for ciprofloxacin have been published earlier (Montero et al., 1996) as well as the macro and microconstants of the *N*-4 alkyl ciprofloxacin derivatives (Hernández-Borrell and Montero, 1997; Vázquez et al., 2001a).

2.5. Octanol/buffer partition coefficient determinations

The experimental partition coefficient (P_{exp}) between *n*-octanol and buffer was determined by a slight modification of the method earlier described (Ross et al., 1992). Briefly, 100 μl of a stock solution of fluoroquinolone (0.2 mg/ml) was diluted with 1.9 ml of appropriate buffer solutions and mixed with 2 ml of octan-1-ol (the organic and aqueous phase was 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 drug concentration. The partition coefficient was calculated as the ratio between molar concentration in octan-1-ol (i.e. ciprofloxacin: $\lambda = 286$ nm and $\varepsilon = 36\,800/\mathrm{M}$ cm) and aqueous phase (i.e. ciprofloxacin: $\lambda = 278$ nm and $\varepsilon = 34\,400/\mathrm{M}$ cm). The total concentration in both phases was measured by spectrophotometry and the experimental partition coefficients (P_{exp}) were determined from:

$$P_{exp} = \left(\frac{C_o}{C_w}\right)\left(\frac{w_w}{w_o}\right) \quad (1)$$

where C_o and C_w refer to the octan-1-ol phase and water phase concentrations, respectively, and w_w and w_o the weight of the aqueous and octanol phases in the samples.

2.6. Lipid membrane extract/buffer partition coefficient determinations

Lipid films (12 mg) were formed on the walls of 10 ml round-bottom flasks following rotary evaporation of chloroform-methanol (50:50, v:v) stock solutions of lipids. Residual solvent was removed by high-vacuum drying overnight. The resulting dried film was redispersed in 1 ml of appropriate buffer solutions with drug added to a final concentration of 10 μg/ml. Three cycles of freezing and thawing alternated with vigorous vortexing were applied for 5 min to allow multilamellar vesicle formation. Liposomes were left to be annealed at room temperature overnight and protected from light. The samples were centrifuged at $150\,000 \times g$ in a Kontron ultracentrifuge for 1 h.

The concentration of the drug in the supernatant was determined spectrophotometrically by UV absorption at the specific wavelength. The experimental or apparent partition coefficients were calculated from:

$$P'_{exp} = \left(\frac{C_T - C_w}{C_w}\right)\left(\frac{w_w}{w_b}\right) \quad (2)$$

where C_T is the concentration of the drug in a control buffer containing a given amount of fluoroquinolone but no lipid, C_w the concentration of the supernatant depleted of fluoroquinolone by the presence of phospholipids and w_w and w_b the weight of the aqueous and phospholipid phases in the sample, respectively. Spectrophotometric determinations were performed at room temperature on a Hewlett-Packard 8451 diode-array spectrophotometer. Absorption cells with a path length of 1 cm were used throughout. The presence or absence of phospholipid in the supernatant was tested using the Barlett method (Barlett, 1959).

2.7. Thermodynamic coefficient and Gibbs energy of partitioning

The thermodynamic partition coefficient (P) is defined as the ratio between the activities of the neutral species in octanol (a HQ°_{o}) and water (a HQ°_{w}):

$$P = \frac{a_{HQ^{\circ}_{o}}}{a_{HQ^{\circ}_{w}}} \approx \frac{[HQ^{\circ}]_o}{[HQ^{\circ}]_w} \quad (3)$$

In ideally dilute solutions activity coefficients are unity, in which case concentrations are used. On the other hand, assuming that only the neutral species undergoes partitioning between both phases, the experimental partition coefficient (P_{exp} or P') determined is defined by:

$$P_{exp} = \frac{[HQ^{\circ}]_o}{[H_2Q^+]_w + [HQ^{\pm}]_w + [HQ^{\circ}]_w + [Q^-]_w} \quad (4)$$

Introducing the four microconstants (see Fig. 1b) and rearranging (Eq. (4)) we obtain,

$$\log P = \log P_{exp} + \log\left(1 + \frac{k_{21}}{k_{11}} + \frac{k_{12}}{[H^+]} + \frac{[H^+]}{k_{11}}\right) \quad (5)$$

where k_{ij} are the microconstants and $[H^+]$ introduces the correction needed for a given pH.

The standard change in Gibbs energy for the process of partitioning of the drug either from the aqueous phase to the octanol or to the lipid phase was then calculated from:

$$\Delta_{trans}G^{\circ} = -2.303RT\log P \quad (6)$$

where R is 8.314 J K/mol. This $\Delta_{trans}G^{\circ}$ represents the change in energy upon transferring one mole of solute from the aqueous to the octanol phase or lipid bilayer.

2.8. Susceptibility tests

The MIC values were determined by the broth-dilution method. Overnight cultures of the bacterial strains in Mueller–Hinton were diluted 1000-fold in fresh broth and 5 μl of the bacterial suspension was inoculated in the same broth containing serial dilutions of the agents. MIC were determined after 18 h of incubation at 37°C as the minimum concentration of antibiotic that inhibits growth.

2.9. Data analysis

Statistical and regression analysis was conducted with the software package Statworks® using a personal computer.

3. Results

3.1. Partition coefficients

The general structure of ciprofloxacin is represented in Fig. 1a. The R at the N-4 position of the piperazinyl group identifies the position where the alkyl substitutes (methyl, ethyl, propyl, butyl, and pentyl, respectively,) were attached. The figure includes the microspeciation scheme (Fig. 1b), from which the k_{ij} can be deduced. The experimental partition coefficients, octanol/buffer and membrane lipid extract/buffer, were used to calculate the $\log P$. Then $\log P$ for each model was represented versus the alkyl length chain using the equation applied to the substitute approach:

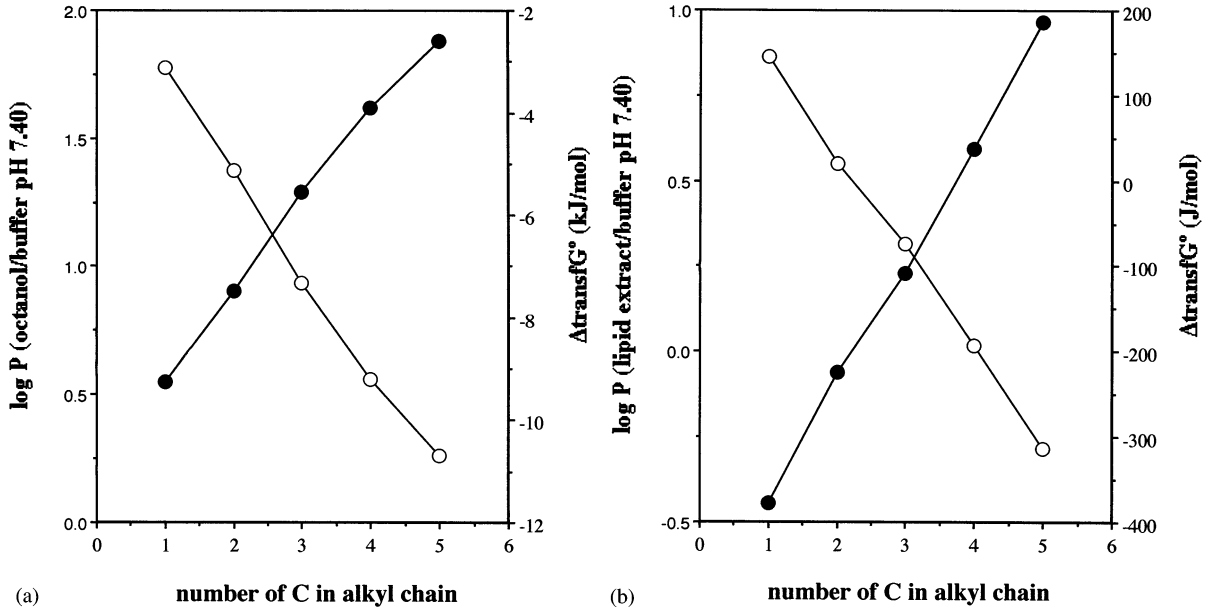


Fig. 2. Representation of log P and Gibbs energy $\Delta G°$ of transfer as a function of the number of C in the alkyl chain substituted in the N-4 position of piperazinyl group, in; (a) octanol/buffer system (b) *E. coli* lipid membrane extract/neutral pH buffer system.

$$\log P_{fq} = \log P_{cip} + \pi n \quad (7)$$

where P_{cip} and P_{fq} are the partition coefficients of the parent compound, ciprofloxacin, and the N-alkyl fluoroquinolone, respectively. The log P and Gibbs energy of transference $\Delta_{trans}G°$ (calculated from Eq. (6)) double plots are shown in Fig. 2a and b and the regression analysis of those data summarised in Table 1. Linear regression were acceptable in all cases but π and $\Delta_{trans}G°$ values obtained from both models were lower from the expected values (Leo et al., 1971).

3.2. Antimicrobial activity

Table 2 summarises the MIC against the microorganisms studied. N-piperazinyl alkyl derivatives were more active against Gram negative than Gram positive bacteria, but *P. aeruginosa*, as has been pointed out, shows low permeability (Yoshimura and Nikaido, 1982). The most active agents were ciprofloxacin and methyl ciprofloxacin and none of the other derivatives was as active as the parent compound. Alkylation of the N-4 position of the piperazinyl group increased the MIC, since MIC values of pentyl derivative were much higher than those of the parent compound, i.e. four-fold in *S. marcescens* NIMA, 16-fold in *E. coli* and almost 28-fold in *P. aeruginosa*.

3.3. Correlation between log P and MIC

Since linear regression analysis of MIC and log P yields poor correlation, parabolic relationships between antibacterial activity and physicochemical properties, including log P, have been applied to other cases (Boyd et al., 1980; Irwing et al., 1987); the following regression equation was, therefore, applied:

Table 1
Linear regression parameters and correlations between the thermodynamic partition coefficients and calculated $\Delta G°$ values of N-4-alkyl-piperazinyl ciprofloxacin derivatives

log P	Π	$\Delta_{trans}G°$ (cal/mol)	r^2
Octanol/buffer	0.338	−459.84	0.995
Lipid membrane extract/buffer	0.347	−472.0	0.999

Table 2
MIC (μg/ml) of ciprofloxacin and their derivatives in different species

Compound	*S. marcescens*	*E. coli*	*P. aeruginosa*	*S. aureus*
Ciprofloxacin	0.125	0.030	0.500	0.250
N-methyl-ciprofloxacin	0.125	0.030	1	0.250
N-ethyl-ciprofloxacin	0.250	0.060	2	0.250
N-propyl-ciprofloxacin	0.250	0.125	4	0.500
N-butyl-ciprofloxacin	0.500	0.250	7	1
N-pentyl-ciprofloxacin	0.500	0.500	14	1

$$\mathrm{MIC} = a \log P^2 + b \log P + C \quad (8)$$

In all cases curves were parabolic in shape. Fig. 3a and b show curves obtained from $\log P_{\mathrm{octanol/buffer}}$ and $\log P_{\mathrm{lipid/buffer}}$, respectively, versus *E. coli* MIC values. The minimum of the curve is centered on the first three compounds of the homologous family (Fig. 1a). Table 3 shows correlation coefficient r and levels of confidence P. A satisfactory correlation for both sets of $\log P$ values was obtained whichever model was used. Although the curves obtained with different MIC were qualitatively similar, quantitative differences were apparent. Among the strains studied, the best parabolic correlation was found between *E. coli* MIC and *P. aeruginosa* in both sets of $\log P$. On the other hand the poorest correlation were found for *S. marcescens* NIMA and *S. aureus*.

4. Discussion

The use of homologous series of compounds allows the study of the contribution of the alkyl chain to partitioning. Our results from the octanol/buffer model are similar to those found in the literature for other homologous series of drugs (Flynn and Yalkowsky, 1972; Ma et al., 1992; Merino et al., 1995). The deviation from expected values is negligible and probably due to the high $\log P$ values of longer alkyl-ciprofloxacin derivatives (particularly pentyl). Presumably, these not behave as an ideally dilute solution as it is conventionally assumed (Ross et al., 1992). Indeed, π values lower than 0.5 can be found in the literature (Flynn and Yalkowsky, 1972) depending on the experimental data used. A refinement of these result could be possibly by using other methods (Rekker and Mannhold, 1992; Mannhold and Dross, 1996). Moreover, the results obtained when our partitioning study was extended to the *E. coli* lipid membrane extract, depend on the influence of other factors. To be known the nature of the system such as the electrical charge (Furet et al., 1992; Vázquez et al., 2001a) and phospholipid variety present in the lipid extract (White et al., 2000). Indeed at pH 4.70, when the cationic microspecies is predominant, we found a higher partitioning (data not shown) than at pH 7.40. Presumably, the electrostatic forces between the positive charge of fluoroquinolone and the negative charge of acidic phospholipids increased the binding (Bedard and Bryan, 1989; Vázquez et al., 2001b).

Enhancement of drug action by introducing chemical modifications in parent compounds is a common strategy. For instance, the increase in the alkyl length of a homologous series of *p*-aminosalycilates leads to increased intrinsic activity of each derivative (Kakemi et al., 1967). The biphasic dependence observed in Fig. 3a and b can be interpreted as a consequence of a transport-controlled process. Other parabolic relationships like those observed here have been reported in QSAR studies of cephalosporins (Boyd et al., 1980) and discussed related to transport phenomena (Irwing et al., 1987).

However, MIC values depend upon both rate of penetration into bacteria and affinity of the antibiotics for their target enzymes, i.e. DNA gyrase and Topoisomerase IV. Thus, under the basis of the mechanism of inhibition of DNA Gyrase (Shen et al., 1989) and assuming that the strains assayed here have similar affinities for the

fluoroquinolones, at a particular length, the *N*-alkyl derivative could not be sterically accommodated at the binding site of the enzyme. This could explain why the minimum MIC corresponded to ciprofloxacin and its methyl derivative, the smaller molecules, and not to the more lipophilic derivatives.

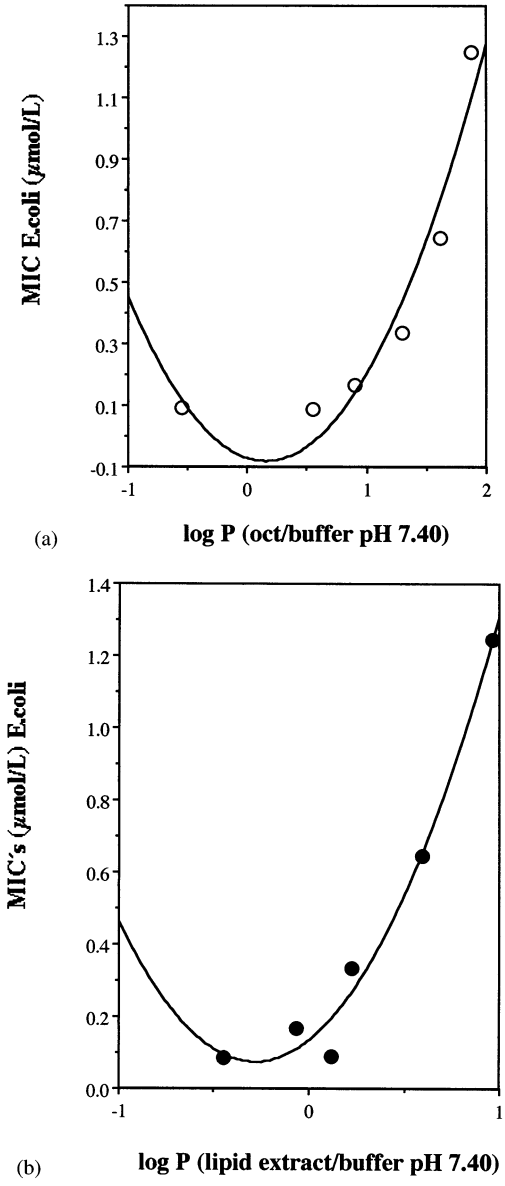


Fig. 3. Correlation between MIC for *E. coli* and log *P* in (a) octanol/buffer system (b) *E. coli* lipid membrane extract/neutral pH buffer system.

Strikingly, the study produced relationships with similar correlation coefficients, when using $\log P_{\text{octanol/buffer}}$ values, in both Gram-negative and *S. aureus* as a representative of Gram-positive species. On the other hand, the analysis showed poor correlation when log *P* values came from the lipid membrane extract/buffer model and MIC from species other than *E. coli*. Although increases in the lipophilicity of the fluoroquinolones increases their activity against some *Mycobacterium* species (Haemers et al., 1990), it appears that in less hydrophobic bacterial species like those studied here, this was not the case. Indeed, this is in agreement with results obtained with *N*-1 phenylquinolone derivatives originally designed to enhance quinolones lipophilicity (Renau et al., 1995) and some benzenesulfonamidefluoroquinolones as well (Nieto et al., 1999). These results are in agreement with the fact that higher partitioning values did not result in an enhancement of the antibacterial activity of the compounds studied here. Consequently, the attachment of those chains at the *N*-4 position of the piperazinyl group could not be the only factor controlling the antibacterial activity (Berlanga et al., 2000a,b).

Insofar as the mechanism of penetration is concerned, it is worth noting that the cell membrane and the outer membrane represent different barriers. Hence, it is known that porins have an important role in determining susceptibility to quinolones in Gram-negative bacteria. Recently we have observed (data not shown), that a porin deficient mutant (Ncip) derived from *S. marcescens* NIMA (Berlanga et al., 2000a,b), accumulated in approx. 12 min more butyl derivative (50 ng/mg cells) than the parent compound (30 ng/mg cells). This could be primarily attributed to an enhanced permeation of the alkyl derivatives through bilayer domains of the outer membrane. Importantly, typical accumulation studies, like those mentioned above, take in account uptake as well as the existence of efflux mechanisms. Thus, even though that our studies of partition end up with negative results for predicting activity it is of interest to note that entry and excretion both are greatly influenced by the hydrophobicity of the antibiotic. Work in progress (manuscript in

Table 3
Parabolic regression analyses of minimum inhibitory concentrations and the thermodynamic correlation coefficients (log P), correlation coefficient (r) and significance ($P>F$) and parameters (a, b, c)

log P	r	$P>F$	a	b	c	
Oct-buffer						
	0.955	0.028	0.4559	0.1463	0.7223	*S. marcercens*
	0.970	0.014	0.0711	0.1232	0.3995	*E. coli*
	0.972	0.014	1.8620	1.7679	10.252	*P. aeruginosa*
	0.953	0.028	0.4562	0.1470	0.7225	*S. aureus*
Lipid/buffer						
	0.906	0.076	0.9780	1.2731	0.6065	*S. marcescens*
	0.991	0.002	0.1350	0.4249	0.7527	*E. coli*
	0.980	0.008	4.1591	11.569	20.978	*P.aeruginosa*
	0.906	0.076	0.9782	1.2734	0.6059	*S. aureus*

preparation) have evidenced a significant decrease in the efflux of the butyl derivative by *S. marcescens* NIMA that was attributed to a lower captation by a recently described efflux pump system (Berlanga et al., 2000a,b). The frequency of isolation of quinolone resistant bacteria is increasing and could have a high impact on the clinical efficacy of these drugs. Hence, the occurrence of a reduced excretion undergone by the butylated compound open new insights in the possible application of the *N*-alkyl derivatives studied here.

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