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Expression of the partition coefficients of a homologous series of 6-fluoroquinolones

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Abstract

In this work the *n*-octanol-water coefficients of a homologous series of N-4 piperazinyl cirprofloxacin were determined at different pH (4.80, 7.40 and 9.10). The thermodynamic partition coefficients were calculated from experimental values and expressed using two diferent models. The ability of both models to account for the partition data were compared with values theoretically predicted. The correlation between the thermodynamic partition coefficients and minimal inhibitory concentrations were also investigated. © 1997 Elsevier Science B.V.

Keywords: Ciprofloxacin; Octanol:buffer partition coefficients; Macroconstants; Microconstants

1. Introduction

Fluoroquinolones, a group of antibiotics historically related to nalidixic acid, are presently under intense research in several fields (Hooper and Wolfson, 1995). Some compounds of this group are active against Gram-negative and Gram-positive bacteria (Wolfson and Hooper, 1985) and appear to be indicated for the treatment of the emerging multi-drug resistant tuberculosis (MDRTB) (Weltman and Rose, 1994). In particular, fluoroquinolones could be used against the *Mycobacterium tuberculosis* and *M. avium-intracellulare* complex, frequently found as a secondary infection in AIDS patients (Young et al., 1986).

The bactericidal action of quinolones is based on their specific inhibition of DNA gyrase (Shen et al., 1989). This intracellular target can be reached by fluoroquinolones because one of the most important features is their ability to enter mammalian and bacterial cells. Although the porin pathway across microbial membranes seems to be the major route of fluoroquinolone entry, it seems that the uptake through the outer membrane also involves passive diffusion through lipid

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regions (Georgopapadokou, 1995). Therefore, the interaction of fluoroquinolones with membrane models is crucial in the study of such a hydrophobic means of permeation, but whichever mechanism predominates it is known that physicochemical properties such as ionization (Ross and Riley, 1992a), lipophilicity (Takácks-Novák et al., 1995), and complexation with metal ions (Ross and Riley, 1992b, 1993; Ross et al., 1993) play a crucial role.

Under biological conditions where the physicochemical conditions (pH, ionic strength and temperature) are imposed, several strategies can enhance fluoroquinolones lipophilicity. Our approach consisted in the introduction of alkyl chains of different lengths (from methyl to butyl) at the N-4 position of the piperazinyl group of ciprofloxacin. Those derivatives are lipophilic and should present an enhanced penetrability in lipid environments and eventually will be able to overcome the efflux-mediated resistance (Kaatz et al., 1993) which involves the action of the membrane protein Nor A, undergone by hydrophilic fluoroquinolones (Takenouchi et al., 1996). On the other hand, it is expected that those derivatives could be sequestered in the bilayer structure eventually promoting drug translocation to the bacterial cytoplasm. Accordingly, the lipophilic enhancement expected by the addition of methylene groups should be beneficial in overcoming the barrier formed by mycolic acids and lipids in mycobacteria (Nikaido et al., 1993).

The aim of this work was to study the partitioning behaviour of a homologous series of N-4 piperazinyl ciprofloxacin between aqueous buffers and *n*-octanol. This information could be exploited in quantitative structure activity relationship studies (QSAR) (Scherrer and Howard, 1977) where log P would be used to evaluate their biological activity, and also in the prediction of their ability to permeate the external barriers of microbes. However, it is known that fluoroquinolones display multiequilibrium in solution due to two proton binding sites, one on the carboxylate of the quinolone nucleus, and the other on the amine of the 7-substituent of the piperazinyl. Consequently four species can be found in solution (Fig. 1) at a given pH: anion (Q^-), zwitterion ($HQ^\pm$), neutral (HQ°) and cation (H_2Q^+). Some authors consider that only the neutral forms are transferred into the *n*-octanol phases (Ross et al., 1992), while others have considered that neutral and zwitterion forms are transferred together (Takácks-Novák et al., 1995). Since one of these two models must be selected, brief descriptions of the procedures are provided in Appendices A and B. The ability of both equations to account for the partition data was studied in this work.

An indirect purpose of this paper was to use the partitioning data to evaluate the ability of fluoroquinolones to be encapsulated in liposomes, and eventually, to enhance their encapsulation efficiencies. For instance, it has been demonstrated that ciprofloxacin encapsulated in liposomes, even at low concentrations, shows greater antimycobacterial activity than the free drug (Majumdar et al., 1992). The possible industrial application of fluoroquinolones encapsulated in liposomes (Montero et al., 1994, 1996) has led to the development of large scale production methods (Carrera et al., 1993; Pons et al., 1995) and to the proposal of complex formulations (Puglisi et al., 1995); the present study could provide valuable information about some of the interactions involved in those formulations.

2. Materials and methods

2.1. Materials

The ciprofloxacin and alkyl derivatives (Table 1) were obtained from CENAVISA Lab., (Reus, Spain). Deionized 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 solutions: acetate buffer, pH 4.80, 0.15 M; Tris hydroximethyl-amino-methane pH 7.40, 0.15 M; borate pH 9.10, 0.15 M; I = 0.15 m. Octanol was HPLC grade from Merck (Barcelona).

2.2. Synthesis of alkyl-derivatives

The alkyl-derivatives were synthesized accordingly a method elsewhere described (Koga et al.,

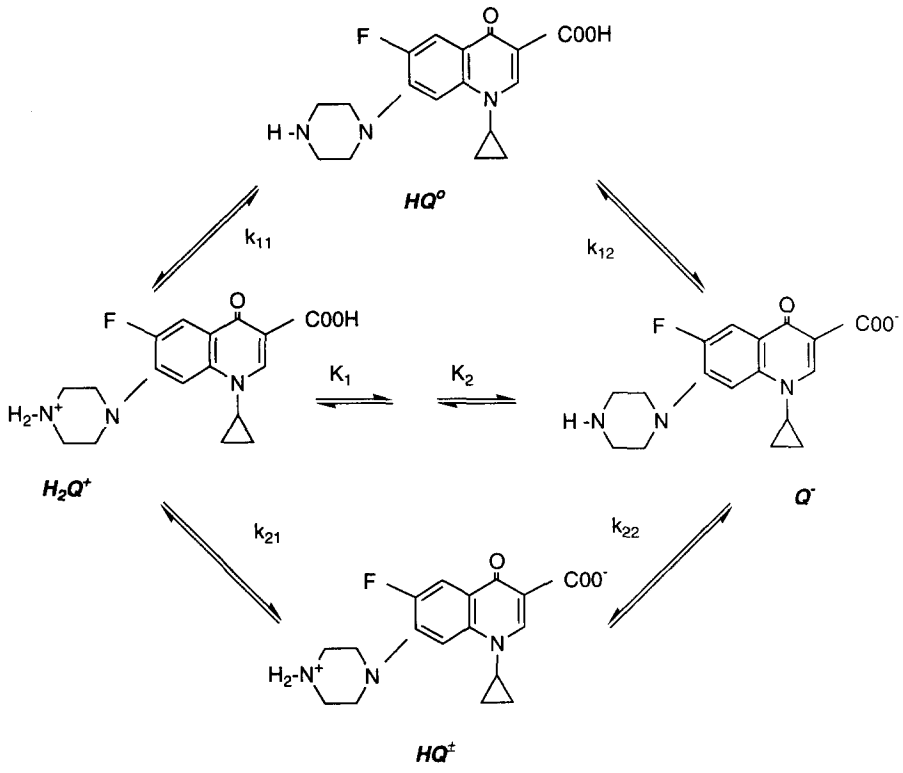


Fig. 1. Microspeciation scheme of ciprofloxacin.

1980). Under continuous stirring, ciprofloxacin (0.010 mols), triethylamine (0.015 mols) and alkyl bromide (0.012 mols) were disolved in 40 ml of dimethylformamide and heated at reflux to 90–95°C for 2 h. Then the resulting solution is dried and the residue resuspended in water. After filtration the product was recrystallized from ethanol. Compounds were judged pure after HPLC and IR determinations.

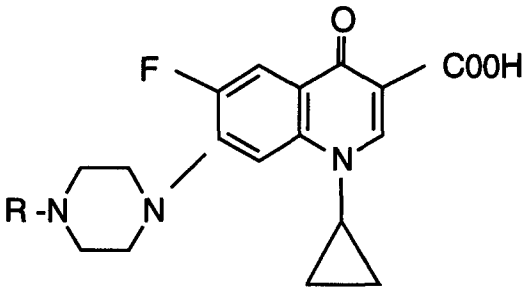
2.3. Susceptibility tests

The minimum inhibitory concentrations, MIC_{90}, were determined following a procedure elsewhere described (Merino et al., 1995). The determination was carried out on 100 strains of *Escherichia coli* using the dilution in the agar plate. The procedure was validated by means of the following typified strains: *Pseudomonas aeruginosa* ATCC 27853, *E. coli* ATCC 29212 and *Staphylococcus aureus* ATCC 29213. The assay was carried out accordingly the National Committee for Clinical Laboratory Standards. The bacterial suspension was inoculated with a Steer's replicator into Müeller-Hinton agar plates prepared with known concentrations of the fluoroquinolones tested covering a suitable range. The plates were cultured at 37°C for 20 h and finally evaluated.

2.4. Determination of macroconstants and microconstants

Details of the experimental procedures and calculations have recently been published (Hernández-Borrell and Montero, 1996). Briefly, while the determination of macroconstants consisted of a conventional acid-base titration, microconstants and microspecie concentrations were obtained by a combination of potentiometric and spectroscopic techniques currently used for amino acids (Edsall and Wyman, 1958; Clement and Hartz,

Table 1
Name, structure, molecular weight, and minimal inhibitory concentration (MIC_{90}) against *E. coli* ATCC25922 of the studied compounds



R alkyl chain	Name	Molecular weight	MIC_{90} (μg/ml)
H	Ciprofloxacin, 1-cyclopropyl-1,4-dihydro-4-oxo-6-fluoro-7-(*N*-piperazinyl)-3-quinolinecarboxylic acid	331.3	0.025
CH_3	*N*-Methylciprofloxacin	345.3	0.025
CH_2CH_3	*N*-Ethylciprofloxacin	359.3	0.050
$(CH_2)_2CH_3$	*N*-Propylciprofloxacin	373.3	0.100
$(CH_2)_3CH_3$	*N*-Butylciprofloxacin	387.3	0.200

1971). The method has been demostrated to be successful in the study of microspeciation of fluoroquinolones (Takácks-Novák et al., 1990) and other drugs (Niebergall et al., 1973; Streng et al., 1976; Sturgeon and Schulman, 1977).

2.4.1. Spectrophotometry

Fluoroquinolone solutions 8×10^{-6} M were prepared in 0.001 M HCl and 0.001 M NaOH with a total ionic strength of 0.5 M. The spectrum of each solution was read in the range of 200 to 400 nm on a Hewlett Packard 8451A diode array spectrophotometer.

2.4.2. Potentiometry

Macroconstants were determined by potentiometry using a Crison micro pH 2001 pH meter equipped with a glass electrode and a calomel reference electrode. The electrode was calibrated with standard Crison buffer solutions pH 4–7.02. Aliquots of 50 ml of 5×10^{-4} M fluoroquinolones in 2×10^{-3} M HCl were titrated by 0.05 M NaOH. Conversely, aliquots of 50 ml of 5×10^{-4} M fluoroquinolones in 2×10^{-3} M NaOH were titrated by 0.05 M HCl. The solutions were neutralized directly in the cell using a micropipette (precision 10 μl). During the titrations the samples were continuously stirred with a magnetic stirrer.

2.5. Octanol-buffer partition coefficient determinations

The experimental partition coefficient (D) was determined between n-octanol and buffer using a method elsewhere described (Ross et al., 1992) with minor modifications. First, 100 μl of a stock solution of quinolone (0.2 mg ml^{-1}) was diluted with 1.9 ml of water and mixed with 2 ml of octan-1-ol (the organic and aqueous phase 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 assayed spectrophotometrically to determine concentration. The partition coefficient

was calculated as the ratio between molar concentration in octan-1-ol (i.e. ciprofloxacin: $\lambda = 286$ nm and $a_m = 36\,800\ M^{-1}\ cm^{-1}$) and aqueous phase (i.e. ciprofloxacin: $\lambda = 278$ nm and $a_m = 34\,400\ M^{-1}\ cm^{-1}$). All values indicated in tables are the mean of three independent measurements.

The thermodynamic partition coefficient (P) is defined as the ratio between the activities of the neutral species in octanol ($a_{HQ^\circ_o}$) and water ($a_{HQ^\circ_w}$),

$$P = \frac{a_{HQ^\circ_o}}{a_{HQ^\circ_w}} \approx \frac{[HQ^\circ]_o}{[HQ^\circ]_w} \quad (1)$$

In ideally dilute solutions concentrations are normally used instead of activities because activity coefficients can be considered negligible.

The standard change in free energy (ΔG°) of partitioning of drug from the aqueous phase to the octanol phase can be calculated by using the following equation:

$$\Delta G^\circ = -2.3RT \log D \quad (2)$$

where R is 2 cal K^{-1} mol^{-1}. This represents the change in free energy upon transferring one mol of solute from the aqueous phase to the organic solvent and is indicative of the spontaneity of the partition process.

3. Results and discussion

The significance of $\log P$ in determining the biological effects of drugs is well established, but its expression for amphoteric zwitterionic compounds is doubtful. Since two forms have been proposed (Ross and Riley, 1992b; Takács-Novák et al., 1992) to express their partition coefficient.

While the method used to measure n-octanol-buffer partition coefficients of fluoroquinolones has been clearly established and upgraded by other investigators (Ross et al., 1992) it has two expressions depending on whether the zwitterionic species are considered as participating in partition (See Appendix A and Appendix B). The experimental partition coefficient (D) can be expressed as the ratio between the sum of the neutral and zwitterionic forms in the organic phase and the sum of concentration of all microspecies in the aqueous phase (Eq. (A-1) Appendix A). Alternatively, it is possible to express the partition coefficient as the ratio between the concentration of the neutral form in the organic phase and the sum of concentrations of all microspecies in the aqueous phase (Eq. (A-3) Appendix B).

The experimental n-octanol-buffer coefficients obtained for ciprofloxacin (Table 2) were close to those reported in literature (Ross and Riley, 1992b). The small differences can be attributed presumably to the analytical method followed and particularly to the fact that fluoroquinolone concentration in octanol phase was determined by mass balance instead of HPLC. Using Eq. (A-2) (Appendix A) and the values of the ionization constants provided in Table 3 an average value of 0.116 ± 0.029 for the thermodynamic partition coefficient (P)[2] was obtained.

The constants of ionization and the microconstants can be found in a previous work (Hernández-Borrell and Montero, 1996) and are summarized in Table 3. Using Eq. (A-9) (Appendix B) and the experimental partition coefficients at each pH (Table 2) a value of $P = 0.7688 \pm 0.089$ for ciprofloxacin was found. The difference from the absolute value found in the literature is due to the different convention followed in this paper (see Appendix B). Actually, Eq. (A-9) has been postulated as the expression of the true partition coefficient (Takács-Novák et

Table 2
Experimental partition coefficients between octanol and different buffers for ciprofloxacin (mean ± S.D., $n = 3$)

pH	D	S.D.
4.80	0.067	0.004
7.40	0.074	0.005
9.10	0.031	0.005

[2] The symbol P used in this work to normalize terminology has the same meaning as K_d used by Ross et al. (1992).

Table 3
Macroscopic and microscopic dissociation constants of the studied compounds

R alkyl chain	pK_1	pK_2	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

al., 1994) but it should be noticed that there is a controversy about its applicability. As can be seen in Table 3, microscopic constants are within a very narrow range, similar to the values shown by the macroscopic constants. This has been already discussed (Ross and Riley, 1992a) and we only suggest here that this may be due to the low precision of potentiometric measurements and the small changes observed in the UV spectrum (Takácks-Novák et al., 1990). On the other hand the method has traditionally been found acceptable in calculating microspeciation constants when differences in pK values were in a narrower range than those for fluoroquinolones (Edsall and Wyman, 1958).

When the experimental method was applied to calculate experimental octanol-buffer coefficients for the compounds of the homologous series an increase was found proportional to the length of the alkyl chain (absolute values not shown). We found that, similarly to the values obtained for other homologous series, N'-methylciprofloxacin showed higher values than those theoretically predicted (Leo, 1993). Normally this is interpreted as a first-element effect which is mainly attributed to specific interactions between these low hydrophobic compounds and the solvent. However to evaluate the contribution of the alkyl chain to the partitioning phenomena the substituent constant approach was used. It was calculated according to the formula:

$$\pi_{CH_2} = \log D_{fq} - \log D_{cip} \quad (3)$$

where D_{fq} and D_{cip} are the partition coefficients of the derivatives and of ciprofloxacin, respectively. For a homologous series, the partition coefficient increases by a factor of 2–4/CH_2 and an average of 0.5/CH_2 is generally accepted. This corresponds to a value of 825 cal/mol for $\Delta G°$ which is the increase in hydrophobicity if the alkyl chain is increased by one CH_2 unit (Tandford, 1973). The correlation between log D and the number of methylene groups can be seen in Fig. 2 along with $\Delta G°$ values obtained from Eq. (2) at different pH values. Fitting those values to the slopes provided the incremental methylene contribution to D and $\Delta G°$ which are summarized in Table 4. As can be

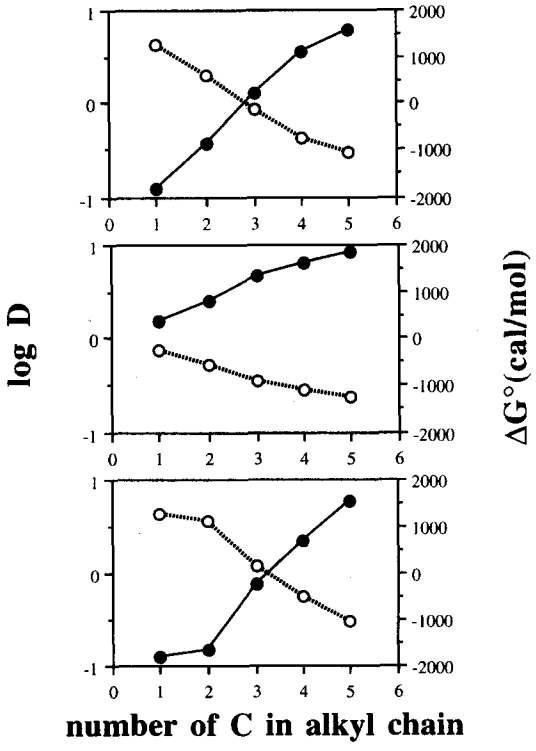


Fig. 2. The dependence of n-octanol-buffer experimental partition coefficients on alkyl chain length of the drug: (a) pH 4.80; (b) pH 7.40, and (c) pH 9.10.

Table 4
Linear regression parameters of correlations between log P and ΔG and alkyl chain length at pH studied

pH	π	$\Delta G°$ (cal/mol)	r^2
4.80	0.47	−647	0.965
7.40	0.18	−253	0.976
9.10	0.45	−623	0.984

seen, π and $\Delta G°$ values were sligthly lower to those theoretically predicted (Leo et al., 1971) but similar to the values obtained studing other homologous series (Ma et al., 1992) at pH 4.70 and 9.10. Conversely significant deviations were obtained at neutral pH. This strong influence of pH on the distribution behaviour should be interpreted in view of fluoroquinolone ionization. When buffer solutions were adjusted to pH 7.40 neutral and uncharged forms predominated (this ratio can be calculated directely from the pKs values (Ross and Riley, 1992a) while positive and negative forms predominated at acid and basic values, respectively. Thus, when there was no net charge a phenomenon of association could not be excluded and consequently deviations from the ideal behaviour of the solution were expected. Conversely, electrostatic repulsions between molecules predominated at pH 4.80 and 9.10. On the other hand, absolute values obtained for the homologous series of ciprofloxacin were similar to those reported in the literature for other homologous series of compounds (Merino et al., 1995).

Once we had examined the effect of the N-alkyl piperazinyl substituent in the parent compound ciprofloxacin we calculated log P for all compounds studied using Eq. (A-2) and Eq. (A-9). We used log P values obtained from Eq. (A-2) and Eq. (A-9) and tried to correlate them with those values theoretically calculated assuming a constant increase of 0.5 for each methylene group added. The fit of the data to the plotted regression equation is better for the log P obtained using the ionization macroconstants than for the log P values obtained by introducing in Eq. (A-9) the microconstants, as can be seen in Fig. 3 (determination coefficients $r^2 = 0.9456$ and $r^2 = 0.8700$,

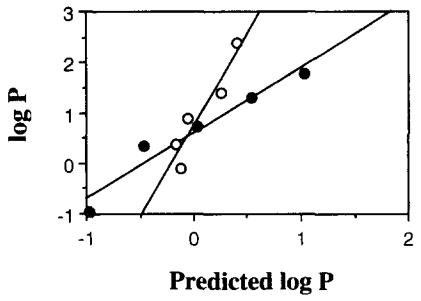


Fig. 3. Simple regression analysis of calculated log P (obtained using Eq. (A-2) (●) and Eq. (A-9) (○)) and predicted log P (obtained considering 0.5 as the constant methylene unit contribution to the partitioning).

respectively). This analysis showed a poor correlation and demonstrated the inability of either equation to predict the expected values showing that neither form has a particular advantage. Certainly, if microspeciation is considered, much more work is necessary. Therefore, we took advantage of the available biological data (Table 1) to address this study to a more fundamental question: the correlation of log P and biological activity.

Obiously, if penetration of the antibiotics through the outer bacterial membrane is related to log P it should also be related to its biological activity. Actually, the antibacterial activity expressed in terms of minimum inhibitory concentration (MIC) seems to obey a parabolic dependence on log P (Boyd et al., 1980) (see Fig. 4). Using the regression equation of the form $\text{MIC} = \log P^2 + b \log P + c$, we obtain $r^2 = 0.9856$ and $r^2 = 0.9733$ and levels of confidence of P =

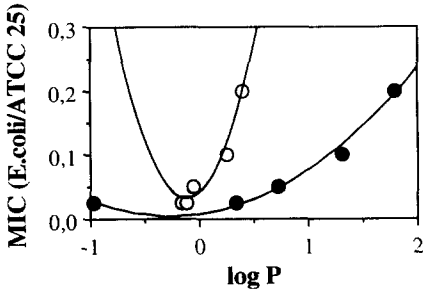


Fig. 4. Parabolic regression analyses of minimum inhibitory concentrations and log P (obtained using Eq. (A-2) (●) and Eq. (A-9) (○), respectively).

0.014 and P = 0.027, respectively, for those log P obtained using Eq. (A-2) and Eq. (A-9). Therefore, we can conclude that the thermodynamic partition coefficient, regardless of the mechanism of cell penetration and expressed in terms of macroconstants or microconstants, reflects the partitioning trends that occur in the membrane envelopes.

Finally, it is well known that octanol-water partition coefficients should be taken only as an indirect measurement of the affinity of drugs for lipid environments. Nevertheless, the more rational approach will be the determination of the lipid-buffer partition coefficients. In that case where even with neutral phospholipids the zwitterion forms could play a role in the particular drug-lipid interaction it seems reasonable to consider the microspeciation of the species. This could be of specific relevance in liposome technology for encapsulation of fluoroquinolones and also in understanding the permeation way through lipid environments of fluoroquinolones in bacteria. The determination of the partition coefficients of the fluoroquinolones between liposomes and buffer is currently in progress in our laboratory.

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Appendix A

The experimental partition coefficient (D) can be defined as:

$$D = \frac{[HQ^{\circ}]_o + [HQ^{\pm}]_o}{[H_2Q^+]_w + [HQ^{\pm}]_w + [HQ^{\circ}]_w + [Q^-]_w} \quad \text{(A-1)}$$

This definition found in the literature (Ross et al., 1992) is based on the assumption that both neutral and zwitterionic species undergo partitioning.

The experimental values will be related to the thermodynamic partition coefficient (P) by

$$\log P = \log D + \log \frac{[H^+]^2 + K_1[H^+] + K_1K_2}{K_1[H^+]} \quad \text{(A-2)}$$

where K_1 and K_2 are the dissociation constants and $[H^+]$ the hydrogen ion concentration.

Appendix B

Alternatively, to the definition used in Appendix A, and assuming that only the neutral species undergoes partitioning between both phases, the experimentally determined partition coefficient can be defined (Takácks-Novák et al., 1995) by

$$D = \frac{[HQ^{\circ}]_o}{[H_2Q^+]_w + [HQ^{\pm}]_w + [HQ^{\circ}]_w + [Q^-]_w} \quad \text{(A-3)}$$

From a scheme of microspeciation (see Fig. 1) the four microconstants in microspecies concentration terms can be written (Alberty and Silbey, 1992), as

$$k_{11} = \frac{[HQ^{\circ}]_w[H^+]}{[H_2Q]_w} \quad \text{(A-4)}$$

$$k_{12} = \frac{[Q^-]_w[H^+]}{[HQ^{\circ}]_w} \quad \text{(A-5)}$$

$$k_{21} = \frac{[HQ^{\pm}]_w[H^+]}{[H_2Q^+]_w} \quad \text{(A-6)}$$

$$k_{22} = \frac{[Q^-]_w[H^+]}{[HQ^{\pm}]_w} \quad \text{(A-7)}$$

where k_{22}, k_{21}, k_{12} and k_{11} are the microconstants of dissociation. Combining Eqs. (A-4), (A-5), (A-6) and (A-7) and Eq. (A-3) yields,

$$D = \frac{[HQ^{\circ}]_o}{\frac{[HQ^{\circ}]_w[H^+]}{k_{11}} + \frac{k_{21}}{k_{11}}[HQ^{\circ}]_w + [HQ^{\circ}]_w + k_{12}\frac{[HQ^{\circ}]_w}{[H^+]}} \quad \text{(A-8)}$$

and introducing the partition thermodynamic partition coefficient (P) Eq. (A-8) can be written in logarithmic form,

$$\log P = \log D + \log\left(1 + \frac{k_{21}}{k_{11}} + \frac{k_{12}}{[H^+]} + \frac{[H^+]}{k_{11}}\right) \quad \text{(A-9)}$$

This expression differs from others (Takács-Novák et al., 1992, 1995) because of the convention followed by those authors who used the inverse of Eqs. (A-4), (A-5), (A-6) and (A-7) (Appendix B) to express ionization constants.

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