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Retention behaviour of quinolone derivatives in high-performance liquid chromatography
Effect of pH and evaluation of ionization constants

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Abstract

This paper examines the effect of solute ionisation on the retention behaviour of a series of quinolones and evaluates their pK_a values using chromatographic data in acetonitrile–water mixtures with acetonitrile percentages of 30, 35, 40 and 50% (v/v). We also compare these pK_a values with those previously obtained in acetonitrile–water mixtures from potentiometric measurements. In doing so, the pH values were measured in the hydroorganic mixture, which was used as the mobile phase, instead of in water, taking into account the effect of activity coefficients. The resulting equations permit the chromatographic determination of the pK_a values of the quinolones in acetonitrile–water mixtures and also permit the prediction of the effect of pH on their chromatographic behaviour. These equations can be combined with those previously derived, which relate retention to the solvent composition of the mobile phase, to establish a general model that relates the elution behaviour of the solute to significant mobile phase properties: composition, pH and ionic strength.

Keywords: pH effects; Retention behaviour; Mobile phase composition; Quinolones; Antibiotics

1. Introduction

Quinolones are an important class of synthetic, orally active, broad-spectrum antimicrobials that are effective against resistant mutants of bacteria [1–3]. Numerous structurally related quinolones have been synthesized and several are in routine clinical use throughout the world. The more potent analogues are represented by molecules supporting a piperazyl moiety.

The antibacterial activity of quinolones is pH-dependent, since they act by inhibition of bacterial DNA gyrase, a process that depends upon both the pH and concentration of the acid. Therefore, the examination of protonation equilibria in quinolone acid solutions is essential in understanding their antibacterial activity. Moreover, the relationships between dissociation constants and structure may prove useful in drug-design studies and in explaining the biopharmaceutical properties of the quinolones.

The widespread use of quinolones in the treatment of systemic infections highlights the need to analyse them and separate their components. Although a quinolone separation may be obtained by trial and error, this may take many attempts and be highly time-consuming. We can minimize the total number of experiments required by using accurate quantitative relationships that can predict the elution of quinolones under different separation conditions. Optimisation of the chromatographic resolution of

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ionogenic solutes in liquid chromatography (LC) is a task that is being actively researched [4–6]. Due to the specific acid–base characteristics of ionogenic solutes, the two most useful optimisation parameters are the pH and the concentration of organic modifier.

The pH of the mobile phase is a major factor influencing the chromatographic behaviour of quinolones given that they contain ionogenic functions such as carboxylic and amino groups. Their retention depends on the percentage of ionized and non-ionized species of the quinolone. Thus, a knowledge of the acid–base dissociation constants of the analytes in acetonitrile–water mixtures, which are usually used as mobile phases, can help to improve the analytical methods and can lead to a better understanding of their biochemical solutions.

In previous studies [7–9], an attempt at optimizing the concentration of organic modifier in the mobile phase, in chromatographic separations, was undertaken, by establishing relationships between the retention parameter and Reichardt's E_T^N scale of solvent polarity. The inclusion of pH as an additional optimization parameter gives rise to several problems [10]. The pH of the mobile phase is usually taken to be the same as that of the aqueous fraction. However, the pK values of the acids used in preparing the buffers are dependent on the solvent composition [11,12], as is the pH of the buffer [13,14]. Sometimes the pH is measured after mixing the buffer with the organic modifier [15]. However, even in this instance, the potentiometric system is usually calibrated with aqueous standards and the pH recorded is not the true pH of the mobile phase. Additionally, the proposed models do not consider the effect of the activity coefficients. This effect can be neglected in water, which has a high dielectric constant, but it may be considerable in acetonitrile–water mixtures [11].

The measurement of pH in acetonitrile–water, the most widely used mobile phase, can be performed in a similar manner to those in water [16], taking into account the pH values previously assigned to primary standard buffer solutions in acetonitrile–water mixtures [13,14], according to the National Institute of Standards and Technology (NIST) multiprimary standard scale [17]. Also, in compliance with International Union of Pure and Applied Chemistry (IUPAC) rules [16–18], the activity coefficients of the species in acetonitrile–water mixtures can be calculated from the ionic strength using the classical Debye-Hückel equation [6].

In this paper, we consider the effect of ionic equilibria on the retention behaviour of a series of quinolones and we evaluate their ionization constant values using chromatographic data, in acetonitrile–water mixtures with acetonitrile percentages of 30, 35, 40 and 50% (v/v). A polystyrene–divinylbenzene copolymer was used as the stationary phase. This polymer is particularly suited to these kinds of studies because of its stability throughout the entire pH range. Experimentally determined pK_a values were compared with those previously obtained using potentiometry in acetonitrile–water mixtures. Thermodynamically valid pH, measured in a hydro-organic mobile phase instead of in water, was used to interpret the role of pH in the chromatographic behaviour of quinolones and the effect of activity coefficients was taken into account. The equations so derived permit the chromatographic determination of the pK_a values of the quinolones in acetonitrile–water mixtures and also permit the prediction of the effect of pH on their chromatographic behaviour. The proposed equations can be combined with those previously derived, which relate retention to the solvent composition of the mobile phase, to establish a general model that relates the elution behaviour of the solute to the significant mobile-phase properties: composition, pH and ionic strength.

2. Experimental

2.1. Apparatus

The chromatographic equipment used consisted of an ISCO Model 2350 pump with an injection valve, a 10-ml sample loop and a variable wavelength V^4 absorbance detector (ISCO) operating at 280 nm, and at 295 nm for ofloxacin. The chromatographic system was controlled by Chemresearch Chromatographic Data Management System Controller software (ISCO) running on a Peceman AT Supermicro personal computer. A Shodex RSpak DS-613 column (150×6 mm I.D.), packed with hydrophobic polystyrene–divinylbenzene gel, was used at ambient temperature. The pH of the mobile phase was

measured with a Crison 2002 potentiometer (0.1 mV) using an Orion 8102 Ross combination pH electrode. All solutions were thermostated externally at 25±0.1°C. The electrode was stabilized in the appropriate acetonitrile–water mixtures prior to the e.m.f. measurements, and the measurements were performed in triplicate, to ensure the stability and reproducibility of the potentiometric system.

2.2. Reagents

All reagents were of analytical grade. Water, with a conductivity lower than 0.05 μS/cm, and acetonitrile (Merck) were of HPLC grade. The eluents were filtered through a 0.22-μm nylon filter membrane (MSI) and degassed ultrasonically before use. The quinolones were obtained from various pharmaceutical firms: norfloxacin (Liade, Boral Qumica), enoxacin (Almirall), fleroxacin (Roche), ofloxacin (Hoescht) and flumequine (Sigma). Working standard solutions of quinolones were prepared in the mobile phase at concentrations of approximately 20 mg l^{-1}. The samples were filtered through a 0.45-μm nylon filter membrane (MSI) before injection.

2.3. Chromatographic procedure

Throughout this study, the mobile phases assayed were acetonitrile–buffer (30:70; 35:65; 40:60; 50:50, v/v). In these media, 25 m*M* phosphoric acid, adjusted to pH values between 3.0 and 11.0 with sodium hydroxide, was used as the buffer component. The flow-rate of the mobile phase was maintained at 1 ml/min. For each quinolone and for every mobile-phase composition and pH considered, the retention time values, t_R, were determined from three separate injections. Capacity factors were calculated from $k'=(t_R-t_0)/t_0$, where t_0 is the retention time of potassium bromide (hold-up time), which was established for each mobile phase composition and pH studied.

2.4. Establishment of pH operational scale

The definition and determination of pH are key questions when using aqueous solvent with an organic modifier. The operational pH in mixed aqueous–organic solvents is usually measured on the assumption that the pH of the mobile phase is the same as that of the aqueous fraction. Thus, errors due to medium effects create uncertainly regarding the true pH [19]. In acetonitrile–water mixtures, the influence of the cosolvent on the pH is substantial [20,21] and, therefore, for successful systematic optimization of the mobile-phase pH, accurate pH measurements in these solvent mixtures are needed.

Operationally, the pH value in an aqueous organic system is determined in the normal manner [22] by comparing the measured e.m.f. value, E_X, with that obtained for a standard buffer solution, E_{PS}, of known pH_{PS} in a solvent of identical composition:

$$pH_X = pH_{PS} + \frac{E_{PS} - E_X}{g} \quad (1)$$

where $g=(\ln 10)\ RT/F$.

As pH_{PS} values have been determined previously in acetonitrile–water mixtures [13,14], in accordance with IUPAC rules [16–18] for the primary standard series of substances proposed by NIST [17], pH values in acetonitrile–water mixtures can be measured using methods similar to those used in water. In this study, we used potassium hydrogen phthalate and phosphate buffers [8,13] as the primary standard buffer reference solutions in the acetonitrile–water mixtures studied.

The molar activity coefficients, *y*, were calculated using the classical Debye-Hückel expression:

$$\log y = \frac{-AI^{1/2}}{1 + a_0 BI^{1/2}} \quad (2)$$

where the values of the Debye-Hückel *A* and *B* constants and the ion size parameter, a_0, in the acetonitrile–water mixtures have been reported previously [23].

The ionic strength, *I*, of the mobile phases used can be calculated for each pH value from charge and mass balances, taking into account the pK_1 and pK_2 values of phosphoric acid at each mobile phase composition [11], the analytical concentration of this acid in the mobile phase, the pH values and the activity coefficients, using an iterative calculation [24].

The pH scale of any hydroorganic solvent mixture is the pH range limited by the zero and pK_{ap} [25], where K_{ap} is the autoprotolysis constant of the

medium. We have previously determined the pK_{ap} values of acetonitrile–water mixtures and derived equations that relate these pK_{ap} values to the percentage of acetonitrile in the mixtures [23]. The pK_{ap} values of the mixtures were found to increase slightly from pure water to a high percentage of acetonitrile [at 70% (w/w) acetonitrile, $pK_{ap} = 16.76$] [23]. For higher acetonitrile concentrations, the increase in pK_{ap} value is sharper, especially after a molar fraction of 0.75 in acetonitrile. This can be explained by the preferential solvation of proton ions in acetonitrile–water mixtures [14].

2.5. Data analysis

Theoretical models describing the dependence of the capacity factor, k', on the pH of the mobile phase, using reversed-phase sorbents, can be derived by considering pH values in the mobile phase, activity coefficients and by taking into account the ionization equilibria of the compounds.

Quinolones are substances with one proton-binding site, such as flumequine, or with two proton-binding sites, such as norfloxacin, enoxacin, fleroxacin and ofloxacin. The capacity factor, k', of any ionizable solute as a function of mobile phase pH can be expressed by considering that the observed capacity factor, k', is a weighted average of the k' of the ionic and neutral forms of the solute [26], according to the molar fractions of these forms in the mobile phase. The overall observed k' for flumequine, with only one carboxylic functional group (Fig. 1), can be given as:

$$k' = x_{HA}k'_{HA} + x_{A^-}k'_{A^-} \quad (3)$$

where k'_{HA} and k'_{A^-} are the capacity factors of the

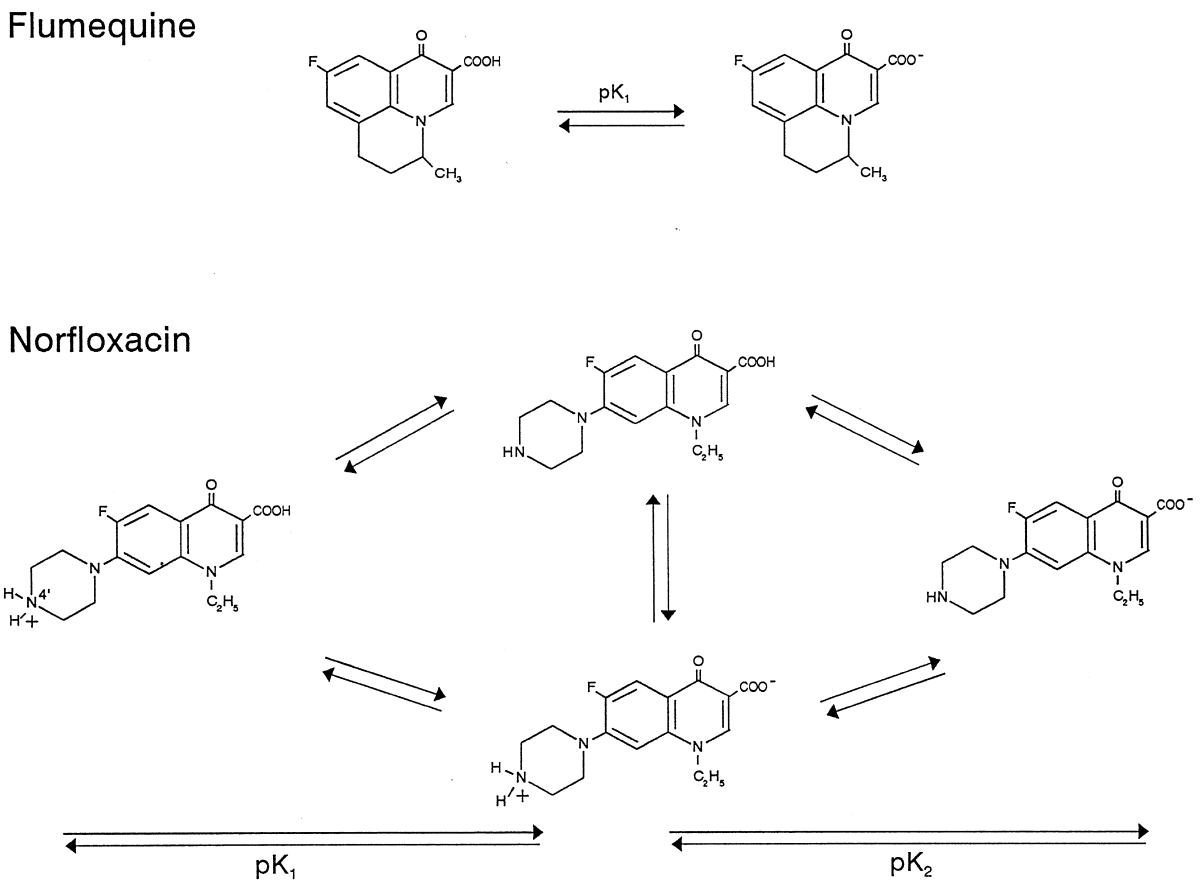

Fig. 1. Protolytic equilibria of flumequine and norfloxacin.

solute in the non-ionized and ionized form, respectively, and x_i is the molar fraction.

Eq. (3) can be written as:

$$k' = \frac{[HA]k'_{HA} + [A^-]k'_{A^-}}{[HA] + [A^-]} \quad (4)$$

dividing by [HA]:

$$k' = \frac{k'_{HA} + \frac{[A^-]}{[HA]} \cdot k'_{A^-}}{1 + \frac{[A^-]}{[HA]}} \quad (5)$$

The protolytic equilibrium of flumequine, as a monoprotic acid, is ruled by the thermodynamic dissociation constant

$$K_a = \frac{[A^-]y\, a_{H^+}}{[HA]} \quad (6)$$

The classic approach neglects activity coefficients and uses the pH value in water instead of the pH value in the mobile phase.

From Eq. (6):

$$\frac{[A^-]}{[HA]} = \frac{K_a}{a_{H^+}y} \quad (7)$$

By replacing Eq. (7) in Eq. (5):

$$k' = \frac{k'_{HA} + k'_{A^-} \cdot \frac{K_a}{a_{H^+}y}}{1 + \frac{K_a}{a_{H^+}y}} \quad (8)$$

For the other quinolones studied, two proton-binding sites need to be considered (Fig. 1). These substances exist in four microscopic protonation forms in solution. The scheme of the interconversion between the four microspecies, as well as the relevant pK_a values are shown in Fig. 1. The pertinent equilibrium constants correspond to the equilibria:

$$^+HBAH \leftrightarrows {}^+HBA^- + H^+ \quad K_{a1}$$

$$^+HBA^- \leftrightarrows BA^- + H^+ \quad K_{a2}$$

The equation that relates the chromatographic retention and the pH of the mobile phase in the case of amphoteric substances such as these quinolones can be derived as in Eq. (8) and is:

$$k' = \frac{k'_{^+HBAH} \cdot \frac{a_{H^+}}{K_{a1}y} + k'_{^+HBA} + k'_{BA^-} \cdot \frac{K_{a2}}{ya_{H^+}}}{\frac{a_{H^+}}{K_{a1}y} + 1 + \frac{K_{a2}}{ya_{H^+}}} \quad (9)$$

Eqs. (8) and (9) were originally elaborated for aqueous solutions only; however, they are of general validity. For aqueous organic solvents, a_{H^+} is the activity of the solvated proton in the mixed aqueous–organic solvent and K_a is the dissociation constant of a compound in the same solvent. These equations have been proposed for cases where the interaction between a solvent and a solute is exclusively controlled by their hydrophobicity. Eventual deviations of the theoretical sigmoidal curves provide evidence of the presence of non-hydrophobic interactions on a tested reversed-phase solvent [27].

The usefulness of such equations can be twofold. They define the equilibria that influence the sorption of organic acids and bases in LC and they permit the prediction of elution behaviour as a function of a minimum number of measurements. That is, the k' can be calculated as a function of pH if K_a and k' values for the different species of the substance are known.

3. Results and discussion

Experiments were carried out to determine the capacity factors, k', of the series of five quinolones studied, over the pH range 3.0 to 11.0. The hydro-organic mixtures used as mobile phases were acetonitrile–water mixtures with percentages of acetonitrile of 30, 35, 40 and 50% (v/v). k' values were determined from three separate injections at every mobile phase composition and at each pH considered. Relative standard deviations lower than 2% were obtained. Experimental k' values are shown in Figs. 2–6.

Standard reference solutions of potassium hydrogen phthalate and phosphate were used in measuring the pH in the acetonitrile–water mobile phase with a commercial combination pH electrode [8]. The pH_{PS} of these standard reference solutions, which are included in the series recommended by the NIST, were previously determined in acetonitrile–water mixtures [13,14]. In addition, good accuracy and

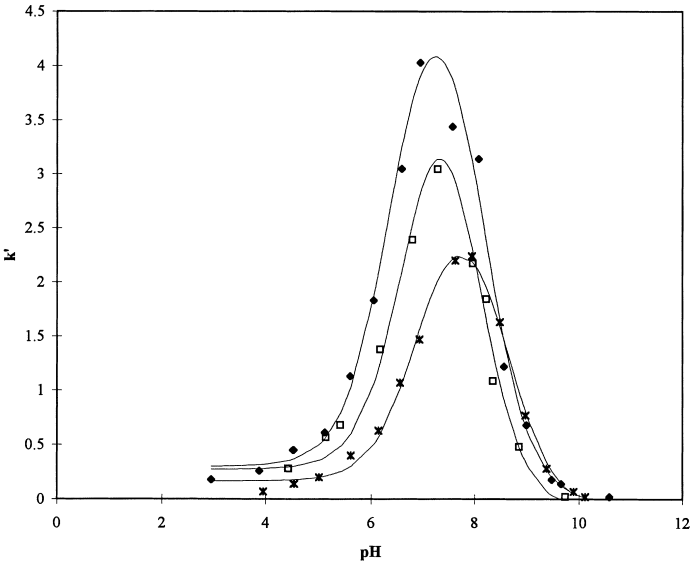

Fig. 2. Plot of the chromatographic capacity factor, k', of enoxacin versus the pH of mobile phases with different percentages of acetonitrile: ♦, 30% (v/v); □, 40% (v/v) and *, 50% (v/v).

rapid stabilization of the potentiometric system were observed, as reported in a previous work [8]. The measurement of pH in the acetonitrile–water mixtures is very convenient since the application of Eqs. (8) and (9) allows the pK_a values of the substances to be obtained.

Four of the quinolones studied have two relevant ionizable functional groups, which means that their

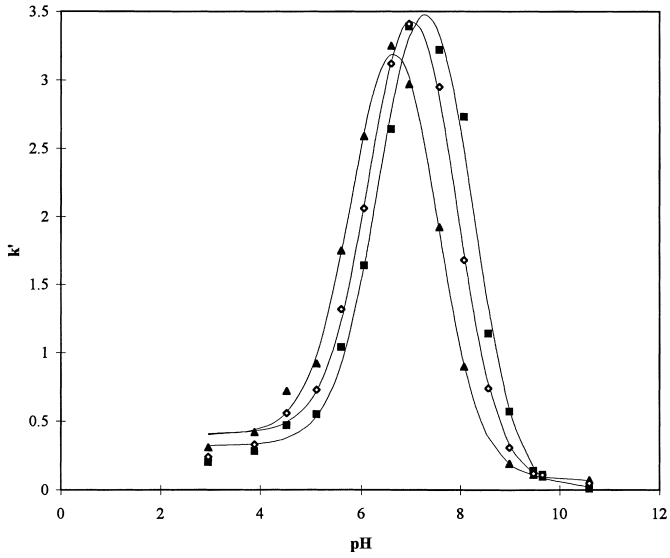

Fig. 3. Plot of the chromatographic capacity factor, k', versus the pH of the mobile phase containing 30% (v/v) acetonitrile: ■, norfloxacin; ▲, fleroxacin and ◇, ofloxacin.

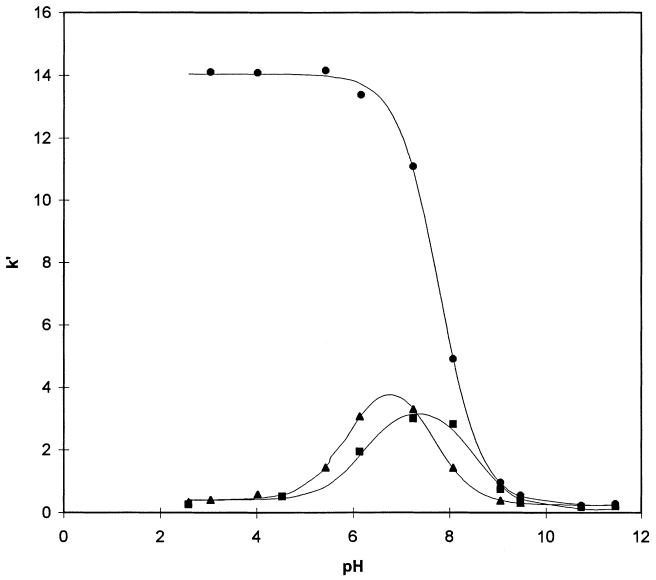

Fig. 4. Plot of the chromatographic capacity factor, k', versus the pH of a mobile phase containing 35% (v/v) acetonitrile. ■, norfioxacin; ▲, fleroxacin and ●, flumequine.

acid–base chemistry involves two protons. In contrast, flumequine has only one relevant ionizable functional group within the pH range of pharmaceutical or physiological importance (Fig. 1).

The octadecylsilica, ODS, stationary phase may be used only in the pH range 2–7, so it was not possible to study the retention of quinolones over the whole pH scale. Thus, we used a polystyrene–divinylbenzene copolymer as the stationary phase. This copolymer is particularly suitable for studying the

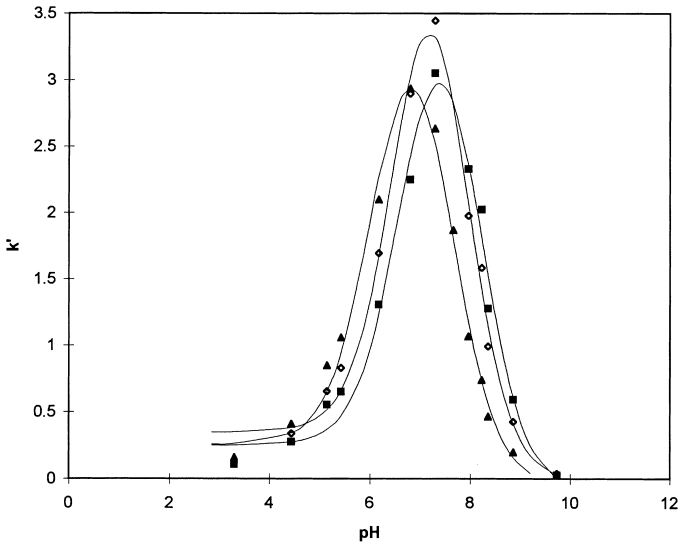

Fig. 5. Plot of the chromatographic capacity factor, k', versus the pH of the mobile phase with 40% (v/v) acetonitrile. Symbols are the same as in Fig. 3.

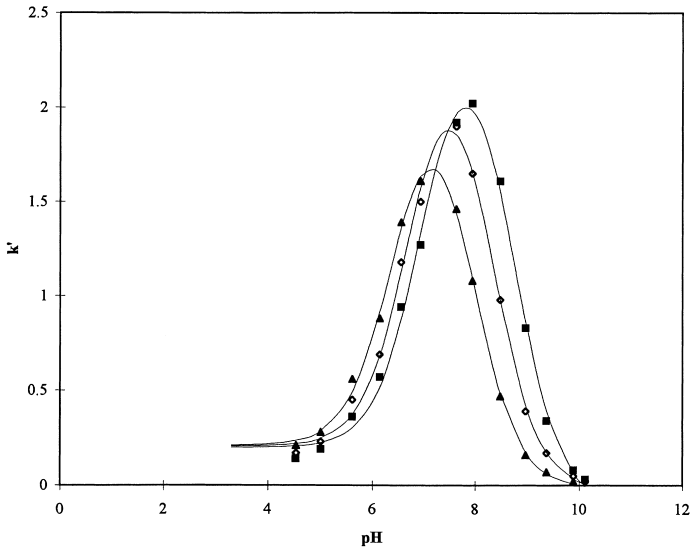

Fig. 6. Plot of the chromatographic capacity factor, k', versus the pH of a mobile phase containing 50% (v/v) acetonitrile. Symbols are the same as in Fig. 3.

effect of pH on chromatographic retention, given its stability throughout the entire pH range.

Eqs. (8) and (9), which relate the capacity factor to pH, were applied to each type of quinolone substance. Each equation was experimentally verified and the pK values of the quinolones studied were determined from the experimental k' values, the pH measurements and the calculated activity coefficient values. The pK values obtained are listed in Table 1 and were calculated using a non-linear least-squares fit of the data. Eqs. (8) and (9) also permit the determination of the capacity factors of the different species of quinolones, and these are given in Table 2.

All quinolones have a carboxyl group, therefore pK_{a1} values can be associated with this carboxylic acid function [28,29] (Fig. 1). The pK_{a2} values can be associated with the presence of a piperazine ring (Fig. 1). Protonation occurs at N_4 of the piperazine

Table 1
pK values of quinolones obtained from chromatographic and potentiometric measurements

Compound		Percentage of acetonitrile							
		30% (v/v)		35% (v/v)		40% (v/v)		50% (v/v)	
		pK_1	pK_2	pK_1	pK_2	pK_1	pK_2	pK_1	pK_2
Norfloxacin	Chromatographic	6.27 (0.08)	8.35 (0.07)	6.14 (0.15)	8.61 (0.15)	6.53 (0.15)	8.28 (0.13)	6.88 (0.06)	8.82 (0.07)
	Potentiometric	7.20 (0 04)	8.78 (0.02)	7.45 (0.05)	8.72 (0.02)			7.81 (0.04)	8.95 (0.05)
Fleroxacin	Chromatographic	5.83 (0.04)	7.58 (0.03)	5.84 (0.05)	7.71 (0.05)	5.96 (0.10)	7.71 (0.09)	6.32 (0.05)	8.05 (0.05)
	Potentiometric	6.59 (0.03)	8.05 (0.02)	6.60 (0.02)	7.94 (0.05)			6.97 (0.02)	8.21 (0.03)
Enoxacin	Chromatographic	6.28 (0 11)	8.28 (0.11)			6.59 (0.15)	8.13 (0.12)	6.81 (0.06)	8.73 (0.07)
	Potentiometric		8.37 (0.04)						8.67 (0.05)
Ofloxacin	Chromatographic	6.10 (0.04)	7.99 (0.03)			6.45 (0.12)	7.95 (0.10)	6.63 (0.06)	8.42 (0.06)
	Potentiometric		8.13 (0.03)						8.58 (0.04)
Flumequine	Chromatographic			7.84 (0.02)					
	Potentiometric			7.60 (0.03)					

Values in parentheses are standard deviations.

Table 2
Chromatographic capacity factors of the protonated species of the quinolones

Compound	Acetonitrile percentage													
	30% (v/v)			35% (v/v)					40% (v/v)			50% (v/v)		
	k'_{+HBAH}	k'_{+HBA^-}	k'_{BA^-}	k'_{+HBAH}	k'_{+HBA^-}	k'_{BA^-}	k'_{HA}	k'_{A^-}	k'_{+HBAH}	k'_{+HBA^-}	k'_{BA^-}	k'_{+HBAH}	k'_{+HBA^-}	k'_{BA^-}
Norofloxacin	0.32	4.22	0.00	0.40	3.56	0.11			0.25	3.90	0.00	0.20	2.56	0.00
	(0.07)	(0.22)	(0.08)	(0.18)	(0.27)	(0.08)			(0.09)	(0.45)	(0.18)	(0.04)	(0.14)	(0.05)
Fleroxacin	0.40	4.06	0.00	0.41	4.72	0.24			0.26	3.83	0.00	0.21	2.20	0.00
	(0.04)	(0.13)	(0.03)	(0.03)	(0.19)	(0.03)			(0.08)	(0.29)	(0.10)	(0.03)	(0.09)	(0.03)
Enoxacin	0.30	5.05	0.00	–	–	–			0.41	4.15	0.00	0.17	2.85	0.00
	(0.11)	(0.39)	(0.10)						(0.11)	(0.48)	(0.15)	(0.04)	(0.13)	(0.05)
Ofloxacin	0.41	4.32	0.00	–	–	–			0.35	4.71	0.00	0.21	2.43	0.00
	(0.04)	(0.11)	(0.03)						(0.09)	(0.46)	(0.14)	(0.03)	(0.11)	(0.03)
							14.04	0.25						
Flumequine	–	–	–				(0.04)	(0.08)	–	–	–	–	–	–

Values in parentheses are standard deviations.

ring over other apparently basic sites, as is confirmed by NMR measurements [29] and by the fact that *N*-acetylnorfloxacin has only one proton-binding group (carboxylate), since the molecule loses amine basicity due to the acetylation of the N atom [29].

The pK_1 values associated with the carboxylic acid function for the compounds studied here were higher than those generally observed with carboxylic acids in water mixtures [11] [e.g. acetic acid in 30% (w/w) acetonitrile has a pK of 5.63]. This decrease in acidity can be attributed to intramolecular H-bond formation with the neighbouring keto function, resulting in stabilization of the protonation species [28]. The formation of an intramolecular hydrogen bond is supported by UV and IR spectral data [30].

The pK_2 values of the secondary amine-type derivatives studied (norfloxacine and enoxacine) are greater than those of the tertiary amines (ofloxacine and fleroxacine). These findings were consistent with reports in the literature for similar secondary and tertiary amines: piperazine, pK=9.71 [31], and *N*-methylpiperazine, pK=8.98 [32], in water. The more water molecules involved in the hydrate sphere of the protonated amine, the greater the stabilization [33]. The protonated form of the secondary amine was stabilized by the greater number of water molecules involved in its hydration sphere in comparison with the corresponding tertiary amine [28].

In a previous paper [34], the variation of the pK_{a1} and pK_{a2} values of quinolones with the percentage of acetonitrile in acetonitrile–water mixtures was studied. The pK_{a1} and pK_{a2} values of a series of quinolones were determined potentiometrically in different acetonitrile–water mixtures. The variations of pK values were different for each substance, although, in general, the pK values increased as the acetonitrile content increased. The pK_{a1} values of quinolones varied even when the percentage of acetonitrile was low, whereas pK_{a2} values showed small changes in the range from 0% to approximately 30% acetonitrile and they increased at higher percentages of acetonitrile. This is explained by the structural features of acetonitrile–water mixtures [34]. Also, the pK values obtained using chromatographic data increased with the percentage of acetonitrile (Table 1). However, preferential solvation by water, in acetonitrile–water mixtures with a molar fraction of acetonitrile that is lower than 0.75, produces lower pK values than expected in these mixtures [21].

In order to confirm the pK values obtained using chromatographic data, we summarize (also in Table 1) the previously obtained pK values of the quinolones in the same solvent mixtures, using the potentiometric method, according to the rules and procedures endorsed by IUPAC [34].

Various authors [35–37] have remarked on the advantages of the LC method for evaluating the ionization constants of substances. Small quantities of compounds are required, poor water-solubility is not a serious drawback and the samples need not be pure. However, as can be deduced from Table 1, the

precision of the pK values of quinolones in acetonitrile–water mixtures determined by the LC method is lower than that found using the potentiometric method. The discrepancy between chromatographic and potentiometric data is greater for pK_{a1} values than for pK_{a2} values, in the case of quinolones with two ionizable functional groups, whereas for flumequine, the discrepancy between chromatographic and potentiometric pK values at 35% (v/v) acetonitrile is not greatly significant from a statistical point of view. The results obtained for flumequine are in accordance with the pK_a values, obtained using LC, for substances with only one carboxylic functional group [38].

The discrepancies between the pK_{a2} values obtained using both methods are, in general, significantly lower than those obtained for pK_{a1} values.

Equations relating chromatographic retention with pH are proposed for those cases where the interaction between the sorbent and the solute was controlled exclusively by their hydrophobicity, that is, no ionic or hydrogen bonding occurred between the solute and the stationary phase. The solvophobic theory, used to derive Eqs. (8) and (9), attributed the retention process to the mobile phase and treated the stationary phase as a passive entity that played no role in the separation process, other than providing a sorptive site for retention [39].

Consideration of the thermodynamics of chromatographic retention indicates that the interaction energy, which is responsible for solute retention, is governed by three competing effects: solute–absorbent interactions, solvent–solute interactions and solvent–absorbent interactions [40]. Carr et al. [41] demonstrated that most of the free energy retention in LC arises from attractive dispersive interactions between the solute and the stationary phase, and not from net repulsive interactions in the mobile phase.

On the other hand, neutral compounds can be analyzed successfully with high levels of efficiency and reproducibility. However, the separation of basic compounds with amino groups often causes many more difficulties [27]. Basic compounds have been described as showing very strong non-hydrophobic interactions [27,40]. If the retention mechanism includes interactions other than those of a hydrophobic nature, the k' versus pH dependence can show different shapes or at least the same deviation from the ideal sigmoidal shape.

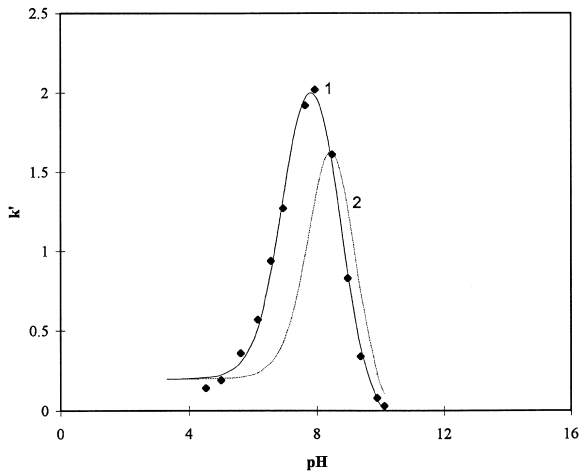

Fig. 7. Experimental, curve 1, and theoretical (potentiometric pK_1 and pK_2 values), curve 2, dependence of k' for norfloxacin at different pH values in acetonitrile–water (50:50, v/v).

Fig. 7 illustrates the theoretical and experimental dependence of k' values on pH. Curves 1 and 2 were constructed as follows: curve 1, experimentally obtained capacity factors for norfloxacin at different pH values in 50% (v/v) acetonitrile–water mixtures were fitted using Eq. (9). Known values of $[H^+]$ (pH measured in the mobile phase) were inserted into Eq. (9) and the k' values of individual species and the K_a values were computed by the non-linear least-squares method. Curve 2, theoretical dependence of k' on pH for a given analyte in the acetonitrile–water mixtures used as mobile phases, as described by Eq. (9). In this curve, K_a values correspond to the previously obtained potentiometric pK_a values, while the k' values of individual species were obtained as described for curve 1.

The retention of quinolones is low in strong acid media (Fig. 7), where the quinolone exists as a single charged cation. As the pH increases, the k' value increases and levels off at the isoelectric point pH. Thus, the equilibrium between the double charged zwitterionic and neutral forms is displaced to the latter form (Fig. 1). If the pH is increased again, the k' values decrease and we have the anionic monocharged species of quinolones (Fig. 1).

It can be shown that the lower degree of concordance between the theoretical and the experimental curve corresponds to the first dissociation of the protonated quinolone. It is assumed that, in acidic

media, the amino groups of quinolones are protonated [28]. This positive monocharged group can have donor–acceptor interactions with the sorbent [42]. Then, the retention in moderately acid media is greater than that predicted theoretically (Fig. 7), and the resulting equilibrium constant is lower than expected. In the same way, at higher pH values, possible repulsion between the electrons of the column and the negatively charged carboxylic group causes the decrease in the experimental k' values, but to a lesser extent that at lower pH values.

In clearly acidic media, proton interactions are also involved and the retention of the protonated amino group can be predicted by the solvophobic model. The ability of a protonated amino solute to reveal nonsolvophobic interactions on the surface of a sorbent depends mainly on its dissociation constant. The strongly basic compounds are the most sensitive probes. The difference between the curve obtained experimentally and the theoretical prediction (Fig. 7) is closely related to the physicochemical characteristics of the solute [27,40]. This is in accordance with the finding that certain departures of sigmoidal k' versus pH curves from the ideal shape were observed only for zwitterionic substances with medium and high pK_{a1} constants [27]. Thus, this problem was not observed in studies concerned with variations in LC retention of the peptide with pH [43] because pK_{a1} values of peptides are about three units.

Knowledge of the pK values of many substances in acetonitrile–water mixtures [12,20,21] permits detailed study to be carried out regarding the influence of the pH of the hydroorganic medium used as the mobile phase on the retention of acidic, basic and zwitterionic substances. Additional aspects will be the subject of other studies.

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