ELSEVIER

Journal of Chromatography A, 871 (2000) 381–389

JOURNAL OF CHROMATOGRAPHY A

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Influence of pH and pK_a values on electrophoretic behaviour of quinolones in aqueous and hydro-organic media

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Abstract

Through correct pH measurements, pK_a and activity coefficient values, a model describing their effect on electrophoretic behaviour of substances is established. The suggested model uses the pH values in the acetonitrile–water mixtures used and takes into account the effect of activity coefficients. The model permits the calculation of acidity constants of analytes in hydro-organic media and also the prediction of the effect of pH on the electrophoretic mobility. The model is tested by determining the dissociation constants of a series of nine quinolones in acetonitrile–water mixtures of 0, 5.5, 10 and 30% (w/w) acetonitrile.

Keywords: Activity coefficients; pH effects; Electrophoretic mobility; Dissociation constants; Quinolones; Antibacterial agents

1. Introduction

A complex series of events, including absorption and distribution of the drug throughout the body while it undergoes metabolic biotransformations, follows the administration of any therapeutic agent. The activity of many biological molecules depends on the presence of charged groups, and, in consequence, dissociation constants can be a key parameter for understanding and quantifying chemical phenomena such as reaction rates, biological activity, or transport and environmental fate [1–3].

Potentiometric, UV spectrophotometric and solubility methods are used in the determination of pK_a values, although they cannot always be applied to compounds that are sparingly soluble in water. Furthermore, these conventional methods do not take into account the purity or the stability of samples, which influence the observed pK_a values [4].

Capillary electrophoresis (CE) has been introduced as a method for convenient and precise pK_a determination [2,4–8]. It only requires small amounts of sample at low solute concentrations, and impurities can be separated. In contrast to potentiometric techniques, the procedure does not require the determination of solute or titrant concentration, but only of migration times. Moreover, the addition of organic solvents to water offers several advantages in CE, the main one being, the improved ability to manipulate selectivity, since mobilities of solutes depend on the nature and ratio of organic solvent and on the pH value of the solution [9,10]. Acetonitrile (MeCN), a good differentiating solvent because of its weakly acidic and basic properties [11,12] and with the added advantages of low viscosity and good UV transparency, which makes it ideal for CE [9,13], has been used in this work.

Quinolones are a family of antibacterial agents

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0021-9673/00/$ – see front matter
PII: S0021-9673(99)01019-5

that act by inhibiting bacterial DNA gyrase. Owing to their favourable antibacterial and pharmacokinetic profile, they are used in clinical applications and in the treatment and prevention of disease in food-producing animals and commercially farmed fish. pK_a values in MeCN–water mixtures are significantly different from those in water [3,8,17–19], but can be obtained from the literature data [14–16]. A good knowledge of the acid–base behaviour of quinolones in MeCN–water mixtures is essential to predict the influence of pH on selectivity and on retention in liquid chromatography (LC) [20] and also to establish and optimise analytical procedures for separation of quinolones by CE.

In this work, a model describing the influence of pH, pK_a and activity coefficients on the electrophoretic behaviour of quinolones in CE is established. This model makes use of correct pH values in MeCN–water mixtures, determined in compliance with IUPAC rules [21,22] since pH of standard reference solutions of NIST are available [23–25] in MeCN–water mixtures, and it also takes into account the effect of activity coefficients. The proposed equations allowed the acidity constants of the analytes in hydro-organic media to be calculated and the effect of pH on the electrophoretic mobility to be predicted. The model was tested by the determination of the dissociation constants of a series of nine quinolones in MeCN–water mixtures with 0, 5.5, 10 and 30% (w/w) of MeCN. Danofloxacin, ciprofloxacin, sarafloxacin, enrofloxacin, difloxacin, flumequine and marbofloxacin were chosen because they are included in the European Union (EU) Council Regulation 2377/90, which establishes regulatory levels for quinolones in food-producing animals [26], and norfloxacin and pipemidic acid were chosen because of their therapeutic importance and widespread use.

2. Experimental

2.1. Apparatus

All CE experiments were performed on a P/ACE System 5500 (Beckman Instruments, Palo Alto, CA, USA) equipped with an autosampler, automatic injector and photodiode array detector. An untreated fused-silica capillary of 47 cm (40 cm from inlet to detector)×75 μm I.D. (Polymicro Technologies Phoenix, AZ, USA) was used. Samples were injected hydrodynamically at 0.5 p.s.i. for 6 s when working with aqueous media and for 3 s when working with MeCN–water mixtures (1 p.s.i.=6894.76 Pa). The experiments were performed at 20 kV at 25±0.1°C. The electropherograms were recorded using a computer program (p/ACE Station 1.0 with interface Golden System) supplied by Beckman. Ciprofloxacin, enrofloxacin, norfloxacin, danofloxacin and marbofloxacin were monitored at 280 nm, sarafloxacin, difloxacin and pipemidic acid at 275 nm, and flumequine at 250 nm. Acetone was also monitored at 280 nm. The pH of the buffer solutions was measured with a Crison 2002 potentiometer (Crison Instruments, Barcelona, Spain) using a Ross electrode 81-02 supplied by Orion Research (Boston, MA, USA).

2.2. Capillary treatment

Before each buffer was changed, the capillary was purged with 1 *M* sodium hydroxide for 20 min, followed by Milli-Q water for 20 min and the appropriate buffer electrolyte for 20 min. The last step was the application of a voltage of 20 kV for 20 min with the capillary filled with buffer solution. Every day the system was first purged with 0.1 *M* NaOH for 5 min followed by water for 15 min and working buffer solution for 20 min. A voltage of 20 kV was also applied for 10 min, with the capillary filled with buffer solution. Between each run the capillary was flushed successively with ultrapure water for 1 min followed by running buffer for 3 min in order to equilibrate the capillary and thereby minimise hysteresis effects. Capillaries were stored overnight filled with working buffer electrolyte.

2.3. Chemicals and reagents

Norfloxacin and flumequine were supplied by Sigma, sarafloxacin and difloxacin were supplied by Abbott Labs., ciprofloxacin was obtained from Lasa Labs., enrofloxacin from Cenavisa, danofloxacin from Pfizer, pipemidic acid was obtained from Prodesfarma Labs., and marbofloxacin from Vet-

oquinol Labs. The structures of the quinolones are shown in Fig. 1.

Phosphoric acid (85%), sodium hydroxide, potassium hydrogenphthalate and acetone were supplied by Merck and acetic acid was obtained from Carlo Erba. All chemicals used in the preparation of buffers and solutions were analytical reagent grade. MeCN, HPLC grade, was supplied by Baker. Water, with a resistivity of 18.2 MΩ cm, was obtained using a Milli-Q water purification system (Millipore, Molsheim, France).

2.4. Preparation of solutions

Solutions of individual quinolones were prepared in 0.05 *M* acetic acid at concentrations of 100 μg/ml and 50 μg/ml for use in acidic and basic aqueous media, respectively, while a concentration of 50 μg/ml was used for all quinolones in working in MeCN–water mixtures. We studied the solubility of quinolones in several media, such as aqueous acetic acid, phosphoric acid, water and MeCN. No significant differences were observed but aqueous acetic acid tended to be the best solvent. Each solution contained acetone at 3% (v/v) as the electroosmotic flow marker [27,28].

2.4.1. Aqueous media

To determine the pK_a values in aqueous solution, several buffer systems in the pH range between 5.25 and 9.00 were chosen. Buffers were prepared with phosphoric acid and adjusted to the appropriate pH with NaOH. The volume of NaOH solution added has been considered negligible comparing to the total volume employed (a maximum of five drops in a 250 ml of buffer prepared). The concentration of the

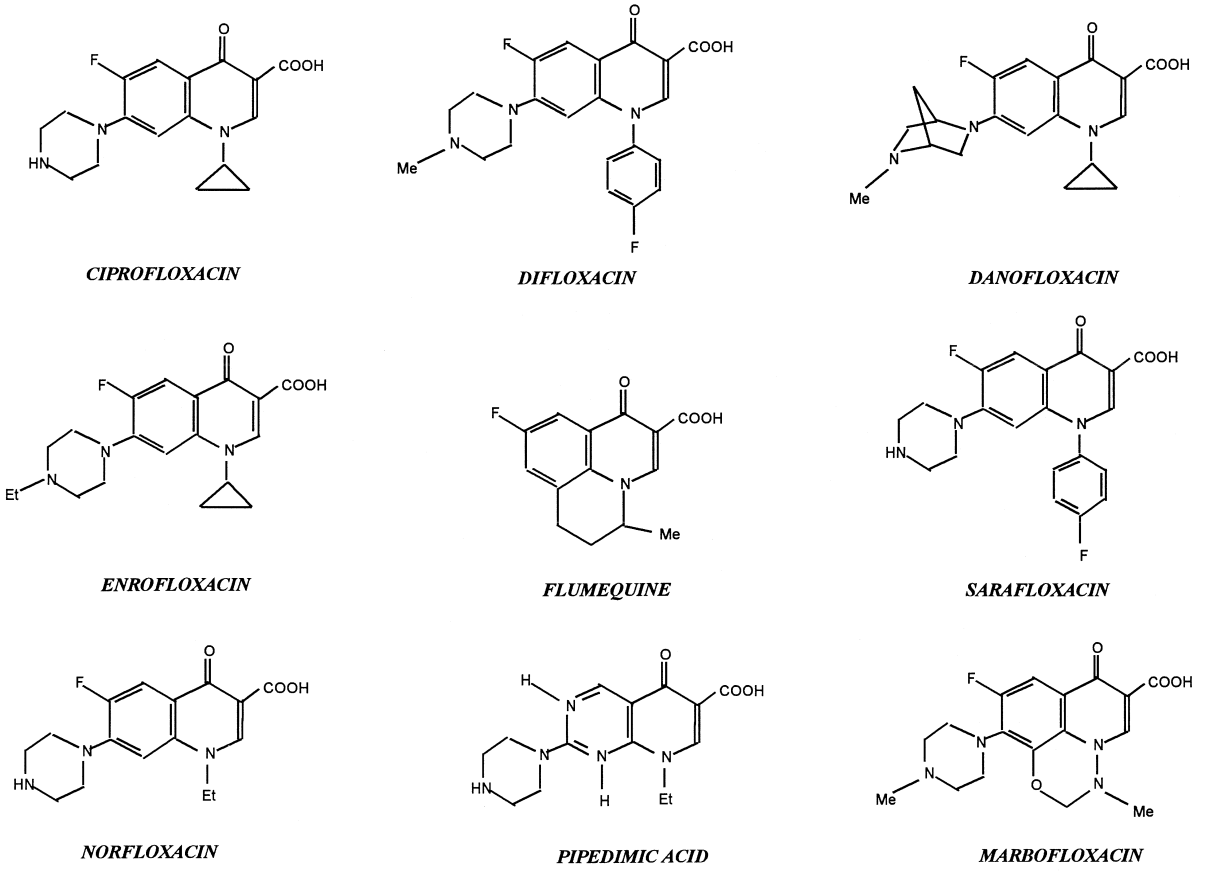

Fig. 1. Structures of quinolones.

buffer was 0.050 *M* and 0.025 *M* in the acidic and basic media, respectively. To determine the electrophoretic mobilities of the fully protonated and deprotonated species a 0.05 *M* phosphate buffer at pH 2 and a 0.025 *M* phosphate buffer at pH 11 were used.

2.4.2. MeCN–water mixtures

The background solvent for the buffer solutions was prepared by mixing water and MeCN with 5.5%, 10% and 30% (w/w) of MeCN. Phosphate working solutions at a concentration of 0.025 *M* in the pH range between 4.0 and 10 were obtained in each MeCN–water mixture by diluting a concentrated solution of phosphoric acid with the appropriate MeCN–water mixture, and adjusting the pH by addition of NaOH. To determine the electrophoretic mobilities of the fully protonated and deprotonated species a 0.025 *M* phosphate buffer at pH 2 and a 0.018 *M* phosphate buffer at pH 11 were used in each MeCN–water mixture. All solutions (quinolones and buffers) were passed through a 0.45-μm filter.

The determination of accurate pH values in MeCN–water mixtures can be performed in a similar way to how they are performed in water, because reference pH values were assigned to buffers solutions in these media [23–25]. Two standard buffers in the different hydro-organic mixtures were used to calibrate the pH meter: sodium hydrogenphosphate plus potassium dihydrogenphosphate buffer (0.025 mol/kg each component) was prepared in 5.5%, 10% and 30% (w/w) of MeCN with pH of 7.15, 7.18 and 7.60 [23–25] and potassium hydrogenphthalate buffer (0.05 mol/kg) with pH of 4.19, 4.32 and 5.02 [23–25] in MeCN–water mixtures with 5.5, 10 and 30% (w/w) of MeCN, respectively.

2.5. Procedures

All solutions were injected in triplicate and monitored at the appropriate wavelength. Injections were repeated for several days until the electrophoretic mobility was constant. The criterion was that the m_e values in three different consecutive days differed by less than $2 \cdot 10^{-6}$ cm^2 s^{-1} V^{-1}. Electrophoretic mobilities were calculated as the difference between the apparent mobility, m_{app}, of each quinolone and mobility of the neutral marker, m_{EOF}, taking into account the length of the capillary and the voltage applied [29]. Each electrophoretic mobility was calculated as the average of at least three replicates.

Eight of the quinolone derivatives studied have two relevant ionisable functional groups, which means that their acid–base chemistry involves two equilibria and in most cases the two pK_a values are close [8,30].

We applied a model of electrophoretic behaviour that allows simultaneous determination of pK_1 and pK_2. This model assumes that the electrophoretic mobility of the substance depends on the mobility and molar fraction of its species [2,4,8]. In general, we can consider for quinolones a protonated species (H_2Z^+) a zwitterionic species (HZ) and a dissociated species (Z^-). The electrophoretic mobility, m_e, can be written as:

$$m_e = x_{H_2Z^+} \cdot m_{H_2Z^+} + x_{HZ} \cdot m_{HZ} + x_{Z^-} \cdot m_{Z^-} \quad (1)$$

where the term corresponding to the intermediate species HZ has no charge and migrates with the electroosmotic flow. Taking $m_{H_2Z^+} = m_a$ and $m_{Z^-} = m_b$, and replacing the terms $x_{H_2Z^+}$ and x_{Z^-} by their expressions for ampholytes: $x_{H_2Z^+} = a_{H^+}^2/(a_{H^+}^2 + K_1 a_{H^+} y + K_1 K_2)$ and $x_{Z^-} = K_1 K_2/(a_{H^+}^2 + K_1 a_{H^+} y + K_1 K_2)$, and assuming that the activity coefficients of the zwitterionic species HZ is 1, we can write:

$$m_e = \frac{a_{H^+}^2 m_a - K_1 K_2 m_b}{a_{H^+}^2 + K_1 a_{H^+} y + K_1 K_2} \quad (2)$$

where y is the activity coefficient and m_a has the opposite sign to m_b.

The proposed model uses the pH values in MeCN–water mixtures instead of water, and takes into account the effect of the activity coefficients, which may be considerable in hydro-organic media but can be neglected in water for dilute solutions (<0.01 *M*) because of the high permittivity of the medium. Similar equations are reported in the literature [4,6], although different approaches were used to consider the activity coefficients.

To apply this model, data pairs of m_e–pH values in each media, water and MeCN–water mixtures with 5.5, 10 and 30% (w/w) of MeCN and the corresponding ionic strength are imported into the

NLREG program [31], in order to calculate dissociation constants for each quinolone.

3. Results and discussion

In order to determine the dissociation constants of quinolones by CE, values of electrophoretic mobilities, m_e, at different pH were obtained by injecting standard solutions of each quinolone and acetone when working with aqueous medium and mixtures of quinolones with acetone when working at 5.5%, 10% and 30% (w/w) MeCN–water media.

Flumequine has one relevant ionisable functional group within the pH ranges of pharmaceutical or physiological importance, corresponding to the carboxylic group. In contrast, the other eight quinolones have two relevant ionisable functional groups, which means that their acid–base chemistry involves two protons, the dissociation of the carboxylic group and the deprotonation of the N_4 of the piperazine ring, at position 7 [30,32].

In order to verify the thermostatting capacity of the systems used, we established the linearity of the plot of current vs. voltage (from 0 to 30 kV), at different pH in each MeCN–water medium used. In all cases Ohm's law was fulfilled and the regression coefficients were greater than 0.990, choosing a working voltage of 20 kV.

Data pairs pH–m_e and the ionic strength over the whole pH range, in all MeCN–water mixtures, were used to determine pK_a values. Initial values for electrophoretic mobility of fully protonated and deprotonated species, m_a, m_b, pK_1, pK_2 and activity coefficients are necessary in order to apply the model. The final values for these parameters were obtained from the fit of the model. The activity coefficients were calculated from the Debye–Hückel equation, although the higher buffer ionic strength are $5\cdot10^{-2}$ *M*, slightly higher than the traditionally accepted upper limits of the simple Debye–Hückel correlation. The Debye–Hückel parameters *A*, and a_0B for all MeCN–water mixtures are shown in Table 1. Also for reader convenience, values of permittivity (ϵ), autoprotolysis constants (pK_{ap}), viscosity (η) and density (ρ), for MeCN–water mixtures studied, are also shown in this table [33–36].

Table 2 shows the pK_a values determined for the series of nine quinolones studied in 0, 5.5, 10 and 30% (w/w) MeCN–water mixtures and the respective standard deviations, *s*, obtained from the application of the model. There are a few pK_a values reported in the literature in MeCN–water media [14–16]. This table also includes some pK_a values previously obtained from potentiometric measurements which are consistent with pK_a values obtained using CE. Also the pK_a values obtained potentiometrically in MeCN–water mixtures in the case of ciprofloxacin (in 10% of MeCN, pK_2 = 8.38, and in 30% MeCN, pK_2 = 8.41) and those for flumequine (in 10% pK_a = 6.90 and in 30% pK_a = 7.78) do not differ significantly from those obtained electrophoretically in this work.

The pK_1 values of quinolones were higher than those generally observed with carboxylic acids in water mixtures [37]. This decrease in acidity can be attributed to an intramolecular H-bond formation with the neighbouring keto function resulting in stabilization of the protonation species [38]. This is supported by UV and IR spectral data [19]. The pK_2 values of quinolones are associated with the deprotonation at N_4 of the piperazine ring [39–43], as confirmed by nuclear magnetic resonance (NMR) [3].

In MeCN–water mixed solvents, the acid dissociation constants for both carboxylic and protonated amino type deprotonations decreased as the solvent

Table 1
Values of autoprotolysis constants (pK_{ap}), permittivity (ϵ), viscosity (η), density (ρ) and Debye–Hückel parameters, *A* and a_0B, at 25°C and weight percentages of acetonitrile in admixtures with water

% (w/w) MeCN	pK_{ap}	ϵ	100η	ρ	*A*	a_0B
0	14.00	78.36	0.890	0.9971	0.5103	1.5000
5.5	14.15	76.62	0.942	0.9885	0.5256	1.5104
10	14.27	75.01	0.968	0.9809	0.5404	1.5206
30	14.92	65.52	0.939	0.9389	0.6476	1.5918

Table 2
pK_a values of quinolones obtained in MeCN–water mixtures

Quinolone	pK_a	% (w/w) MeCN			
		0	5.5	10	30
Ciprofloxacin	pK_1	5.86 (0.05)	6.13 (0.05)[a]	6.10 (0.06)	6.84 (0.03)
	pK_2	8.24 (0.07)	8.13 (0.10)	8.30 (0.06)	8.44 (0.04)
Enrofloxacin	pK_1	5.88 (0.03)	5.82 (0.05)	6.12 (0.05)	6.81 (0.02)
	pK_2	7.74 (0.03)	7.83 (0.05)	7.89 (0.04)	8.04 (0.02)
Norfloxacin	pK_1	5.94 (0.05)	6.26 (0.05)[a]	6.17 (0.07)	6.88 (0.03)
	pK_2	8.22 (0.07)	8.08 (0.08)	8.29 (0.06)	8.47 (0.04)
Danofloxacin	pK_1	6.07 (0.06)	5.73 (0.03)	6.04 (0.03)	6.82 (0.03)
	pK_2	8.56 (0.07)	8.40 (0.04)	8.64 (0.03)	8.76 (0.05)
Difloxacin	pK_1	5.66 (0.04)	5.68 (0.06)	6.24 (0.09)	6.45 (0.07)
	pK_2	7.24 (0.06)	7.43 (0.06)	7.37 (0.07)	7.68 (0.08)
Sarafloxacin	pK_1	5.62 (0.08)	5.78 (0.04)	6.27 (0.04)	6.72 (0.04)
	pK_2	8.18 (0.09)	8.11 (0.04)	8.26 (0.05)	8.28 (0.05)
Marbofloxacin	pK_1	n.d.[b]	n.d.	6.16 (0.06)	6.63 (0.03)
	pK_2	n.d.	n.d.	8.02 (0.08)	8.11 (0.04)
Pipemidic acid	pK_1	5.42 (0.03)	5.58 (0.03)[a]	5.76 (0.03)[a]	n.d.
	pK_2	8.18 (0.09)	n.d.	8.43 (0.05)[a]	8.45 (0.04)[a]
Flumequine	pK_1	6.61 (0.03)	7.21 (0.05)	6.91 (0.04)	7.71 (0.03)

[a] Values obtained from potentiometric measurements.
[b] n.d.: not determined in this work.

became enriched in the organic component, Table 2. It is difficult to interpret the variations of pK_1 and pK_2 of quinolones with the percentage of MeCN in the mixtures. However, the variation in pK_1 values corresponding to dissociation of carboxylic acid are greater than the variations obtained for pK_2, which are little influenced as the solvent is enriched in MeCN. This can be explained taking into account that in the dissociation of neutral or anion acids, charges are created [$HA \leftrightarrow H^+ + A^-$ or $HA^{n-} \leftrightarrow H^+ + A^{(n-1)-}$] and in solvents with similar acidity, like MeCN–water mixtures, the electrostatic interaction overwhelms the specific solute–solvent interactions. Hence, pK_a values increase with the percentage of MeCN in accordance with the expression $pK_a = A + B/\epsilon$ [44,45], where A and B are constants for a given substance. Although the change in ϵ values between water and MeCN–water at 30% are small, Table 1, the variation of pK_1 values of quinolones in the range of percentage studied are of about one unit. However, in the dissociation of a monocharged cation acid (such as the ammonium ions in the N_4' of quinolone piperazine ring), there is no change in the number of charges ($HA^+ \leftrightarrow H^+ + A$) and the change in the dielectric constant of the medium does not affect the dissociation process. In this instance, the dissociation depends only on the solvation of the different species by the solvents of the mixture. Thus, pK_2 values of quinolones show low changes in the range from 0% to 30% (w/w) of MeCN (variations in less than 0.5) [14,44]. In consequence, for all the quinolones studied the pK_1 and pK_2 values come closer when the percentage of MeCN is increased.

Considering the equation of the model and the great variation of pK_1 in relation to pK_2 that we have obtained experimentally, we can plot a theoretical curve of electrophoretic mobility vs. pH for a hypothetical zwitterionic quinolone with a pK_2 value of 8.4 and study the influence of a variation of pK_1 on the theoretical curve (Fig. 2). When pK_a values for the substance are different enough (more than 0.2) two inflection points are obtained. When there is

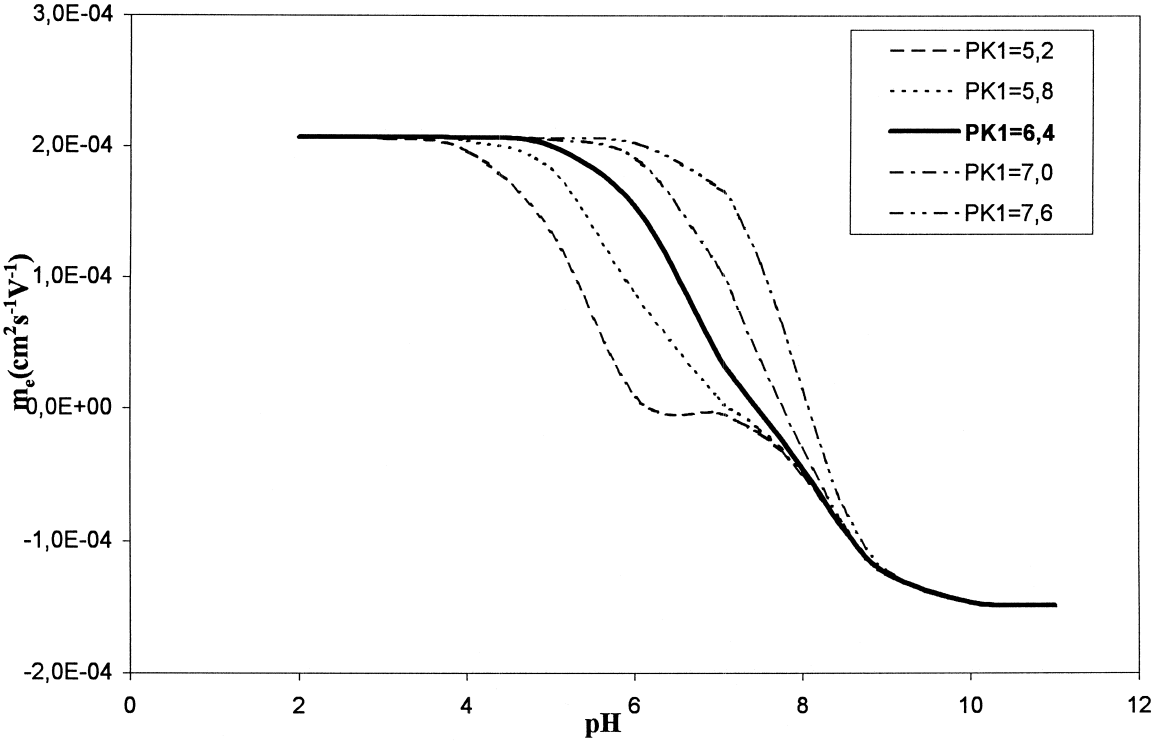

Fig. 2. Effect of the variation of pK_1 values on the electrophoretic behaviour of a substance (pK_2 = 8.4).

a decrease in the difference between the two dissociation constants, that is to say, values of pK_1 and pK_2 are closer, we has only one inflection point.

In Fig. 3, we have plotted, as an example, experimental data pairs of m_e–pH for enrofloxacin for each MeCN–water mixture studied up to 30% (w/w) of MeCN and the fit of these data with the proposed model. There is a good fit between the model and the experimental data of electrophoretic mobility in all media. In general, the pK_a values increase when the solvent is enriched in the MeCN and, the curve is displaced to higher pH values. However, this Fig. shows that in water, when the pK_a values of sarafloxacin are more different, two inflection points are obtained, while when MeCN content of the mixture increases the two infection points merge into one as can be observed in the theoretical curve (Fig. 2). On the other hand, an increase in the percentage of MeCN produces a decrease of the m_a values and a slight rise of m_b values. Similar behaviour in pK_a, m_a and m_b values were observed for all the quinolones studied.

The coherence between the pK_a values obtained in MeCN–water mixtures using CE and the values obtained using the potentiometric method, the low standard deviations of the results and the good fit between the theoretical and experimental electrophoretic data prove the applicability of the proposed model for the determination of dissociation constants, with the inherent advantages of the electrophoretic technique.

Acknowledgements

Financial support of DGICYT, Project PB95-0966, of the Spanish Government and project 1997SGR0089 by Comisionat per Universitats i Recerca of the Catalan Government are gratefully acknowledged. The authors thank Abbott S.A.,

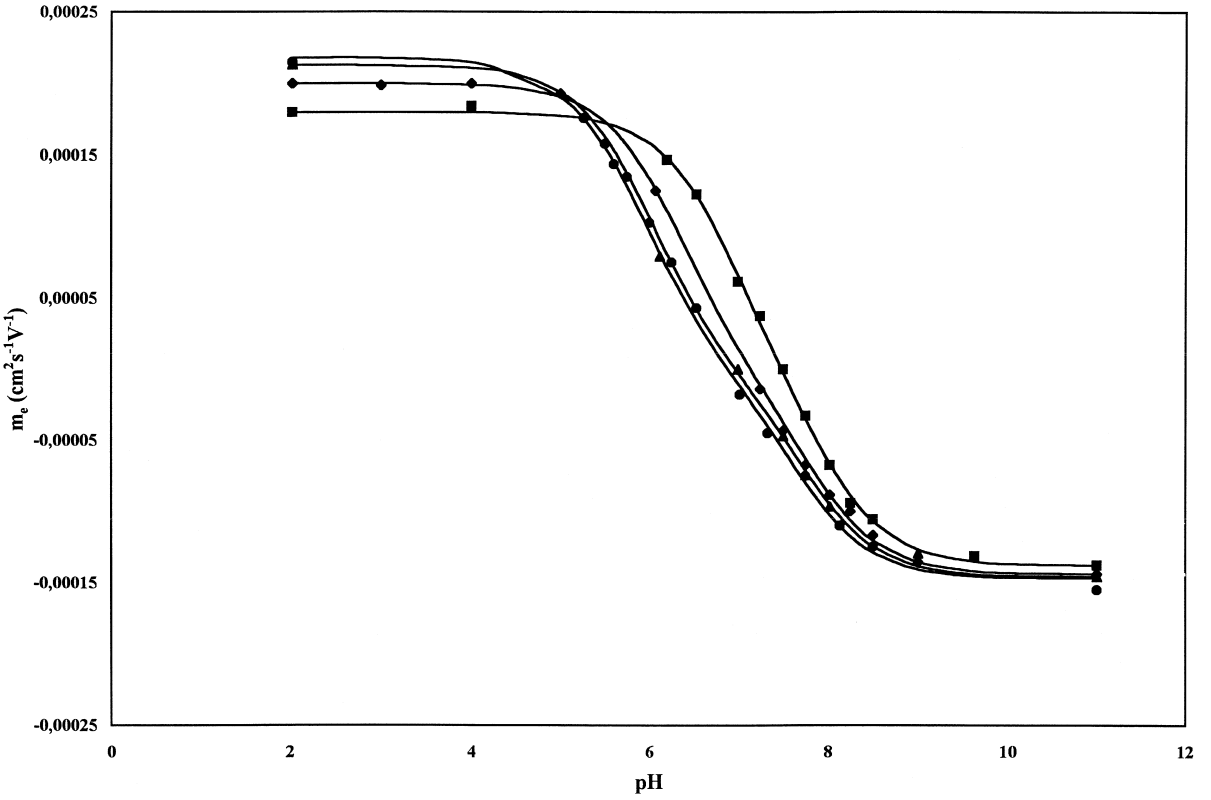

Fig. 3. Plot of experimental and predicted mobilities of enrofloxacin vs. pH at the studied percentage of MeCN in water: (●) 0% (w/w) MeCN, (▲) 5.5% (w/w) MeCN, (♦) 10% (w/w) MeCN, (■) 30% (w/w) MeCN.

Cenavisa S.A., LASA, Pfized S.A. and Vetoquinol Laboratories for the kindly donation of the quinolone standards.

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