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Evaluation of electrophoretic method versus chromatographic, potentiometric and absorptiometric methodologies for determing pK_a values of quinolones in hydroorganic mixtures

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Abstract

The advantages of using capillary electrophoresis (CE) over other methodologies for determining pK_a values of drugs in hydroorganic media are discussed. The focus of the discussion based upon the pK_a values of a series of quinolones determined in acetonitrile (MeCN)–water mixtures by CE, liquid chromatography, potentiometric, and spectrophotometric methods. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Quinolones; Dissociation constants; Acetonitrile–water mixtures; Electrophoretic mobility; Liquid chromatography; Potentiometry; UV–Visible spectrophotometry

1. Introduction

Quinolones comprise a most interesting group of antibiotics whose bacterial action is based on their anti-DNA gyrase activity. These drugs are suitable for the treatment of systematic infections as well as urinary tract infections. They all possess a carbonyl group in position 4, and are often referred to as 4-quinolones [1,2]. An increase in their antibacterial activity is greatly influenced by the addition of the 6-fluoro and 7-piperazinyl group to the molecule. The activity of many biological molecules such as quinolones depends on the presence of charged groups. Consequently, the dissociation constant can be a key parameter for understanding and quantifying chemical phenomenon or biological activity as the passage of many drugs into cells and across other membranes is a function of pH in the internal environment and the pK_a of the drug [3].

Capillary electrophoresis (CE) has been introduced as a method for convenient and precise aqueous pK_a determination [3–7]. The advantages of using CE to determine accurate thermodynamic pK_a values of compounds are numerous: CE requires small amounts of sample at low

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solute concentration, and the procedure does not require solute measurement or titrant concentrations like potentiometric techniques, but only of migration times. Calculations are straightforward and independent of solute purity, since impurities can be separated from the solutes of interest [5,6]. Moreover, CE permits pK_a determination in aqueous solutions without difficulties which is not the case for liquid chromatography (LC), in which the retention could be significant without the addition of an organic modifier [8–10].

The use of non-aqueous solvents in general, and binary water–organic solvent systems in particular, extends the range of aqueous CE applications. This provides a more versatile electrophoretic separation, since it is possible to work in a medium with different dielectric constants, polarities, densities, viscosities and acid–base properties [11–13]. Other advantages of non-aqueous CE are improved solubility of analytes with low solubility in pure aqueous buffer solutions, and low operating currents when a voltage is applied [14]. As a result, less Joule heat is produced in non-aqueous CE conditions than in buffered aqueous systems, allowing much higher electrical field strengths than those currently used in CE [15].

Various aqueous–organic mixtures permit good separation procedures and evaluation of pK_a using CE [15–18]. Acetonitrile (MeCN) and its mixtures with water are widely used in non-aqueous CE, due to the excellent characteristics of the pure solvent. MeCN is a very weak base and acid and therefore it is a good differentiating solvent for both bases and acids. Furthermore, it has a low viscosity and good UV transparency, which makes it ideal for non-aqueous CE [14,19]. The use of MeCN–water mixtures in CE requires the correct measurement of pH in these media. Measurements are performed in a similar way to those performed in water using IUPAC standardization rules. In previous works [20–22], the standard pH values were assigned in MeCN–water mixtures for primary reference buffer solutions of the NIST scale.

This paper focuses on using CE over other methodologies for determining the pK_a of drugs in hydroorganic media.

2. Experimental

2.1. Apparatus

In order to obtain potentiometric pK_a values, emf values of the potentiometric cell were measured with a CRISON 2002 potentiometer (± 0.1 mV) using a Radiometer G202C glass electrode and a reference Ag/AgCl electrode prepared according to the electrolytic method and directly immersed in the solution to avoid the residual liquid junction potentials [23]. The glass electrode was stored in water when not in use and soaked for 15–20 min in MeCN–water mixture before potentiometric measurements were taken. The stabilization criterion for the emf readings was 0.2 mV within 150 s. In all instances the electrode system gave stable and reproducible potentials within 5 min. The reference electrode was stable for 3 months of continual work. The standard emf of the cell, E^0, is the average of at least 15 standardizations. The standardization of the electrode system was carried out with solvent media or electrodes were changed and the constancy of E^0 values was ensured by continual surveillance and periodical calibrations. The titrant was added from a Metrohm 655 Dosimat autoburette. The cell was thermostatted externally at 25 ± 0.1°C. The potentiometric assembly was automatically controlled with a microcomputer.

All CE experiments were performed on a P/ACE System 5500 (Beckman Instruments, Palo Alto, CA) equipped with an autosampler, automatic injector and photodiode array detector. An untreated fused-silica capillary of 47 cm × 75 μm ID, 40 cm from inlet to detector, (Polymicro Technologies Phoenix, AZ) was used. Samples were injected hydrodynamically at 0.5 psi for 6 s when working with aqueous media and for 3 s when working with MeCN–water mixtures. 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 chromatographic equipment used consisted on an ISCO Model 2350 pump with an injection valve, a 10 μl sample loop and a variable wavelength V^4 absorbance detector (ISCO) operating at 280 nm for all quinolone except for ofloxacin that is monitored at 295 nm. 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 ID), packed with hydrophobic polystyrene–divinylbenzene gel, was used at ambient temperature.

The UV–Vis spectra, obtained to determine absorptiometric pK_a values, were recorded at each pH value using a Perkin–Elmer λ19 spectrophotometer equipped with 1 cm path length cell. The system was organized in a closed loop circuit with a continuous flow of 25°C thermostatted solutions using a Watson-Marlow 505 DU peristaltic pump. The titrant was added from a Metrohm 655 Dosimat autoburette.

All the emf or pH values using UV, LC and CE methodologies were measured with a CRISON 2002 potentiometer (Crison Instruments, Barcelona, Spain) and a Ross electrode 81-02 supplied by Orion Research Incorporated (Boston, MA).

2.2. Chemicals and reagents

Unless otherwise indicated, analytical reagent grade chemicals were used. Quinolones used in this work are shown in Fig. 1 and were purchased from different laboratories: ofloxacin (Hoescht), pipemidic acid and enoxacin (Prodesfarma-Almirall), norfloxacin, flumequine and oxolinic acid (Sigma), fleroxacin (Roche), ciprofloxacin (LASA), enrofloxacin (Cenavisa), danofloxacin (Pfizer), difloxacin and sarafloxacin (Abbot), marbofloxacin (Vetoquinol), nalidixic acid and cinoxacin (Impex Química).

Acetonitrile, HPLC grade, was supplied by Baker and Merck. Water, with a resistivity of 18.2 MΩ cm, was obtained using a Milli-Q water purification system (Millipore, Molsheim, France).

2.2.1. Potentiometric measurements

Stock 0.1 and 0.02 mol/l potassium hydroxide (Carlo-Erba) solutions were prepared in each MeCN–water mixture with an ion-exchange resin [23] to avoid carbonation and standardized volumetrically against potassium hydrogen phthalate. Stock 0.1, 0.03 and 0.01 mol/l hydrochloric acid (Merck) solutions were prepared and standardized against tris(hydroxymethyl)aminomethane (Merck).

2.2.2. Electrophoretic measurements

Phosphoric acid (85%), sodium hydroxide, potassium hydrogenphthalate and acetone were supplied by Merck and acetic acid was obtained from Carlo-Erba. Solutions of individual quinolones were prepared in acetic acid 0.05 M at concentrations of 100 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 working in MeCN–water mixtures. Each solution contained acetone at 3% (v/v) as the electroosmotic flow marker [24,25].

In 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 0.050 M are used in aqueous acidic media and 0.025 M for use in basic aqueous and MeCN–water mixtures. Working buffer solutions were used in the pH range between 4 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 phosphate buffer of 0.025 M at pH 2 and a phosphate buffer of 0.018 M at pH 11 were used in each MeCN–water mixture. All solutions (quinolones and buffers) were passed through a 0.45 μm filter.

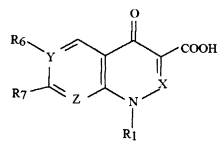
Two standard buffers in the different hydroorganic mixtures were used to calibrate the pH-meter [10,26]: (1) sodium hydrogenphosphate plus potassium dihydrogenphosphate buffer (0.025 mol/kg each component) and (2) potassium hydrogenphthalate buffer (0.05 mol/kg) were prepared in 5.5%, 10%, 30% (w/w) of MeCN [20–22].

2.2.3. Chromatographic measurements

Working standard solutions of quinolones were prepared in the mobile phase in concentrations of approximately 20 mg/l. The samples were filtered through a 0.45 μm nylon filter membrane (MSI) before injection. Eluents were filtered through a 0.22 μm nylon filter membrane (MSI) and degassed ultrasonically before use. Two standard buffers in the different hydroorganic mixtures were used to calibrate the pH-meter [10,26]: (1) sodium hydrogenphosphate plus potassium dihydrogenphosphate buffer (0.025 mol/kg each component) and (2) potassium hydrogenphthalate buffer (0.05 mol/kg) were prepared in 25%, 30%, 34.2% and 43.8% (w/w) of MeCN [20–22].

2.2.4. Spectrophotometric measurements

Stock solutions of 6×10^{-3} M hydrochloric acid (Merck) and 0.1 M sodium chloride were prepared in each MeCN–water mixture and standardized potentiometrically. Solutions of individual quinolones were prepared at concentration level of 4×10^{-4} and 0.1 M in sodium chloride to adjust the ionic strength and standardized potentiometrically.



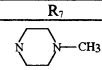
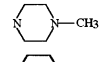
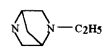
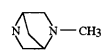
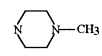
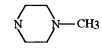
Quinolone		Substituents at position					
		R_1	X	Y	R_6	R_7	Z
Ofloxacin	OFL		CH	C	F		CH
Pipemidic acid	PIP	$-C_2H_5$	CH	N	-		N
Norfloxacin	NOR	$-C_2H_5$	CH	C	F		CH
Enoxacin	ENO	$-C_2H_5$	CH	C	F		N
Fleroxacin	FLE	$-CH_2-CH_2F$	CH	C	F		CF
Ciprofloxacin	CIP		CH	C	F		CH
Flumequine	FLU		CH	C	F	H	CR_1
Enrofloxacin	ENR		CH	C	F		CH
Danofloxacin	DAN		CH	C	F		CH
Difloxacin	DIF		CH	C	F		CH
Sarafloxacin	SAR		CH	C	F		CH
Marbofloxacin	MAR		CH	C	F		$-R_1$
Nalidixic acid	NAL	$-C_2H_5$	CH	C	H	CH_3	N
Cinoxacin	CIN	$-C_2H_5$	N	C	$-R_7$		CH
Oxolinic acid	OXO	$-C_2H_5$	CH	C	$-R_7$		CH
Piromidic Acid	PIR	$-C_2H_5$	CH	N	H		N

Fig. 1. Structure of quinolones studied.

2.3. Procedures

2.3.1. Potentiometric method

The pK_a values of quinolones were determined by titration of the appropriate solutions of quinolones in the MeCN–water mixtures studied. These MeCN–water mixtures contained a measured excess of HCl solution using KOH solution as titrant, according to the criteria endorsed by the IUPAC [27,28].

pK_a values were obtained from systematic measurements of the emf of the cell:

$$\mathrm{Pt/Ag/AgCl/HA + A + Cl^-}$$

$-$ in MeCN–water/Glass electrode

where HA and A are the acid and basic species, respectively, involved in the dissociation equilibrium studied. The emf, E, of this cell is directly related to the activity of the hydrogen and chloride ion in solution:

$$E = E^0 + k \log(a_{H^+} a_{Cl^-}), \quad (1)$$

where E^0 is the standard emf of the cell and $k = (\ln 10)RT/F$ [23]. Taking into account the general expression for the dissociation equilibria studied [29]:

$$K_a = \frac{c_A y_A c_{H^+} y_{H^+}}{c_{HA} y_{HA}}, \quad (2)$$

the functional equation which permits pK_a calculation is obtained:

$$\mathrm{p}K_a = \frac{E^0 - E}{k} + \log \frac{c_{HA} y_{HA} c_{Cl^-} y_{Cl^-}}{c_A y_A}, \quad (3)$$

where c_{HA} and c_A are the molar concentrations of acidic and basic species, respectively; c_{Cl^-} is the molar concentration of the ion chloride and y_x is the molar activity coefficient of the species x, these values can be calculated through the classical Debye–Hückel equation [23,30,31]. Potentiometric procedure is described in detail elsewhere [21,32,33].

2.3.2. Electrophoretic measurements

Each electrophoretic mobility was calculated as the average of at least three replicates [34]. 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 [3,4,7]. In general, we can consider for quinolones, a protonated species (H_2A^+), a zwitterionic species (HA), and a dissociated species (A^-). The electrophoretic mobility, m_e, can be written as:

$$m_e = x_{H_2A^+} m_{H_2A^+} + x_{HA} m_{HA} + x_{A^-} m_{A^-} \quad (4)$$

where the term corresponding to the intermediate species HA has no charge and migrates with the electroosmotic flow. Taking $m_{H_2A^+} = m_a$ and $m_{A^-} = m_b$, replacing the terms $x_{H_2A^+}$ and x_{A^-} by their expressions for ampholytes (4), and assuming that the activity coefficients of the zwitterionic species HA is 1, we can write:

$$m_e = \frac{a_{H^+}^2 + m_a - K_1 K_2 m_b}{a_{H^+}^2 + K_1 a_{H^+} y + K_1 K_2}, \quad (5)$$

where y is the activity coefficient and m_a has the opposite sign to m_b. To apply this model, data pairs of m_e–pH values in each media, 0%, 5.5%, 10% and 30% (w/w) of MeCN are imported into the NLREG program [35].

2.3.3. Chromatographic measurements

Throughout this study, the mobile phases assayed were MeCN-buffer (30:70, 35:65, 40:60, 50:50; v/v), adjusted to pH values between 3 and 11 [9]. 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. The capacity factor, k', of any ionizable compound 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 [36], according to the molar fractions of these forms in the mobile phase. The overall observed k' for flumequine, with only one carboxylic functional group, can be given as [37]:

$$k' = x_{HA} k'_{HA} + x_{A^-} k'_{A^-} \quad (6)$$

where k'_{HA} and k'_{A^-} are the capacity factors of the solute in the non-ionized and ionized form, respectively, and x_i is the molar fraction. The pro-

tolytic equilibrium of flumequine, as a monoprotic acid, is ruled by the thermodynamic dissociation constant $K_a = [A^-]ya_{H^+}/[HA]$. Replacing the terms x_{HA} and x_{A^-} by their expressions for ampholytes, and substituting we obtain:

$$k' = \frac{k'_{HA^+}k'_{A^-}\frac{K_a}{a_{H^+}y}}{1+\frac{K_a}{a_{H^+}y}}. \quad (7)$$

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. (7) and is:

$$k' = \frac{k'_{H_2A^+}\frac{a_{H^+}}{K_1y} + k'_{HA} + k'_{A^-}\frac{K_2}{a_{H^+}y}}{\frac{a_{H^+}}{K_1y} + 1 + \frac{K_2}{a_{H^+}y}}. \quad (8)$$

These equations are of general validity. For aqueous organic solvent, a_{H^+} is the activity of the solvated proton in the mixed aqueous–organic solvent, K_1 and K_2 are the dissociation constants of a compound in the same solvent and $k'_{H_2A^+}$, k'_{HA} and k'_{A^-} are the capacity factors of the fully protonated species, amphoteric species, and the fully deprotonated species of the quinolones, respectively.

2.3.4. Spectrophotometric measurements

Experimental data were obtained from the titration of a solution of quinolone at a constant ionic strength of 0.1 M in NaCl and adding small amounts of acid or base to change the pH in the range 3–12. The titration vessel was thermostatted at 25°C. At each pH, UV–Vis spectra were recorded with 1 nm resolution in order to obtain different spectra around the maximum λ for each quinolone. For example, flumequine spectra are obtained from 220 to 270 nm and enrofloxacin from 250 to 300 nm. Previously, the calibration of the electrodic system was made by adding of stock solution of HCl to MeCN–water with the adequate percentage of MeCN. Data were processed using the program STAR [38] which calculates stability constants and molar absorptivities of pure species by multilinear regression. It can work with up to 50 different spectra having 50 different wavelengths each.

3. Results and discussion

Potentiometric pK_a values were obtained from a series of various measurements, making a total of more than 5600 independent measurements over the solvent interval explored. Table 1 shows the dissociation constant values of quinolones previously determined [39,40], using the PKPOT program [32], in different MeCN–water mixtures up to 70% (w/w) of MeCN and the respective standard deviation. Some values of pK_a of quinolones in water are also included in Table 1 [39,40].

In order to obtain pK_a of quinolones using CE methodologies, data pairs pH–m_e and the ionic strength over the whole pH range, in all MeCN–water mixtures, were used. 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 previously proposed model [4,17]. The final values for these parameters were obtained from the fit of the model using the NLREG program [35]. As an example, in Fig. 2 we have plotted experimental data pairs of m_e–pH for sarafloxacin in the mixtures studied, and the fit of the data with the proposed theoretical model, obtaining a good relationship between them. These curves show two inflexion points, in general due to the two dissociation constants of quinolones. In MeCN–water with high percentages of MeCN, where pK_1 and pK_2 values are close, the two inflexion points merge into one. In general, the pK_a values increase when the solvent is enriched in the MeCN and, the curve is displaced to higher pH values. 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 is obtained for all the quinolones studied. Values of pK_a for quinolones using CE methodologies are shown in Table 1. The values in parentheses are the standard deviation obtained in the fit of the data [4,17]. The CE values asterisked were obtained in this work.

Capacity factors were obtained over a pH range of 3–11 in order to determine pK_a of quinolones using LC methods. For example, experimental k' values are shown in Fig. 3 for norfloxacin, flerox-

Table 1
pK_a values of quinolones obtained by potentiometric, chromatographic, electrophoretic and spectrophotometric methods in MeCN–water media[a]

	0%		5.5%		10%		16.3%		25%		30%		34.2%		40%		43.8%		50%		70%	
	pK_1	pK_2	pK_1	pK_2	pK_1	pK_2	pK_1	pK_2	pK_1	pK_2	pK_1	pK_2	pK_1	pK_2	pK_1	pK_2	pK_1	pK_2	pK_1	pK_2	pK_1	pK_2
Ofloxacin		8.11^{b}(0.02)	6.20^{b}(0.03) 6.29^{c}(0.09)	8.13^{c}(0.08)		8.17^{b}(0.03)			6.10^{d}(0.04)	8.13^{b}(0.03) 7.99^{d}(0.03)		8.35^{b}(0.04)	6.45^{d}(0.12)	7.95^{d}(0.10)		8.37^{b}(0.05)	6.63^{d}(0.06)	8.58^{b}(0.04) 8.42^{d}(0.06)		8.76^{b}(0.04)		9.87^{b}(0.02)
Pipemidic acid	5.42^{e}(0.03)	8.18^{e}(0.07)	5.58^{b}(0.03) 5.79^{c}(0.03)	8.52^{c}(0.03)	5.76^{b}(0.03)	8.43^{b}(0.05)						8.45^{b}(0.04)				8.41^{b}(0.04)				8.65^{b}(0.04)		9.49^{b}(0.03)
Norfloxacin	6.22^{b}	8.38^{b}	6.26^{b}(0.05)	8.40^{b}	6.57^{b}(0.06)	8.48^{b}(0.03)	6.81^{b}(0.03)	8.58^{b}(0.01)	7.20^{b}(0.04)	8.78^{b}(0.02)	7.45^{b}(0.05)	8.72^{b}(0.02)	6.53^{d}(0.15)	8.28^{d}(0.13)		8.76^{b}(0.05)	7.81^{b}(0.04)	8.95^{b}(0.059)	7.98^{b}(0.03)	9.05^{b}(0.04)		10.01^{b}(0.02)
	5.94^{e}(0.05)	8.22^{e}(0.07)	6.56^{e}(0.13)	8.08^{e}(0.08) 8.38^{e}(0.08)	6.17^{e}(0.07)	8.29^{e}(0.06)			6.27^{d}(0.08)	8.35^{d}(0.07)	6.88^{e}(0.03) 6.14^{d}(0.15)	8.47^{e}(0.04) 8.61^{d}(0.15)					6.88^{d}(0.06)	8.82^{d}(0.79)				
Enoxacin	6.00^{b}	8.50^{b}	6.20^{b}(0.02)			8.51^{b}(0.02)			6.28^{d}(0.11)	8.28^{d}(0.11)		8.38^{b}(0.05)	6.59^{d}(0.15)	8.13^{d}(0.12)		8.61^{b}(0.05)	6.81^{d}(0.06)	8.67^{b}(0.05) 8.73^{d}(0.07)		8.95^{b}(0.04)		9.81^{b}(0.02)
Fleroxacin	5.46^{b} 5.55^{c}	8.00^{b}	5.71^{b}(0.02)		5.93^{b}(0.05)	7.95^{b}(0.06)	6.17^{b}(0.04)	8.07^{b}(0.03)	6.59^{b}(0.03) 5.83^{d}(0.04)	8.05^{b}(0.02) 7.58^{d}(0.03)	6.60^{b}(0.02) 5.84^{d}(0.05)	7.94^{b}(0.05) 7.71^{d}(0.05)	5.96^{d}(0.10)	7.71^{d}(0.09)		8.06^{b}(0.04)	6.97^{b}(0.02) 6.32^{d}(0.05)	8.21^{b}(0.03) 8.05^{d}(0.05)	7.11^{b}(0.03)	8.39^{b}(0.03)		9.44^{b}(0.04)
Ciprofloxacin	6.09^{b}	8.62^{b}	6.13^{b}(0.05)	8.13^{e}(0.10)	6.10^{e}(0.06)	8.38^{b}(0.04)					6.84^{e}(0.03)	8.41^{b}(0.04)				8.61^{b}(0.04)				8.95^{b}(0.05)		9.84^{b}(0.03)
	5.86^{e}(0.05)	8.24^{e}(0.07)	6.43^{e}(0.11)	8.49^{e}(0.11)		8.30^{e}(0.06)						8.44^{e}(0.04)										
Flumequine	6.50^{e,f}(0.05)				6.90^{b}(0.04)		7.09^{b}(0.03)		7.60^{b}(0.03)		7.78^{b}(0.02)				8.11^{b}(0.02)				8.66^{b}(0.02)		9.85^{b}(0.02)	
	6.42^{c}				6.91^{e}(0.04)						7.71^{e}(0.03) 7.84^{d}(0.02) 7.56^{e,f}(0.01)											
Enrofloxacin	5.88^{e}(0.03)	7.74^{e}(0.03)	5.82^{e}(0.05)	7.83^{e}(0.05)	6.12^{e}(0.05)	7.89^{e}(0.04)					6.81^{e}(0.02)	8.04^{e}(0.02)										
											6.95^{f,e}(0.01)	8.38^{e,f}(0.01)										
Danofloxacin	6.07^{e}(0.06)	8.56^{e}(0.07)	5.73^{e}(0.03)	8.40^{e}(0.09)	6.04^{e}(0.03)	8.64^{e}(0.03)					6.82^{e}(0.03)	8.76^{e}(0.05)										

Table 1 (*Continued*)
pK_a values of quinolones obtained by potentiometric, chromatographic, electrophoretic and spectrophotometric methods in MeCN–water media[a]

	0%		5.5%		10%		16.3%		25%		30%		34.2%		40%		43.8%		50%		70%	
	pK_1	pK_2	pK_1	pK_2	pK_1	pK_2	pK_1	pK_2	pK_1	pK_2	pK_1	pK_2	pK_1	pK_2	pK_1	pK_2	pK_1	pK_2	pK_1	pK_2	pK_1	pK_2
Difloxacin	5.66[e](0.04)	7.24[e](0.06)	5.68[e](0.06)	7.43[e](0.06)	6.24[e](0.09)	7.37[e](0.07)					6.45[e](0.07)	7.68[e](0.08)										
Saraflox acin	5.62[e](0.08)	8.18[e](0.09)	5.78[e](0.04)	8.11[e](0.04)	6.27[e](0.04)	8.26[e](0.05)					6.72[e](0.04)	8.28[e](0.05)										
Marbofloxacin	5.69[e,f](0.11)	8.02[e,f](0.16)			6.16[e](0.06)	8.02[e](0.08)					6.63[e](0.03)	8.11[e](0.04)										
Nadilixic acid	6.01[e,f](0.05)				6.57[b](0.04)						7.42[b](0.04)				7.76[b](0.04)				8.31[b](0.02)		9.55[b](0.03)	
Cinoxacin					5.05[b](0.03)						5.87[b](0.04)				6.13[b](0.04)				6.72[b](0.02)		7.85[b](0.03)	
Oxolinic acid	6.78[e,f](0.04) 6.87[c]																					

[a] Potentiometric values (POT) are obtained from Refs. [39,40]. Electrophoretic values (CE) are obtained from Ref. [17]. Chromatographic values (LC) are obtained from Ref. [9].Spectrophotometric values (UV–Vis) are obtained from Refs. [41,42].
[b] Potentiometric pK_a values.
[c] UV–Vis pK_a values.
[d] Chromatographic pK_a values.
[e] Electrophoretic pK_a values.
[f] Values determined in this work.

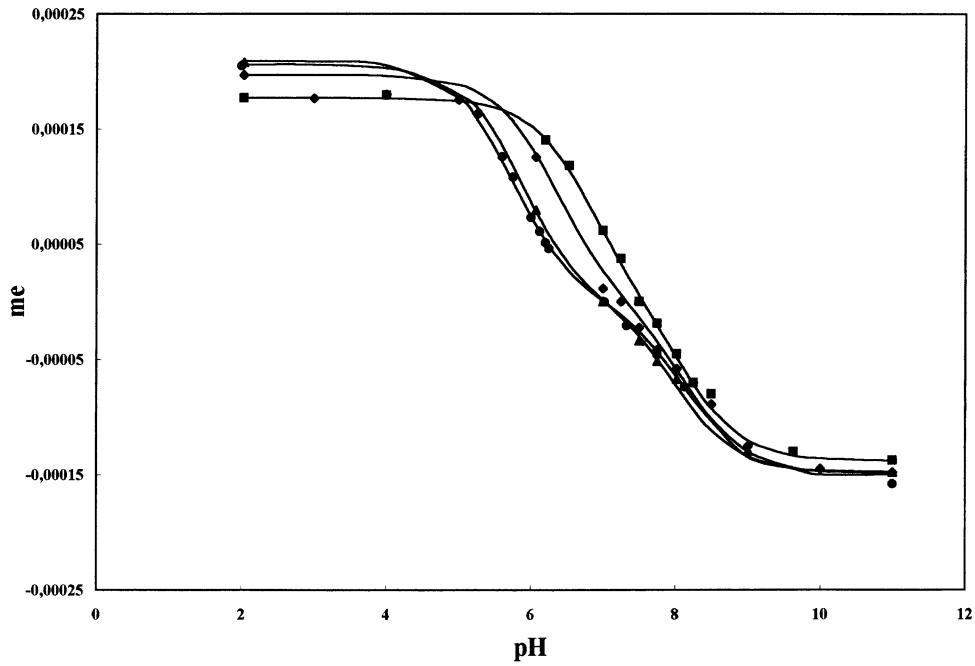


Fig. 2. Plot of experimental and theoretical mobilities of sarafloxacin versus pH at the studied percentage of MeCN in water: (●) 0% (w/w) MeCN, (▲) 5.5% (w/w) MeCN, (◆) 10% (w/w) MeCN, (■) 30% (w/w) MeCN.

acin and flumequine versus the pH values of a mobile phase containing 30% (w/w) MeCN. Eqs. (7) and (8), which relate the capacity factor to pH, were applied to each type of quinolone. Each equation was experimentally verified and the pK_a values of the substances studied were determined from the following: experimental k' values, the pH measured in the hydroorganic media, and the calculated activity coefficient. The pK_a values were calculated using a non-linear least-squares fit of the data and are shown in Table 1 [9].

For the determination of pK_a values using UV methodology, values of absorbance were obtained over a pH range of 3–12. Fig. 4 shows values of absorbance for enrofloxacin between 250 and 300 nm at 30% (w/w) of MeCN. The data was processed using the STAR program [38] in order to obtain the pK_a values for substances using an iterative procedure. The spectrophotometric pK_a values obtained in this work for quinolones are shown in Table 1 with some values obtained from the literature [41,42].

From Table 1 it is deduced that the variation of pK_a values with the percentage of MeCN were different for each substance, although, in general, the pK_a values increased as the MeCN content increased. The pK_1 values of quinolones varied even when the percentage of MeCN was low, whereas pK_2 values showed small changes in the range from 0% to approximately 30% MeCN and they increased at higher percentages of MeCN. This is explained by the structural features of MeCN–water mixtures [40].

Traditionally, potentiometric methods have been employed in the determination of pK_a values. However, these methods often cannot be applied to compounds that are barely soluble in water. In order to obtain accurate pK_a values, these conventional methods needs to take into account the purity of reagents and analytes that invariably influence the observed pK_a values [3,18]. Moreover, potentiometric method requires a relatively high quantity of substance, time-consuming solvent preparation for carbonate-free solutions, the standardization of high purity titrant

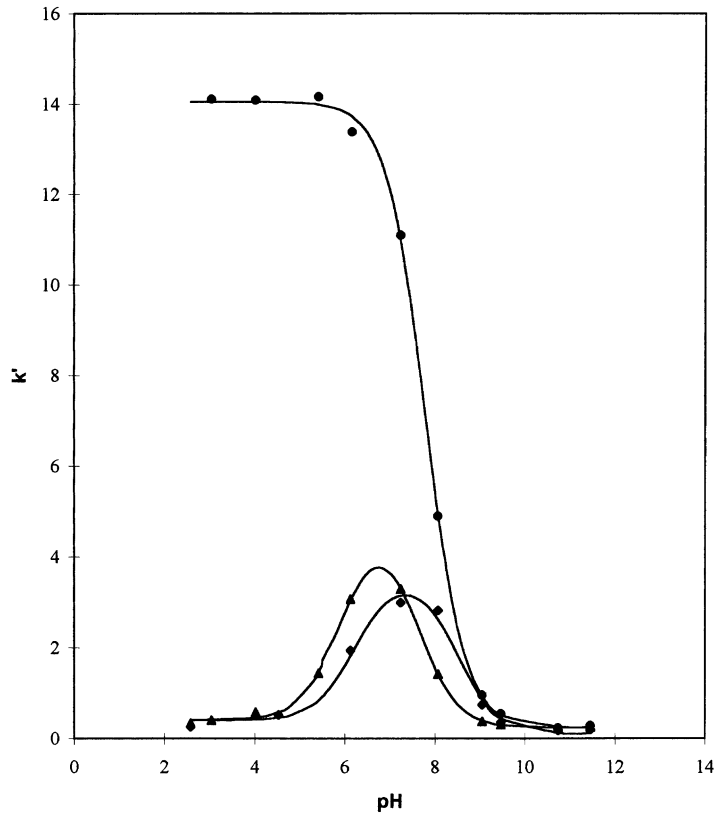


Fig. 3. Plot of the chromatographic capacity factor, k' versus the pH of a mobile phase containing 30% (w/w) MeCN: (■) norfloxacin, (▲) fleroxacin, (●) flumequine.

and the time and effort to calibrate the electrodic system. UV-spectrophotometric method hinges on the neutral and ionic species having different spectra. When this criterion is met, excellent precision is obtained [5,38].

Liquid chromatography is also used as a technique for the determination of dissociation constants because small quantities of compounds are required, poor water solubility is not a serious drawback, and samples do not need to be pure. However, in general, precision of the pK_a is lower than when using potentiometric, spectrophotometric and electrophoretic methods [43–46] as is shown in Table 1. The discrepancy between pK_a values obtained from the different methods is greater for pK_1 values than for pK_2 values, in the case of quinolones with two ionizable functional groups. Whereas for flumequine at 30% (w/w) MeCN, the discrepancy between values is not appreciatively significant from a statistical point of view. Equation relating chromatographic reten-

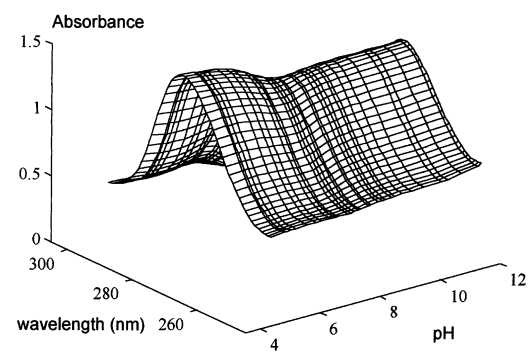


Fig. 4. Plot of experimental absorbance values of enrofloxacin versus λ as a function of pH in MeCN–water mixture with 30% (w/w) of acetonitrile.

tion with pH, 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 an absorptive site for retention [47]. Carr et al. [48] demonstrated that most of the free energy retention in LC arises from the attractive dispersive interactions between the solute and the stationary phase, and not from net repulsive interactions in the mobile phase. However, basic compounds have been described as showing very strong non-hydrophobic interactions [48,49]. 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 [9]. It is assumed that, in acidic media, the amino groups of quinolones are protonated. This positive monocharged group can have donor–acceptor interactions with the sorbent [50]. Then, the retention in moderately acid media is greater than that predicted theoretically, and the resulting equilibrium constant is lower than expected. This fact explains the differences in pK_a values of quinolones obtained by LC method and pK_a values obtained by other methodologies. The most important drawback for LC methodology is that it is not possible to determine pK_a values in hydroorganic mixtures with low contents of organic solvent because of the high retention times obtained. One of the disadvantages of the LC method is that the pH range of the mobile phase, and therefore the range of pK_a values that can be determined, are limited by the stability of the column packings. For example, silica-based reversed phase packing cannot operate above pH 8 due to deterioration of the silica support. Several authors [51] recommend columns with styrene–divinylbenzene copolymer [9], since it is stable in the alkaline medium as well as in the acidic medium as opposed to the silica based reversed phase packing. Some compounds, however, show excessive retention on styrene–divinylbenzene copolymers so it is necessary to add a considerable amount of organic solvent to the mobile phase in order to obtain reasonable retention times. This means that it is difficult to obtain pK_a values in totally aqueous media using LC methods.

Recently, CE has been introduced as a method for convenient and precise pK_a determination [3,4,7,17,52]. The method relies on the principle that solute exhibits an electrophoretic mobility continuum versus pH. In its neutral state, the solute has no mobility, in its fully charged state, it has its maximum mobility. Intermediate mobilities are a function of dissociation equilibrium and can be determined by regression analysis [4,7,17]. This method offers several advantages as it requires only small amounts of sample at low solute concentrations, the procedure does not require measurement of solute or titrant concentrations, only migration times. Calculations are straightforward and independent of the solute purity and is a universal technique for determining pK_a values in a wide pH range [4,5,17,52,53] as well as presenting a high sensitivity and selectivity relative to potentiometry and spectrophotometry. The precision obtained in the determination of pK_a values of quinolones are similar to those obtained in potentiometric methods and, in general, better than those obtained in LC or spectrophotometric methodologies (Table 1). The possible general application of the CE methodology to any ionizable compound in any aqueous or hydroorganic media, in addition to the advantages described in this work, make the CE technique a powerful tool to determine the pK_a values of drugs.

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