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Prediction of retention behaviour and evaluation of pK_a values of peptides and quinolones in liquid chromatography

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Abstract

The present paper examines the effect of the solute ionisation on the retention behaviour in liquid chromatography of a series of peptide and quinolone compounds of biological interest, using acetonitrile–water media as mobile phases and a polymeric-based stationary phase. Polymeric columns with polystyrene–divinylbenzene (PS–DVB) polymer show advantages over silica-based reversed-phase packings since the former are stable in a wide pH range. $^{S}_{S}pK_a$ values have been evaluated using chromatographic data in acetonitrile–water mixtures with acetonitrile percentages of 30, 35, 40 and 50% (v/v) for quinolones and 12.5 and 20% (v/v) for peptides. The quinolones show great retention on PS–DVB phase stationary. It was thus necessary to work with a higher acetonitrile content in the mobile phase than for the less retained peptides. The pH values were measured in the hydroorganic mixtures, used as mobile phases, instead of in water and account was taken of the effect of activity coefficients. The derived equations permit the chromatographic determination of $^{S}_{S}pK_a$ values of the peptides and quinolones in acetonitrile–water mixtures by fitting it to the experimental data in a nonlinear least-square procedure and also permit the prediction of the effect of $^{S}_{S}$pH on their chromatographic behaviour. We have also compared the obtained $^{S}_{S}pK_a$ values with those previously obtained in acetonitrile–water mixtures from potentiometric measurements.

Keywords: Retention behaviour; Dissociation constants; Peptides; Quinolones

1. Introduction

Advances in recombinant DNA have resulted in an increasing number of peptide and protein research drugs. Likewise, solid-phase peptide synthesis (SPPS) has produced many peptides of pharmaceutical interest in a wide molecular-mass range. Many areas of research in pharmacology and food science are currently engaged in the isolation, separation or purification of peptides [1]. On the other hand, the quinolones constitute a family of antibacterial agents whose use is already extensive in both human and veterinary clinics. The quinolones are bactericides and act by inhibiting bacterial DNA-girase [2]. Numerous structurally related quinolones have been synthesised and several are in routine clinical use throughout the world. The more potent analogues are represented by molecules supporting a piperazyl moiety. The wide use of the quinolones for treatment of systemic infections may require a greater need of analysis and separation methods for these components. Liquid chromatography (LC) is

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the most widely used analytical technique for the analysis and separation of different compounds like those under discussion here. The present paper describes testing of the potential value of polymeric columns like polysterene–divinylbenzene (PS–DVB) to work at high pH.

Although a desired separation may be obtained by trial and error, this may take many attempts. The total number of experiments can be minimised by using accurate quantitative relationships capable of predicting elution of compounds under different separation conditions. The optimisation of chromatographic resolution of ionogenic solutes in LC is a task that has been actively researched [3–5]. Due to the specific acid–base characteristics of ionogenic solutes, the two most useful optimisation parameters are the pH and the organic modifier concentration. An approach for optimizing organic modifier concentration in the mobile phase, during chromatographic separations, has been tackled in previous works [6–8] by establishing relationships between the retention parameter and Reichardt's E_T^N scale of solvent polarity. The pH of the mobile phase is a major factor that influences the chromatographic behaviour of the biological compounds studied here as they contain ionogenic functions such as carboxylic and amino groups. Their retention depends on the percentage of ionised and non-ionised species of these compounds. This makes it possible to obtain the dissociation constants from the variation of the retention with the pH. Thus, a knowledge of acid–base dissociation constants of the analytes in hydroorganic mixtures which are usually used as mobile phases, can help to improve the analytical methodology and can lead to a better understanding of their biochemical properties.

Prediction of chromatographic behaviour of ionizable compounds, is greatly aided by relationships between retention factor, dissociation constants and pH [9,10]. However, the inclusion of pH as an additional optimisation parameter using hydroorganic mixtures raises several problems [11]. Thermodynamically valid pH and pK_a values must be used to interpret ionic equilibria in LC. Moreover, because of the high permitivity of the medium, activity coefficients can be neglected in water for dilute solutions (<0.01 M), but the coefficients must be considered in hydroorganic mixtures.

Several procedures are used to measure the mobile phase pH. The most common procedure is to measure the pH of the aqueous buffer before mixing it with the organic modifier, ${}^{w}_{w}$pH. A more rigorous procedure, recommended by the IUPAC, is to measure the pH of the mobile phase after mixing the aqueous buffer and the organic modifier. In this instance, the electrode system used to measure pH can be calibrated either with aqueous buffers ${}^{S}_{w}$pH, or with buffers prepared in the same solvent composition used as mobile phase, ${}^{S}_{S}$pH. This requires knowledge of the pH value of reference buffers prepared in different aqueous–organic solvent mixtures. Following IUPAC definitions and recommendations the three pH values can be related to the pH scales, ${}^{w}_{w}$pH, ${}^{S}_{w}$pH and ${}^{S}_{S}$pH, respectively [18]. pH measurements in acetonitrile–water, the most widely used mobile phase are based upon the operational definition of pH, in which pH is estimated by:

$$\mathrm{pH_x} = \mathrm{pH_s} + \frac{E_S - E_X}{K_g} \quad (1)$$

where the unknown pH of solution x, $\mathrm{pH_x}$, is related to the pH of a standard reference solution, $\mathrm{pH_s}$ and the e.m.f. values of the potentiometric cell containing the standard, E_s, and the unknown solution, E_x. K_g must be used for practical measurements, usually carried out in cells with glass electrodes, and corresponds to the practical slope of the E versus pH function [12]. Therefore, if a potentiometric cell used to measure the pH of an aqueous organic solvent s (e.g. an LC mobile phase) is standardised with the buffers prepared in the same solvent mixture, s, the standard state for the proton activity is the solvent mixture, s, and the ${}^{S}_{S}$pH quantity will be obtained. But if the electrode system is calibrated with aqueous buffers, the standard state is water and the ${}^{S}_{w}$pH quantity will be obtained when the pH of the test solution in solvent is measured.

As $\mathrm{pH_S}$ values have been previously determined in acetonitrile–water mixtures [14,15], in accordance with the IUPAC rules [16,17] for the primary standard series of substances proposed by NIST [13], ${}^{S}_{S}$pH values in acetonitrile–water mixtures can be measured. In this study we used potassium hydrogen phthalate and phosphate buffers [16,18] as primary standard buffer reference solutions in the acetoni-

trile–water mixtures studied. The molar activity coefficients, γ, were calculated using the classical Debye–Hückel expression:

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

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 [19].

The ${}^{S}_{S}$pH scale of any hydroorganic solvent mixture is the pH range limited by the zero and ${}^{S}_{S}pK_{ap}$ [19] where ${}^{S}_{S}K_{ap}$ is the autoprotolysis constant of the medium. We have determined ${}^{S}_{S}pK_{ap}$ values of acetonitrile–water mixtures and derived equations that relate these ${}^{S}_{S}pK_{ap}$ values with the percentage of acetonitrile in the mixtures [19]. The ${}^{S}_{S}pK_{ap}$ values of the mixtures increases slightly from pure water to high percentage of acetonitrile (at 70% (w/w) of acetonitrile ${}^{S}_{S}pK_{ap} = 16.76$) [19]. For higher contents of acetonitrile, the ${}^{S}_{S}pK_{ap}$ value increase is steeper, 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. In this way, in the water-rich region of acetonitrile–water mixtures ($x_{MeCN} \leq 0.15$) and in the microheterogeneity region ($0.15 \leq x_{MeCN} \leq 0.75$) preferential solvation by water of proton ion increases continuously. This is consistent with the values obtained for preferential solvation, δ_w, of proton ion by water in acetonitrile–water mixtures. At $x_{MeCN} \geq 0.75$, the water–acetonitrile interactions that could be discounted in the middle range now become important. This may be considered as a region in which preferential solvation by water decreased [15,20].

The present work considers the effect of ionic equilibria on the retention behaviour of a series of peptides (Gly–Gly–Val, Gly–Gly–Ile, Ala–Leu–Gly, Gly–Gly–Phe) and quinolones (norfloxacin, enoxacin, fleroxacin, ofloxacin and flumequine). The structures are show in Fig. 1. Ionisation constants were evaluated using chromatographic data, in acetonitrile–water mixtures with percentages of acetoni-

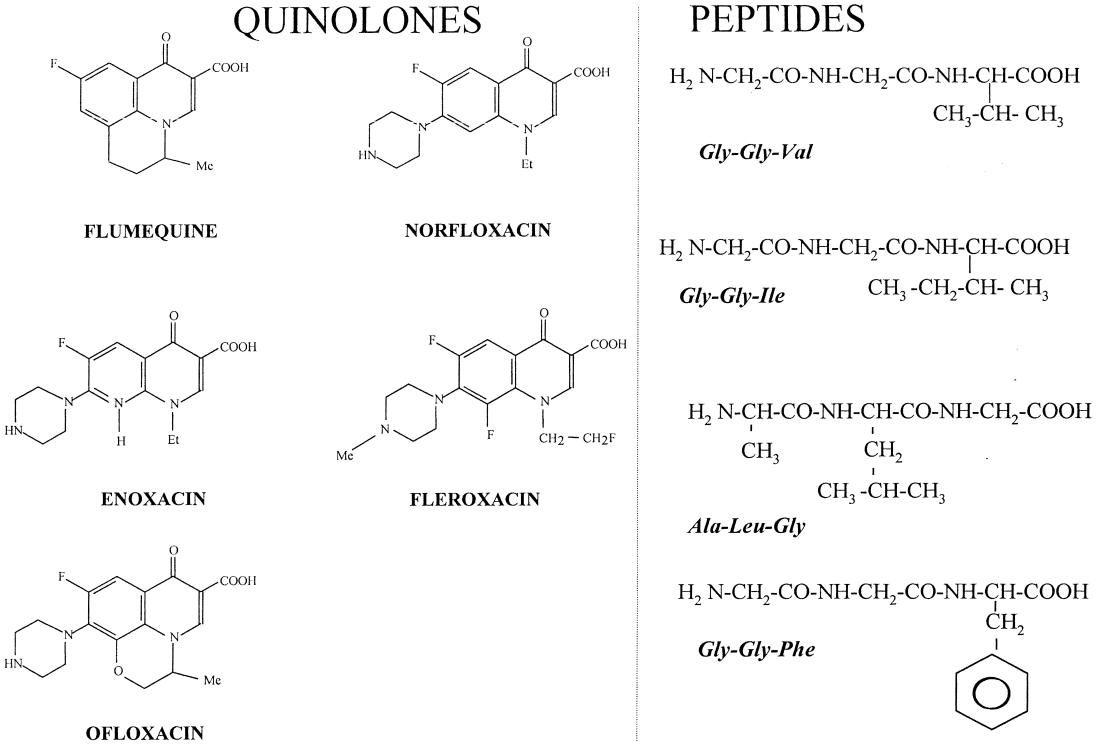

Fig. 1. Structures of the peptides and quinolones studied.

trile of 30, 35, 40 and 50% (v/v) for quinolone compounds and 12.5 and 20% (v/v) for peptide compounds and employing a polystyrene–divinylbenzene (PS–DVB) polymer as stationary phase because of its stability throughout the entire pH range. Comparisons of the experimentally determined ${}^{S}_{S}pK_a$ values with those previously obtained using potentiometry in acetonitrile–water mixtures are made. Thermodynamically valid ${}^{S}_{S}pH$, measured in the hydroorganic mobile phase instead of in water, were used to interpret the role of pH in the chromatographic behaviour of these compounds and the effect of activity coefficients was taken into account.

2. Experimental

2.1. Reagents

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. All reagents were of analytical grade. The peptides (Gly–Gly–Val, Gly–Gly–Ile, Ala–Leu–Gly, Gly–Gly–Phe) were purchased from Sigma.The quinolones were obtained from different pharmaceutical firms: norfloxacin (Liade, Boral Química), enoxacin (Almirall), fleroxacin (Roche), ofloxacin (Hoescht) and flumequine (Sigma). Working standard solutions were prepared in the mobile phase at concentrations of approximately 100 mg l^{-1} for peptides and 20 mg l^{-1} for quinolones. The samples were filtered through a 0.45-μm nylon filter membrane (MSI) before injection.

2.2. Apparatus

Chromatographic measurements were carried out on equipment consisting of an ISCO Model 2350 pump with an injection valve with a 10-μl sample loop and a variable-wavelength V^4 absorbance detector (ISCO) operating at 214 nm for peptide compounds and 280 nm for the quinolone compounds except for ofloxacin, which was detected 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. The column employed was a Shodex RSpak DS-613 column, 150×6 mm I.D., packed with hydrophobic PS–DVB gel and 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 stabilised in the appropriate acetonitrile–water mixtures prior to the e.m.f. measurements, which were performed in triplicate to ensure stability and reproducibility of the potentiometric system.

2.3. Chromatographic procedure

The mobile phases assayed were acetonitrile–water (12.5:87.5 and 20:80) with 0.1% phosphoric acid for peptides and acetonitrile–buffer (30:70, 35:65, 40:60 and 50:50) for quinolones 25 m*M* phosphoric acid being the buffer component. The mobile phase ${}^{S}_{S}pH$ values were adjusted between 2.5 and 11.0 with sodium hydroxide. The flow-rate of the mobile phase was maintained at 1 ml/min. For each compound and for every mobile phase composition and ${}^{S}_{S}pH$ considered, the retention time values, t_R, were determined from three different injections. Retention factors were calculated from $k = (t_R - t_0)/t_0$, where t_0 is the retention time of the potassium bromide (hold-up time) which was established for each mobile phase composition and ${}^{S}_{S}pH$ studied.

2.4. Data analysis

Theoretical models describing the dependence of the retention factor, *k*, on the pH of the mobile phase, using reversed-phase sorbents, can be derived considering ${}^{S}_{S}pH$ values and activity coefficients and taking into account the ionisation equilibria of the compounds.

The studied peptides are substances with two proton-binding sites. 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 retention factor, *k*, of any ionizable solute as a function of mobile phase ${}^{S}_{S}pH$ can be expressed considering that the

observed retention factor, k, is a weighted average of the k of the ionic and neutral forms of the solute [21] 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. 2, can be given as:

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

where k_{HA} and k_{A^-} are the retention factors of the solute in nonionised and ionised form, respectively and x_i is the molar fraction in nonionised and ionised form as indicated in Eqs. (4) and (5), respectively:

$$x_{HA} = \frac{a_{H^+}\gamma}{a_{H^+}\gamma + K_a} \quad (4)$$

$$x_{A^-} = \frac{K_a}{a_{H^+}\gamma + K_a} \quad (5)$$

taking into account Eqs. (4) and (5), Eq. (3) can be written as:

$$k = \frac{a_{H^+}\gamma k_{HA} + K_a k_{A^-}}{a_{H^+}\gamma + K_a} \quad (6)$$

or

$$k = \frac{k_{HA} + k_{A^-}\dfrac{K_a}{a_{H^+}\gamma}}{1 + \dfrac{K_a}{a_{H^+}\gamma}} \quad (7)$$

The classical approach neglects activity coefficients and uses the pH value in water instead of the pH value in the mobile phase. The molar activity coefficients, y, were calculated using the classical Debye–Hückel expression, Eq. (2). The ionic strength, I, of the used mobile phases can be calculated at every $^{S}_{S}$pH value from charge and mass

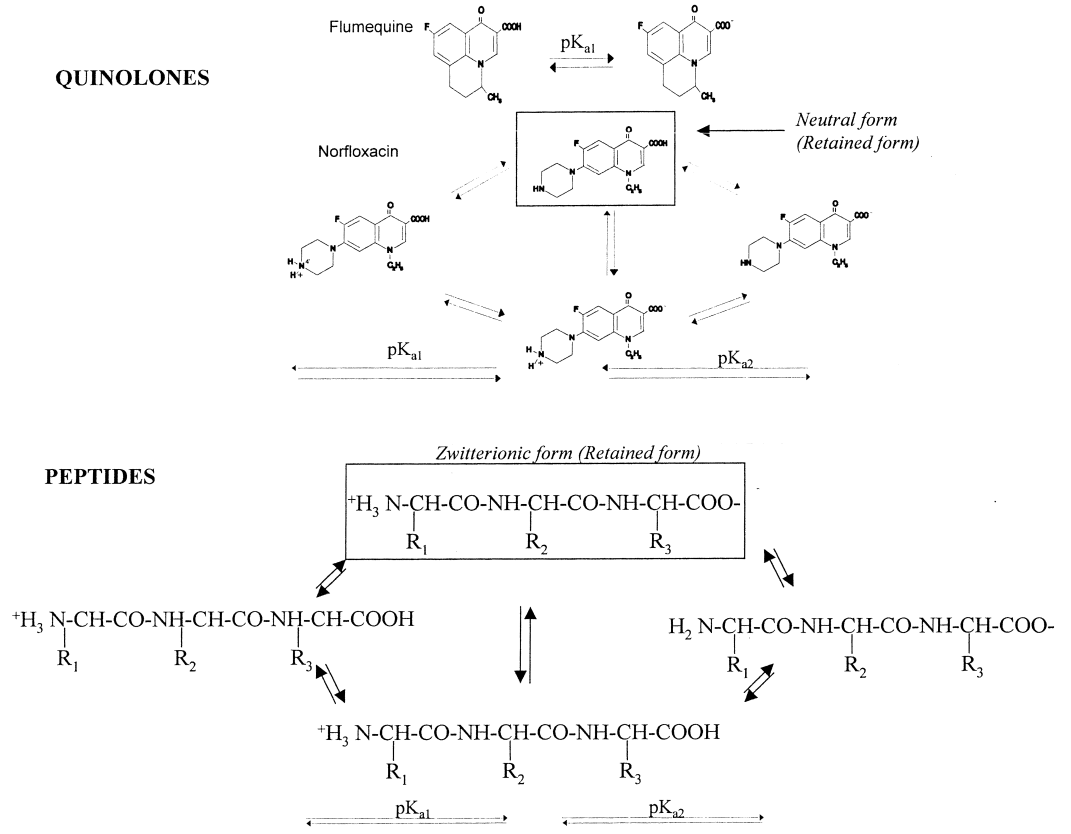

Fig. 2. Protolytic equilibria of peptides and quinolones studied. The predominant retained form of each compound is also indicated.

balances, taking into account the ${}^{S}_{S}pK_{a1}$ and ${}^{S}_{S}pK_{a2}$ values of phosphoric acid at each mobile phase composition [22], the analytical concentration of this acid in the mobile phase, the ${}^{S}_{S}pH$ values and the activity coefficients, involving the use of an iterative calculation [23].

For the peptides and other quinolones studied except for flumequine, two proton-binding sites should be considered. These substances exist in four microscopic protonation forms in solution. The scheme of the interconversion between the four microspecies, as well as their functional groups are shown in Figs. 1 and 2. The pertinent dissociation constants correspond to the equilibria:

$$^{+}HB\,AH \quad \leftrightarrows \quad {}^{+}HB\,A^{-} + H^{+} \quad K_{a1}$$
$$^{+}HB\,A^{-} \quad \leftrightarrows \quad BA^{-} + H^{+} \quad K_{a2}$$

The equation which relates the chromatographic retention and the pH of the mobile phase in the case of amphoteric substances, such as these peptides and quinolones, can be derived in a similar way to Eq. (7) and is:

$$k = \frac{k_{^{+}HBAH}\dfrac{a_{H^{+}}}{K_{a1}\gamma} + k_{^{+}HBA^{-}} + k_{BA^{-}}\dfrac{K_{a2}}{a_{H^{+}}\gamma}}{\dfrac{a_{H^{+}}}{K_{a1}\gamma} + 1 + \dfrac{K_{a2}}{a_{H^{+}}\gamma}} \qquad (8)$$

Eqs. (7) and (8) were originally elaborated only for aqueous solutions; 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 give evidence of the presence of adsorption-like retention mechanism or non-hydrophobic interactions [24–27].

The usefulness of such equations is 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 at each pH if K_a and k values for the different species of the substance are known.

3. Results and discussion

Experimental k values of the series of peptides and quinolones studied were determined from three different injections at every mobile phase composition and at each ${}^{S}_{S}pH$ considered. Relative standard deviations lower than 2% were obtained.

The peptides and four of the quinolones studied have two relevant ionizable functional groups, which means that their acid–base chemistry involves two protons (see Figs. 1 and 2). In contrast, flumequine has only one relevant ionizable functional group within the pH range of pharmaceutical or physiological importance, Fig. 2.

A PS–DVB copolymer is used in our study as stationary phase. This copolymer is particularly suited to study the effect of pH on chromatographic retention, because the octadecylsilica, ODS, stationary phase may only be used in the pH range from 2 to 7, it was therefore not possible to study the retention of peptides and quinolones over the whole pH scale [26,27].

Retention behaviour for all the studied compounds can be described by Eqs. (7) and (8). At a given pH, these equations relate the retention factor of the fully protonated, zwitterionic or deprotonated species and the dissociation constants, taking into account the effect of the ionic strength. The retention factor for some of the compounds were plotted against the mobile phase ${}^{S}_{S}pH$ in Fig. 3. Symbols stand for the experimental data and solid lines indicate the best nonlinear regression fits for each substance, using Eq. (8).

Each equation was experimentally verified and the ${}^{S}_{S}pK_a$ values of compounds studied were determined from the experimental k values, the ${}^{S}_{S}pH$ measurements and calculated activity coefficient values. The obtained ${}^{S}_{S}pK_a$ values are listed in Table 1 and were calculated by using a non-linear least-squares fit of the data (coefficient correlations ≥ 0.99). Eqs. (7) and (8) also permit the determination of the retention factors of the different peptides and quinolones species, given in Table 2.

Peptides and quinolones have a carboxyl group, therefore pK_{a1} values can be associated with this carboxylic acid function [9,28,29] (Fig. 1). The pK_{a2} values can be associated to the presence of a terminal amino group for the peptides and to the presence of a

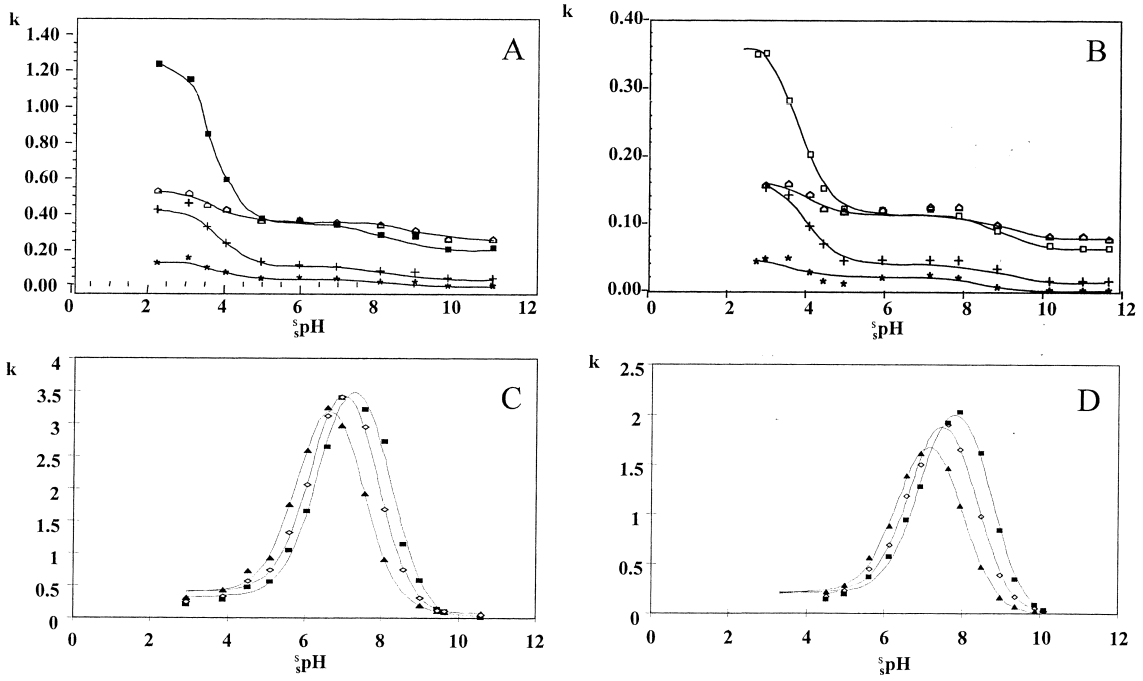

Fig. 3. Plots of k vs. $^{S}_{S}$pH for peptides and quinolones: (A) 12.5% (v/v) of acetonitrile in the mobile phase, ■, Gly–Gly–Phe; △, Ala–Leu–Gly; +, Gly–Gly–Ile; ☆, Gly–Gly–Val. (B) 20% (v/v) of acetonitrile in the mobile phase; □, Gly–Gly–Phe; △, Ala–Leu–Gly; +, Gly–Gly–Ile; ☆, Gly–Gly–Val. (C) 30% (v/v) of acetonitrile in mobile phase, ■, norfloxacin; ▲, fleroxacin, ◇, ofloxacin. (D) 50% (v/v) of acetonitrile in the mobile phase, the symbols are the same as indicated in (C).

piperazine ring for the quinolones (Fig. 2) [20,23]. Protonation occurs at N_4 of the piperazine ring over other apparently basic sites. This is proven 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 N atom [29].

The pK_{a1} values of di- and tripeptides being greater than the corresponding pK_{a1} of monomeric amino acid, can be explained by the fact that in monomeric amino acids the carboxylic and amino groups are adjacents [31]. In effect, pK_{a1} values corresponding to the C-terminal carboxylic group obtained are approximately one pK_a unit greater than the relevant values of the given monomeric amino acid showing the same order (Gly, $^{w}_{w}$pK_{a1} = 2.35; Val, $^{w}_{w}$pK_{a1} = 2.24; Ile, $^{w}_{w}$pK_{a1} = 2.33 and Phe, $^{w}_{w}$pK_{a1} = 2.58) [31].

The pK_{a1} values associated with the carboxylic acid function for the quinolones studied here were higher than those generally observed with carboxylic acids in hydroorganic mixtures (e.g. acetic acid in 30% (w/w) of acetonitrile–water mixture has a $^{S}_{S}$pK_{a1} = 5.63) [22]. This decrease in acidity can be attributed to an intramolecular H-bond formation with the neighbouring keto function resulting in stabilisation of the protonated species [28]. The formation of an intramolecular hydrogen bond is supported by UV and IR spectral data [32].

The pK_{a2} values of the secondary amine type derivatives studied (norfloxacine, enoxacine and ofloxacine) are greater than those of the tertiary amines (fleroxacine). These findings were consistent with reports in the literature for similar secondary and tertiary amines: piperazine, $^{w}_{w}$pK_{a1} = 9.71 [33], and *N*-methylpiperazine, $^{w}_{w}$pK_{a1} = 8.98 [34]. The more water molecules involved in the hydrate sphere of the protonated amine, the greater the stabilisation [35]. The protonated form of the secondary amine was stabilised by the greater number of water molecules involved in its hydration sphere when compared with the corresponding tertiary amine [28].

Table 1
pK_a values of peptides and quinolones obtained from chromatographic and potentiometric methods; the values between parentheses are the standard deviations

Compound	Method	Acetonitrile percentage							
		12.5% (v/v)		20% (v/v)					
		${}^{S}_{S}pK_{a1}$	${}^{S}_{S}pK_{a2}$	${}^{S}_{S}pK_{a1}$	${}^{S}_{S}pK_{a2}$				
Gly–Gly–Phe	Chromatographic	3.60 (0.02)	8.29 (0.11)	3.75 (0.05)	9.03 (0.29)				
	Potentiometric	8.02 (0.02)	8.02 (0.01)	3.59 (0.04)	8.11 (0.02)				
Ala–Leu–Gly	Chromatographic	3.67 (0.12)	9.08 (0.24)	3.98 (0.27)	9.16 (0.30)				
	Potentiometric	3.62 (0.02)	8.12 (0.01)	3.78 (0.03)	8.23 (0.01)				
Gly–Gly–Ile	Chromatographic	3.80 (0.01)	8.34 (0.06)	3.95 (0.10)	9.06 (0.48)				
	Potentiometric	3.55 (0.04)	8.09 (0.04)	3.79 (0.01)	8.17 (0.01)				
Gly–Gly–Val	Chromatographic	3.80 (0.08)	8.35 (0.20)	3.64 (0.41)	8.56 (0.40)				
	Potentiometric	3.54 (0.02)	8.08 (0.01)	3.73 (0.05)	8.16 (0.03)				
		30% (v/v)		35% (v/v)		40% (v/v)		50% (v/v)	
		${}^{S}_{S}pK_{a1}$	${}^{S}_{S}pK_{a2}$	${}^{S}_{S}pK_{a1}$	${}^{S}_{S}pK_{a2}$	${}^{S}_{S}pK_{a1}$	${}^{S}_{S}pK_{a2}$	${}^{S}_{S}pK_{a1}$	${}^{S}_{S}pK_{a1}$
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 (0.04)					
	Potentiometric			7.60 (0.03)					

Although ${}^{S}_{S}pK_a$ values of peptides and quinolones obtained in acetonitrile–water mixtures increase with the percentage of acetonitrile, these ${}^{S}_{S}pK_a$ values are lower than expected considering the high ${}^{S}_{S}pK_a$ values expected in the neat solvent acetonitrile [36]. The variation of ${}^{S}_{S}pK_a$ values of these compounds in acetonitrile–water mixtures could be explained because preferential solvation occurs in these media [14,20] and is attributable to structural features of these mixtures [37,38]. Preferential solvation in acetonitrile–water mixtures produces lower ${}^{S}_{S}pK_a$ values than expected because of the preferential solvation by water [14].

In previous papers [23,30], the variation of the ${}^{S}_{S}pK_a$ values obtained using potentiometric techniques of peptides and quinolones with percentage of acetonitrile in acetonitrile–water mixtures was studied. The variation of ${}^{S}_{S}pK_a$ values is different for each substances although, in general, the ${}^{S}_{S}pK_a$ values increase in direct proportion with acetonitrile content, (see Table 1 and Fig. 4). ${}^{S}_{S}pK_{a1}$ values of peptides and quinolones vary from low acetonitrile percentages, whereas ${}^{S}_{S}pK_{a2}$ values show low changes in the range 0 to ~30% of acetonitrile and they increase at higher percentages of acetonitrile. This is explained by the structural features of acetonitrile–water mixtures [23,30].

In order to confirm the determined ${}^{S}_{S}pK_a$ values using chromatographic data, Table 1 also summarises the previously obtained ${}^{S}_{S}pK_a$ values of the peptides and quinolones in the same solvent mixtures, using the potentiometric method [23,30], according to the rules and procedures endorsed by IUPAC [16]. The advantages of using LC to determine accurate thermodynamic ${}^{S}_{S}pK_a$ values of compounds are numerous: LC requires small amounts of sample at low solute concentration and the procedure does not require solute measurement

Table 2
Chromatographic retention factors of the protonated, zwitterionic and deprotonated species of the studied compounds; values between parentheses are standard deviations

Compound	Acetonitrile percentage													
	12.5% (v/v)			20% (v/v)										
	k^{+}_{HBAH}	$k^{+}_{HBA^-}$	k_{BA^-}	k^{+}_{HBAH}	$k^{+}_{HBA^-}$	k_{BA^-}								
Gly–Gly–Phe	1.27 (0.01)	0.35 (0.01)	0.20 (0.01)	0.38 (0.01)	0.11 (0.01)	0.06 (0.01)								
Ala–Leu–Gly	0.53 (0.01)	0.36 (0.01)	0.26 (0.01)	0.16 (0.01)	0.11 (0.01)	0.08 (0.01)								
Gly–Gly–Ile	0.43 (0.01)	0.11 (0.01)	0.04 (0.01)	0.17 (0.01)	0.04 (0.01)	0.01 (0.01)								
Gly–Gly–Val	0.13 (0.01)	0.04 (0.01)	0.00 (0.01)	0.05 (0.01)	0.02 (0.01)	0.00 (0.01)								
	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^-}
Norfloxacin	0.32 (0.07)	4.22 (0.22)	0.00 (0.08)	0.40 (0.18)	3.56 (0.27)	0.11 (0.08)	–	–	0.25 (0.09)	3.90 (0.45)	0.00 (0.18)	0.20 (0.04)	2.56. (0.14)	0.00 (0.05)
Fleroxacin	0.40 (0.04)	4.06 (0.13)	0.00 (0.13)	0.41 (0.03)	4.72 (0.19)	0.24 (0.03)	–	–	0.26 (0.08)	3.83 (0.29)	0.00 (0.10)	0.21 (0.03)	2.20 (0.09)	0.00 (0.03)
Enoxacin	0.30 (0.11)	5.05 (0.39)	–	–	–	–	–	–	0.41 (0.11)	4.15 (0.48)	0.00 (0.15)	0.17 (0.04)	2.85 (0.13)	0.00 (0.05)
Ofloxacin	0.41 (0.04)	4.32 (0.11)	–	–	–	–	–	–	0.35 (0.09)	4.71 (0.46)	0.00 (0.14)	0.21 (0.03)	2.43 (0.11)	0.00 (0.03)
Flumequine	–		–	–	–	–	14.04 (0.04)	0.25 (0.08)	–	–	–	–	–	–

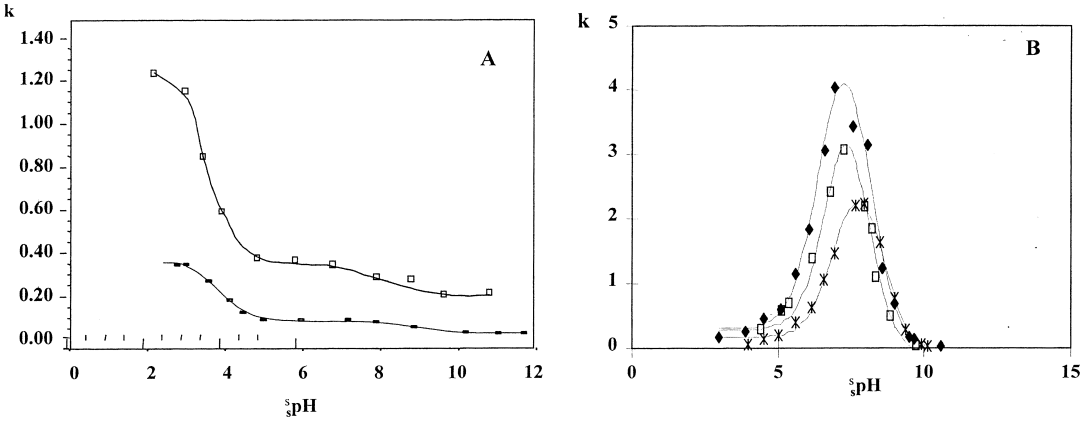

Fig. 4. Plots of k vs. $^{S}_{S}$pH with different percentages of acetonitrile: (A) Gly–Gly–Phe, (□) 12.5% (v/v) and (■) 20% (v/v); (B) enoxacin, (♦) 30% (v/v), (□) 40% (v/v) and (*) 50% (v/v).

or titrant concentration as do potentiometric techniques, but only retention times. Calculations are independent of solute impurities, since impurities can be separated from the solutes of interest on the LC column. However, as can be deduced from Table 1, the precision of ${}^{S}_{S}pK_a$ values in MeCN–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 ${}^{S}_{S}pK_{a2}$ values than for ${}^{S}_{S}pK_{a1}$ values, in the case of peptides and greater for ${}^{S}_{S}pK_{a1}$ values than for ${}^{S}_{S}pK_{a2}$ values, in the case of quinolones with two ionizable functional groups, whereas for flumequine the discrepancy, between chromatographic and potentiometric ${}^{S}_{S}pK_{a1}$ values at 35% (v/v) of acetonitrile, is not highly significant.

Equations derived that relate chromatographic retention with ${}^{S}_{S}pH$, have been proposed for cases where the interaction between the sorbent and the solute is exclusively controlled by their hydrophobicity, that is, no ionic or hydrogen bonding interaction occurs between the solute and the stationary phase. The solvophobic theory, used to derived Eqs. (7) and (8), attributed the retention process to the mobile phase and treats the stationary phase as a passive entity that plays no role in the separation process, other than providing a sorptive site for retention [39].

Consideration of the thermodynamics 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's group [25,41] demonstrated that most of the free energy retention in LC arises from attractive interactions between the solute and the stationary phase, not from net repulsive interactions in the mobile phase. Likewise, the retention of compound in the polymeric columns used is markedly influenced by the solute hydrogen bond donor acidity and exhibits a more adsorption-like retention mechanism than do other studied phases [25].

On the other hand, separation of basic compounds with amino groups often cause many more difficulties [24]. If the retention mechanism includes interactions other than hydrophobic ones, the k versus ${}^{S}_{S}pH$ dependence can show different shapes or at least deviation from the ideal sigmoidal shape.

The retention of peptides is relatively high in pronounced acid media, Figs. 3 and 4, where the peptide exists as a single charged cation. When ${}^{S}_{S}pH$ increases, the k value decreases and levels off at the isoelectric point ${}^{S}_{S}pH$. If ${}^{S}_{S}pH$ is increased again, the k value decreases and we have the anionic monocharged species of peptides, Fig. 2. In the case of quinolones, the retention is relatively low at pronounced acid media, Figs. 3 and 4, where the quinolone exists as a single charged cation. When ${}^{S}_{S}pH$ increases, the k value increases and levels off at the isoelectric point ${}^{S}_{S}pH$. Thus, the equilibrium between the double charged zwitterionic and neutral forms is displaced to the latter form for the quinolones, Fig. 2, in contrast with the case of the peptides. If ${}^{S}_{S}pH$ is increased again, the k value decreases and we have the anionic monocharged species of quinolones, Figs. 3 and 4.

It is assumed that in acidic medium the amino groups of peptides and quinolones are protonated [28]. These positive monocharged group can have donor–acceptor interactions with the sorbent [41]. However, proton interactions are also involved and compete with protonated species. In clearly acid media, proton interactions are very important 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. If the compounds present acid–base equilibria in clearly acid media, like the peptide series studied, the influence of donor–acceptor interactions between protonated form and sorbent should be low.

For the quinolones, the proton concentration is lower at pH values near of their pK_{a1}, and the interactions of the protonated quinolone with the sorbent are stronger than in the case of the peptides due to the weak competition of the proton at these pH values. It is claimed that the retained species for the quinolones is the neutral form, although the predominant species in the mobile phase must be the zwitterionic form. The strong adsorption of the neutral form on this column and the donor–acceptor interactions of the amino protonated form would explain the abnormal behaviour of quinolones and in

consequence the lower concordance between the theoretical and the experimental values corresponding to the first dissociation constant of the protonated quinolone.

The peptides are very polar compounds and interactions with the sorbent are not influenced to the same degree as with the quinolones. This is due to the strong competition of the proton by the donor–acceptor interactions with the sorbent since in these cases ${}^{S}_{S}pK_{a1}$ values are nearly three units and the proton concentration is relatively high. This finding is consistent with reports that sigmoidal k versus ${}^{S}_{S}pH$ curves depart from the ideal shape only for zwitterionic substances with medium or high pK_{a1} constants [24]. Thus, this problem was not observed in studies concerning variations of the peptide LC retention with the ${}^{S}_{S}pH$ [26] and other substances with low pK_a values.

The results obtained for flumequine are in accordance with the ${}^{S}_{S}pK_{a1}$ values, obtained using LC, for substances with only one carboxylic functional group [42]. In these substances only a small repulsion can be expected between the sorbent and the carboxylate group using polymeric columns as in this work; possible weak nonhydrophobic interactions between carboxylic groups and sorbent could also exist in accordance with Carr et al. [25]. This interaction could explain the slightly higher chromatographic ${}^{S}_{S}pK_{a1}$ values of flumequine than the potentiometric ones.

The lower concordance between the potentiometric and chromatographic ${}^{S}_{S}pK_{a2}$ values for peptides could be explained by the existence of nonhydrophobic interactions of the protonated amino group of the predominant zwitterionic form with the sorbent. Moreover, account must be taken of the fact that the very small retention factor values of peptides at high ${}^{S}_{S}pH$ values implies a relatively high experimental error. In contrast with quinolone substances, the predominant form in the macroscopic equilibria is the zwitterionic form but the neutral form is the retained form in the sorbent. The possible repulsion between the sorbent and the negatively charged carboxylic compound could explain that chromatographic ${}^{S}_{S}pK_{a2}$ values of quinolones are slightly lower than the potentiometric ones. This can be attributed to a possible repulsion between the sorbent and the negatively charged carboxylic compound that could cause the decreases in the experimental chromatographic k values. Thus, chromatographic ${}^{S}_{S}pK_{a2}$ values of quinolones are slightly lower than the potentiometric ones.

In conclusion, in this work a model describing the effect of pH on retention in polymeric column by LC was evaluated. The suggested model employs the ${}^{S}_{S}pH$ value in the acetonitrile–water mixture used as mobile phase, instead of ${}^{w}_{w}pH$ and takes into account the effect of the activity coefficients. The usefulness of the proposed equations is twofold. First, they permit the determination of the acidity constants in the hydroorganic mobile phase, constants which influence the retention behaviour of ionizable compounds in LC, and second, the prediction of the optimum ${}^{S}_{S}pH$ because selectivity and resolution can be easily predicted [26], on the basis of a few experimental data. The disagreement in some cases of chromatographic dissociation constants with the potentiometric ones can be explained according to the characteristics of the acid–base solute and of the sorbent.

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