

## Determination of Protonation Constants of Some Tetracycline Antibiotics by Potentiometry and LC Methods in Water and Acetonitrile-Water Binary Mixtures

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Uma estimativa acurada das constantes de dissociação de antibióticos tetraciclina em misturas binárias acetonitrila-água é muito importante para muitas técnicas de separação, como cromatografia líquida e eletroforese capilar que usam essas misturas de solvente. Neste estudo, os valores de  $pK_a$  de cinco tetraciclina (tetraciclina, oxitetraciclina, clortetraciclina, doxiciclina e metaciclina) foram determinados em água e em misturas binárias (10%, 20% e 30% (v/v)), através de potenciometria e cromatografia líquida. Os resultados obtidos mostram uma tendência similar para os valores de  $pK_a$  dos cinco antibióticos tetraciclina, os quais aumentam com o aumento da concentração dos modificadores orgânicos, resultando numa relação linear entre os valores de  $pK_a$  e a fração molar de acetonitrila. Os valores obtidos para  $pK_a$  em meio aquoso foram comparados com os valores preditos pelo programa SPARC.

An accurate estimation of dissociation constants of tetracycline antibiotics in acetonitrile-water binary mixtures is very important for several separation techniques such as liquid chromatography and capillary electrophoresis that use these solvent mixtures. In this study, the  $pK_a$  values of five tetracyclines (tetracycline, oxytetracycline, chlortetracycline, doxycycline and metacycline), have been determined in water and acetonitrile-water binary mixtures (10%, 20% and 30% (v/v)) by potentiometry and liquid chromatography (LC). The obtained results show a similar trend for the  $pK_a$  values of five tetracycline antibiotics as they increase with increasing concentration of organic modifier, which allows a linear relationship between  $pK_a$  values and mole fraction of acetonitrile to be obtained. The  $pK_a$  values obtained in aqueous medium have been compared with the values predicted by the SPARC on-line  $pK_a$  calculator.

**Keywords:** tetracyclines, dissociation constants, potentiometric titration, LC

### Introduction

The dissociation constant ( $pK_a$ ) of a drug molecule is a key parameter in absorption, distribution, metabolism, excretion and toxicity researches because it governs solubility, absorption, distribution, and elimination of substances. Also, the  $pK_a$  values constitute important data for thorough understanding of certain chemical phenomena such as biological uptake, and the binding of these molecules to environmental matrices and forming chelates with metallic cations. The drugs'  $pK_a$  data are applied to estimate the major species of pharmaceuticals present in the environment (usually in neutral pH range) and dosage-form development. Particularly, these obtained  $pK_a$  values can be used for the development of separation methods

of tetracyclines on various separation techniques such as liquid chromatography (LC) and capillary electrophoresis (CE).<sup>1,2</sup>

There are lots of  $pK_a$  determination techniques for compounds of pharmaceutical or biological interest such as potentiometric titrations,<sup>3-7</sup> UV-Vis,<sup>8,9</sup> LC<sup>10-12</sup> and CE methodology<sup>13-16</sup> and software computational prediction.<sup>17-19</sup> Among these techniques, the potentiometric titration is a more general method and it does not require the presence of chromophore groups for  $pK_a$  determination. If due care is taken, this technique is accurate and has good reproducibility. Fast and automated instruments are commercially available for potentiometry; however, their disadvantages include the requirements to use milligram amount of pure substances and the use of carbonate free titrants. Also, UV-Visible absorption spectrometry has still been used widely by the help of improved computer

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programs for the determination of dissociation constants. Moreover, the use of computer programs for the refinement of dissociation constants allows the different  $pK_a$  values in polyprotic substances to be determined, even when they are very close.<sup>19,20</sup>

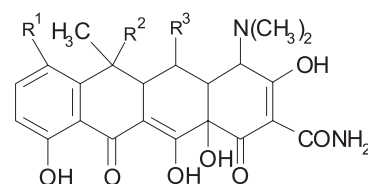
In the last decade, a new procedure has been developed when LC and CE methodologies are used for determination of ionization constants in combination with a diode array detector (DAD) for absorbance measurements.<sup>11,21,22</sup>

Traditionally, water has been considered the solvent which best represents the biological conditions. However, hydro-organic mixtures such as acetonitrile-water (MeCN-water), have been found suitable because they show simultaneously a low polar character and a partially aqueous content, as do all biological systems. MeCN can dissolve a wide majority of organic acids more effectively than water and in many cases it is a more suitable solvent for the determination of the dissociation constants. MeCN behaves as a weaker base and as a much weaker acid than water. It has a relatively high dielectric constant ( $\epsilon = 36$ ) and a small autoprotolysis constant ( $pK_s = 33.6$ ). Accordingly, MeCN acts as a strongly differentiating solvent with a modest solvating power for many polar ionic solutes.<sup>23</sup>

Tetracycline antibiotics (TCs) are broad-spectrum medicinal drug compounds active against a number of gram-positive and gram-negative bacteria. TCs have been successfully used worldwide in both veterinary and human medicine. These antibiotics are widely used as veterinary drugs for food-producing animals because of their broad-spectrum activity and cost effectiveness.<sup>3,24</sup> The commonly used TCs in medicine are tetracycline (TC), oxytetracycline (OTC), chlortetracycline (CTC), doxycycline (DC) and metacycline (MTC). TCs consist of 4 fused rings with substitutions on positions 5, 6 and 7. As we can deduce in Figure 1, the structure of TCs are very similar. They are amphoteric compounds with characteristic pH values and form crystalline hydrates and salts with acids and bases. The general formula contains three distinct acid groups—the tricarbonyl-methane, the ammonium cation and the phenolic diketone group. The protonation sites of TCs are shown in Figure 2.

The  $pK_a$  values of TCs are either not known accurately or not available at all. Only a limited number of studies related with  $pK$  values of studied TCs are found in the literature. However, some data in literature differ considerably, number of  $pK_a$  values and the methods used for their determination are not adequately described (temperature, ionic strength, solvent media, etc.).<sup>2,3,24-27</sup> In order to overcome the lack of information related with the acid-base equilibria of this kind of compounds

in MeCN-water binary mixtures, the  $pK_a$  values of five TCs (tetracycline, oxytetracycline, chlortetracycline, doxycycline and metacycline) in water and MeCN-water binary mixtures have been determined by means of potentiometric and chromatographic (LC and LC-DAD) measurements. The potentiometric determinations were carried out in water, 10%, and 20% (v/v) MeCN content, whereas the chromatographic determinations of  $pK_a$  values were at 10%, 20%, and 30% (v/v) MeCN content. The  $pK_a$  values obtained will be useful for the development and optimization of separation methods of TCs based on liquid chromatography in MeCN-water mixtures. The results obtained for  $pK_a$  in water have been compared with those predicted by SPARC on-line calculator.<sup>28,29</sup> This program estimates several physico-chemical properties of organic compounds on the basis of their molecular structure.



Compounds	R <sup>1</sup>	R <sup>2</sup>	R <sup>3</sup>
Tetracycline	H	OH	H
Chlortetracycline	Cl	OH	H
Oxytetracycline	H	OH	OH
Doxycycline	H	H	OH
Metacycline	H	=CH <sub>2</sub>	OH

Figure 1. Molecular structures of studied compounds.

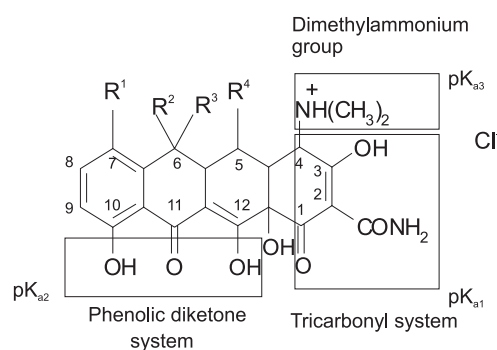


Figure 2. Structural groupings in the tetracyclines.

## Experimental

### Chemicals and reagents

TC, OTC, CTC, DC and MTC were purchased from Sigma and were used without further purification. Water, with conductivity  $\geq 0.05 \mu\text{S cm}^{-1}$  was obtained with a Milli

Q water purification system (Milli Pore Corp.) and used to all solutions. MeCN was of HPLC grade and used as the organic component of the mobile phase. Potassium hydrogen phthalate was dried at 110 °C before use (Fluka). The pH values of the mobile phases were measured against a 0.05 mol kg<sup>-1</sup> potassium hydrogen phthalate solution as primary reference standard solution, dissolved in the appropriate MeCN-water medium. This standard has been selected because their pH values at different MeCN percentages (up to 70% (v/v)) are already known.<sup>30</sup> The use of organic solvent-water mixtures requires the correct measurements of pH in these media. Measurements are performed in a similar way to those performed in water using IUPAC standardization rules.<sup>31,32</sup> Stock 0.025 mol L<sup>-1</sup> carbonate-free potassium hydroxide solutions were prepared in each MeCN-water mixture from 1 mol L<sup>-1</sup> potassium hydroxide solution (Titrisol, Merck) by dilution. Hydrochloric acid solutions were prepared by dilution from 1 mol L<sup>-1</sup> hydrochloric acid (Titrisol, Merck) in each MeCN-water mixture. The ionic strength of all working solutions was adjusted at 0.1 mol L<sup>-1</sup> with potassium chloride (Merck).

The alkali titre and absence of carbonate were periodically checked by pH-metry, using the appropriate Gran function<sup>33</sup> against primary standard oven-dried potassium hydrogen phthalate. Acid solutions prepared from hydrochloric acid were titrated against standardized potassium hydroxide.<sup>34</sup>

Stock standard solutions of TCs were freshly prepared in water at concentrations of approximately 200 mg L<sup>-1</sup> and stored in amber bottles in a refrigerator (4 °C). Working solutions were diluted with the corresponding mobile phase to 10 mg L<sup>-1</sup>. These solutions were passed through a 0.45 µm nylon filter membrane (MSI) before injections. The hold-up time,  $t_m$ , was measured for every mobile phase composition by injection of 0.01% (m/v) potassium bromide solution. The retention factors,  $k$ , for all the compounds at each mobile phase assayed were determined from three different injections of the TCs.

### Apparatus

In order to obtain the pK<sub>a</sub> values using potentiometric technique, potential values of the potentiometric cell were measured with a Model Mettler-Toledo MA 235 pH/ion analyser with combined glass electrode system (± 0.1 mV). The cell was thermostated externally at 25 °C ± 0.1 with a cooler system water bath (HETO CBN 8-30 and temperature control unit HETO HMT 200) and the test solution was stirred magnetically under a continuous stream of purified nitrogen. Triplicate titrations were carried out in the double-wall Pyrex titration cell of 80 mL capacity.

A chromatographic system consisted of Shimadzu Model LC 10 ADVP pump with an auto injector (SIL 10 AD VP) and diode array detector system (SPDM 10 A DAD) was used for studies. This equipment has column oven (CTO 10 AVP) and degasser system (DGU 14 A). Phenomenex Luna RP C-18 column (250 mm × 4.6 mm i.d., 5 µm) was used as stationary phase. Flow rate was 0.8 mL min<sup>-1</sup> and the column temperature was 25 °C. The compounds were injected using isocratic system and 20 µL volume was injected. The effluent was monitored at 271 nm.

### Procedures

#### Potentiometric procedure

The glass electrode was stored in water when not in use and soaked for 20-25 min in MeCN-water mixture before potentiometric measurements. The stabilization criterion for the potential readings was 0.2 mV within 120 s. In all instances, the electrode system gave stable and reproducible potentials within 3 min. The standardization of the electrode system was carried out, each time in MeCN-water mixtures (background solution) studied by Gran's method.<sup>33</sup> For this purpose, the electrode was immersed in these mixtures (20 mL and 0.1 mol L<sup>-1</sup> ionic strength). Usually, 10 or 12 additions of hydrochloric acid were enough for E<sup>0</sup> to be accurately determined. The potential was allowed to stabilize after each addition of acid and the potential values obtained were used to calculate the standard potential of the cell, E<sup>0</sup>.

The pK<sub>a</sub> values of TCs were determined by titration of the appropriate solutions of these compounds (2.74 × 10<sup>-3</sup> mol L<sup>-1</sup> for tetracycline) in water, 10% (v/v), and 20% (v/v) MeCN-water, using KOH solution as titrant, in 0.1 mol L<sup>-1</sup> ionic strength (KCl). Carbon dioxide dissolved in titrate solution was purged out by nitrogen gas. The TCs solution was completely mixed with a magnetic stirrer. Titration was carried out in triplicate at constant temperature (25 ± 0.1 °C) to ensure reproducibility. Drop wise addition of KOH solution (100 µL) was performed by calibrated Biohit-Proline pipettors (Biohit Corp. Finland) and the titrations were continued up to pH 12.

#### Chromatographic method

Formic acid (pH 2.5; 3.0; 3.54; 4.0; 4.5; 5.0) was preferred as buffer component because of its appropriate pK<sub>a</sub> values and the symmetrical peak shape of the TCs in this buffer solution. The pH of the mobile phase was adjusted between 2.5 and 6.0 with sodium hydroxide. Potassium hydrogen phthalate solutions (0.05 mol kg<sup>-1</sup>) dissolved in the appropriate MeCN-water medium were

used as reference standard value (RSV) for calibration of pH/ion analyzer. pH measurement of the mobile phases were performed in triplicate to ensure stability and reproducibility of the potentiometric system.

For each TCs and for every mobile phase composition and pH considered, the retention time values,  $t_{Ri}$ , were determined from three different injections. Retention factors ( $k_i$ ) were calculated from equation 1:

$$k_i = (t_{Ri} - t_m) / t_m \quad (1)$$

where  $t_m$  indicates the retention time of the potassium bromide (hold-up time), which was measured by injection of 0.01% potassium bromide solution in water for each mobile phase composition and pH studied. The  $pK_a$  values associated with tricarbonyl system were determined by using the NLREG program.<sup>35</sup>

#### Liquid chromatography-diode array detection

A novel method based on the absorbance spectra at the maximum of chromatographic peak, obtained with DAD has been applied to calculate the  $pK_a$  values. This method allows comparing the values of dissociation constants obtained from chromatographic retention of tetracyclines at different pH values and those obtained from absorbance spectra at the maximum of the chromatographic peak. It is possible to constitute a valuable means of obtaining better precision. Absorbance spectra were recorded between 190 and 300 nm and then processed by modified STAR (Stability Constants by Absorbance Readings) program.<sup>11,21,36</sup>

#### SPARC calculations

The SPARC program uses algorithms based on fundamental chemical structure theory that combines principles of quantitative structure activity relationships (QSAR), linear free-energy relationships (LFER) and perturbation theory from quantum chemistry.<sup>28,29</sup> This program estimates the macroscopic and microscopic  $pK_a$  of any organic compound solely from its chemical structure. Macroscopic  $pK_{a1}$ ,  $pK_{a2}$  and  $pK_{a3}$  values of studied compounds were calculated by this program. SPARC is available free of charge on the internet.

## Results and Discussion

The obtained  $pK_a$  values of tetracyclines by different methodologies, together with literature data and the values predicted by SPARC are given in Table 1. The first dissociation constant  $pK_1$ , is generally attributed to tricarbonyl system and nearly between 3.0-3.5 in water. The second dissociation constant  $pK_2$  is due to the phenolic

diketone system being about 7 and the third dissociation constant  $pK_3$  assigned to the protonated dimethylamino group (about 9).

Potentiometric  $pK_a$  values were determined from several series of independent measurements. One series of measurements for one titration of TC in water using KOH solution as titrant is shown in Figure 3. Two steps were clearly observed in the potentiometric curve, the first relating to the tricarbonyl system, the second to the ionization of phenolic diketone system. The third step was not clearly seen as expected and it is related to the dimethylammonium group. From the potentiometric data, the equilibrium constant for TC gave a value for  $pK_{a1}$ ,  $pK_{a2}$  and  $pK_{a3}$  of  $3.35 \pm 0.03$ ,  $7.29 \pm 0.03$  and  $9.88 \pm 0.03$ , respectively. The data obtained for TCs from the literature data are in good agreement. The calculation of  $pK_a$  values requires an iterative cycle for each point of the potentiometric titration at which electromotive force (e.m.f.) was measured and were carried out by using the program PKPOT.<sup>20</sup> This program allows the thermodynamic acid-base constants in aqueous and non-aqueous media to be determined, taking into account the activity coefficient of the species and by this program we could determine  $pK_{a1}$ ,  $pK_{a2}$  and  $pK_{a3}$  values of tetracyclines. This program refines the thermodynamic equilibrium constants in order to minimize the sum of squared differences ( $U_{emf}$ ) between the experimental electromotive force (e.m.f.) values and those calculated on the basis of the mass balances for hydrogen and tetracycline, the equilibrium constants, the cell standard potential and the activity coefficients:

$$U_{emf} = \sum_i^m \sum_j^n (emf_{i,j,exp} - emf_{i,j,calc})^2 \quad (2)$$

where m is the number of titrations and n the number of experimental points in each titration.

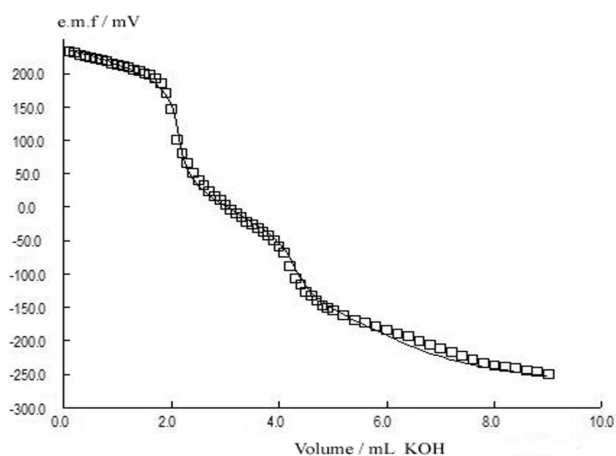
For LC technique, data pairs of pH and retention factors and the ionic strength over pH range of 2.5-4.5 were used. The k values were determined from three separate injections at every mobile phase composition at each pH considered. Relative Standard deviations were lower than 2%. CTC, DC and MTC could not be eluted from column in 10% (v/v) MeCN-water mixture. Plots of the retention factor (k) values *versus* pH of the mobile phases are presented in Figure 4 for tetracyclines in 30% (v/v) MeCN.

Typical sigmoidal curves were obtained, showing the dependence of the analyte capacity factors upon the pH of the mobile phase. In general, the retention of the investigated analytes decreased with increasing of the pH. The observed retention factors *versus* pH of the mobile phases were fitted to the following equation (Boltzman

**Table 1.** Dissociation constants of tetracyclines obtained from the literature, SPARC program, potentiometric and LC methods in water, 10%, 20% and 30% (v/v) MeCN-water binary mixtures

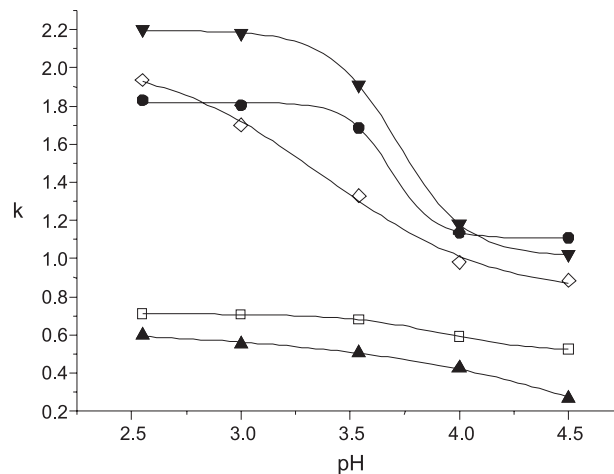
Compounds	SPARC	Lit.	Potentiometry			LC			LC-DAD		
			Water	10% (v/v)	20% (v/v)	10% (v/v)	20% (v/v)	30% (v/v)	10% (v/v)	20% (v/v)	30% (v/v)
TC	pK <sub>a1</sub> pK <sub>a2</sub> pK <sub>a3</sub>	3.32(0.30) <sup>a</sup>									
		7.78(0.05) <sup>a</sup>									
		9.58(0.30) <sup>a</sup>									
	3.01	3.30 <sup>b</sup>	3.35(0.03)*	3.47(0.02)	3.62 (0.07)	3.77(0.23)	3.98(0.09)	4.15(0.21)	3.09(0.05)	3.69(0.02)	4.00(0.03)
	5.79	7.70 <sup>b</sup>	7.29(0.03)	7.24(0.03)	7.28 (0.07)	---	---	---	---	---	---
	8.55	9.50 <sup>b</sup>	9.88(0.03)	10.00(0.09)	10.25 (0.08)	---	---	---	---	---	---
CTC	pK <sub>a1</sub> pK <sub>a2</sub> pK <sub>a3</sub>	3.36(0.04) <sup>c</sup>									
		7.77(0.02) <sup>c</sup>									
		9.27(0.02) <sup>c</sup>									
	3.95	7.55(0.02) <sup>a</sup>	3.25(0.02)	3.30(0.05)	3.34(0.04)	---	3.89(0.28)	4.07(0.32)	---	3.71(0.03)	3.90(0.01)
	5.59	9.33(0.30) <sup>a</sup>	6.72(0.02)	6.74(0.01)	6.67(0.01)	---	---	---	---	---	---
	7.94	3.30 <sup>b</sup>	8.84(0.06)	9.19(0.05)	9.33 (0.05)	---	---	---	---	---	---
OTC	pK <sub>a1</sub> pK <sub>a2</sub> pK <sub>a3</sub>	7.40 <sup>b</sup>									
		9.30 <sup>b</sup>									
		3.22(0.30) <sup>a</sup>									
	3.79	7.46(0.03) <sup>a</sup>	3.53(0.01)	3.62(0.03)	3.71(0.04)	3.53 (0.06)	3.99(0.01)	4.15(0.30)	3.36(0.04)	3.46(0.02)	3.90(0.03)
	5.51	8.94(0.30) <sup>a</sup>	7.25(0.03)	7.19(0.02)	7.20(0.04)	---	---	---	---	---	---
	8.46	3.30 <sup>b</sup>	9.58(0.07)	9.73(0.06)	9.83 (0.05)	---	---	---	---	---	---
DC	pK <sub>a1</sub> pK <sub>a2</sub> pK <sub>a3</sub>	7.30 <sup>b</sup>									
		9.10 <sup>b</sup>									
		3.02(0.30) <sup>a</sup>									
	3.58	3.02(0.30) <sup>a</sup>	3.50(0.04)	3.62(0.08)	3.71 (0.08)	---	4.05(0.01)	4.08(0.14)	---	3.57(0.04)	3.82(0.02)
	5.74	7.97(0.15) <sup>a</sup>	7.07(0.06)	7.20(0.05)	7.43 (0.05)	---	---	---	---	---	---
	9.67	9.15(0.07) <sup>a</sup>	9.13(0.09)	9.10(0.05)	9.38(0.02)	---	---	---	---	---	---
MTC	pK <sub>a1</sub>	3.55	---	---	---	---	3.80(0.09)	4.04(0.18)	---	3.43(0.04)	3.92(0.02)
	pK <sub>a2</sub>	5.68	---	---	---	---	---	---	---	---	---
	pK <sub>a3</sub>	9.53	---	---	---	---	---	---	---	---	---

\*Parenthesis are standard deviations; <sup>a</sup>pK<sub>a</sub> values are obtained in water from Reference 3 by potentiometric method. <sup>b</sup>pK<sub>a</sub> values are obtained in water from Reference 26 by multiwavelength spectrophotometry. <sup>c</sup>pK<sub>a</sub> values are obtained in water from Reference 24 by potentiometric method.



**Figure 3.** Plot of potentiometric titration of  $2.74 \times 10^{-3} \text{ mol L}^{-1}$  tetracycline (TC) in water with  $0.025 \text{ mol L}^{-1}$  KOH. Solid lines indicate the theoretical values calculated by PKPOT program.

sigmoid  $Y = \text{bottom} + (\text{top} - \text{bottom}) / [1 + \exp(V_{50} - X) / \text{slope}]$ , using a non-linear least square program (Origin 7.0). Computer generated plots of  $k$  versus pH were obtained and the pH value at the inflection point ( $V_{50}$ ) was taken as valuable index of pK<sub>a1</sub> (Table 1).

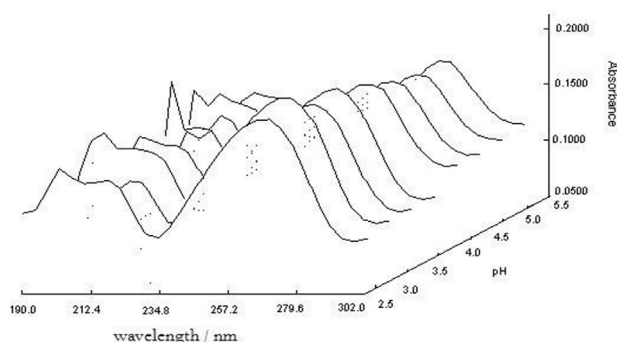


**Figure 4.** Fitted sigmoidal equation of studied compounds in 30% (v/v) MeCN-water binary mixture, A)  $\blacktriangledown$ : doxycycline,  $\diamond$ : metacycline,  $\bullet$ : chlortetracycline,  $\square$ : tetracycline,  $\blacktriangle$ : oxytetracycline.

The pK<sub>a</sub> values were calculated using a non-linear least-square fit of the data using the program NLREG<sup>35</sup> and are shown in Table 1. This is a general purpose program, where the function to be minimized and the parameters to be estimated can be defined by means of the built-in program

editor. In our case, the input data include the experimental retention factors ( $k_{\text{exp}}$ ), the measured pH and the calculated activity coefficients, whereas the dissociation constants and the retention factors of the neutral and anionic species are treated as parameters to be optimized. Starting values for these parameters must also be included. The ionic strength was determined from the amount of sodium hydroxide added to obtain the desired pH of the mobile phase, and from the dissociation constant of the buffer in the more acidic solutions (corresponding to formic acid buffers).

The LC-DAD technique does not use retention factors; instead of them it uses the spectra measured by the diode-array detector, but the procedure will be affected by the errors in the pH measurement. The comparison between  $\text{pK}_a$  values obtained from retention and absorbance allows a better understanding of dissociation equilibria to be reached, with the additional advantage that both kinds of data can be obtained in the same experimental run. The spectral data obtained from STAR program corresponding to the  $\text{pK}_a$  determination of doxycycline in 30% (v/v) MeCN are presented in Figure 5.



**Figure 5.** Plot of experimental absorbance values of doxycycline obtained by LC-DAD method in 30% (v/v) MeCN-water mixture.

The program refines the equilibrium constants, using the Gauss-Newton algorithm, until a minimum value in the sum of the squared differences between the experimental and calculated absorbance values for each spectra and wavelength ( $U_{\text{abs}}$ ) is obtained:

$$U_{\text{abs}} = \sum_{i=1}^{ns} \sum_{j=1}^{nw} (A_{i,j,\text{exp}} - A_{i,j,\text{calc}})^2 \quad (3)$$

where  $ns$  and  $nw$  indicate the number of spectra and wavelengths, respectively.  $A_{i,j,\text{exp}}$  and  $A_{i,j,\text{calc}}$  are the experimental and calculated absorbance values for the wavelength  $j$  in the spectrum  $i$ . The calculated absorbances are obtained in three steps: the program first solves the mass balances for each spectrum according to the guessed equilibrium constants and experimental conditions; then, a multiple linear regression procedure is applied in order

to determine the molar absorbances of each unknown species, and finally the individual absorbance values are re-calculated from the guessed species concentration and the corresponding molar absorbances.

Among these techniques, the potentiometric method is a high precision technique to determine  $\text{pK}_a$  values of substances. It is commonly used due to its accuracy and the commercial availability of fast, automated instruments. However, its shortcomings include the requirements to use a milligram of pure compounds and a mixture of aqueous buffers. It needs a minimum solubility of the compound studied (about  $10^{-3}$  mol  $\text{L}^{-1}$ ). This is due to the fact that the electrode system is calibrated before each potentiometric titration,<sup>33</sup> and the pH electrode remains into the solution during all the titration. Because of that the calibration parameters are the same for all titration data corresponding to the same experimental run. To avoid errors, especially for measurements at neutral-to-high pH, carbonate-free solutions must be prepared laboriously.

LC is used as a powerful technique for the determination of dissociation constants, as it requires only a small quantity of compounds, studied samples do not need to be pure and poor water solubility is not a serious drawback. This method does not include measuring solute or titrant concentrations, just retention times. Also, calculation is straightforward and independent of solute purity. However, the standard deviations of  $\text{pK}_a$  values are higher than those obtained by potentiometric and LC-DAD methods. One of the most important disadvantages of the LC method is that the pH of the mobile phase and, therefore, the range of  $\text{pK}_a$  values that can be determined are limited by the stability of the column package. Moreover, due to the large retention times observed, it is not easy to determine  $\text{pK}_a$  values in water and aqueous-organic mixtures with low contents of organic solvent. Nevertheless, as the main objective of  $\text{pK}_a$  determination by LC is the optimization of chromatographic separations, this method is perfectly adequate for this purpose.

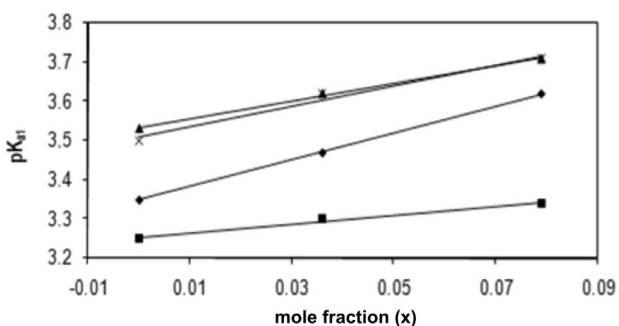
In recent years, a new procedure has been developed when LC and CE methodologies are used for  $\text{pK}_a$  determination in combination with a diode-array detector (DAD) for absorbance measurements. In these cases,  $\text{pK}_a$  values can be determined from the absorbance spectra obtained at the maxima of chromatographic or electrophoretic peaks. The advantages of these proposed methods lie in the fact that the application of both methods does not increase the analysis time and enables the confirmation of results.

By comparison in LC methodology, the pH of the mobile phase is measured against the pH of a standard; the drawback of this procedure is the possibility of small changes in the electrode response every time it is immersed in a different solution. Moreover, small errors in the

preparation of the standard will be noted as systematic deviations. Additionally, the retention factors (*k*) used to estimate the  $pK_a$  are derived from the retention time of the solute and the hold-up time, giving lower precision.

A further comparison between the  $pK_a$  values obtained in this work in aqueous medium and those given in the literature and predicted by SPARC shows that the values corresponding to the first  $pK_a$  are generally in good agreement. The remarkably good results obtained by SPARC should be mentioned, because, except in the case of CTC, predicted values for tricarbonyl system ( $pK_{a1}$ ) are within 0.3  $pK_a$  units from real experimental values. Results are not so good for the dissociation constants of the phenolic diketone system ( $pK_{a2}$ ), as the differences between the results obtained in this work and predicted by SPARC are higher but those available in the literature are similar, particularly for  $pK_{a3}$ , in which case they may exceed 0.5  $pK_a$  unit.

The relationships between  $pK_{a1}$  values obtained and mole fractions are shown in Figure 6. The equations between  $pK_{a1}$  values and mole fraction, of organic modifier are shown in Table 2.



**Figure 6.**  $pK_{a1}$  values of TCs versus mole fractions of MeCN. Symbols: ◆: tetracycline, ■: chlortetracycline, ×: doxycycline ▲: oxytetracycline.

**Table 2.** The equations between  $pK_{a1}$  values and mole fractions of organic modifier

Compounds	Equation	Regression coefficient
TC	$pK_{a1} = 3.42 (0.04) x + 3.35 (0.01)$	$R = 0.999$
CTC	$pK_{a1} = 1.13 (0.13) x + 3.25 (0.01)$	$R = 0.993$
OTC	$pK_{a1} = 2.27 (0.12) x + 3.53 (0.01)$	$R = 0.999$
DC	$pK_{a1} = 2.64 (0.35) x + 3.51 (0.02)$	$R = 0.991$

\*x represents the mole fraction of organic modifier (MeCN).

The values obtained in this work by potentiometry, LC and LC-DAD for the first  $pK_a$  are in good agreement, but LC values are a bit higher from the others. Table 2 shows that the variation of potentiometric  $pK_{a1}$  values of TCs are increased linearly when the content of MeCN is increased.

$pK_2$  values are nearly constant and for most cases  $pK_3$  values are increased by the increasing of the organic modifier. The change of  $pK_a$  values of TCs in MeCN-water mixtures could be explained by the fact that preferential solvation by water exists in MeCN-water mixtures.<sup>37</sup> Although the variations of the physico-chemical properties of solutes in mixed solvents can be described by the Kamlet-Taft solvatochromic parameters<sup>38</sup> or the Dimroth-Reichardt ET polarity parameter,<sup>39</sup> it has been found that in several water-organic co-solvent mixtures, such as acetonitrile-water<sup>30</sup> (up to 70% acetonitrile),  $pK_a$  values of a given substance show a linear relationship with the mole fraction of organic co-solvent. This is indicated by the following expression:

$$pK_{a,\phi} = pK_{a,w} + \phi \Delta pK \quad (4)$$

where  $pK_{a,w}$  indicates the dissociation constant in water,  $\phi$  the mole fraction of organic cosolvent,  $\Delta pK$  the slope of the linear relationship, and  $pK_{a,\phi}$  the  $pK_a$  at the corresponding composition.

## Conclusions

This paper presents the first study dealing with the determination of  $pK_a$  values of TCs by potentiometry and LC methods in MeCN-water binary mixtures and gives a possibility for deeper analysis of the processes which take place during LC analysis of TCs. The chromatographically determined  $pK_a$  values are the most appropriate for predicting the effect of eluent pH on retention and hence for optimization of separation methods based on especially reversed phase liquid chromatography. Also, the important data extracted from this exploration can be used for other pharmacokinetic, pharmacological or technological studies concerning these compounds.

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## References

1. Poole, C. F.; Poole, S. K.; *Chromatography Today*, Elsevier: Amsterdam, 1991.
2. Babić, S.; Horvat, A. J. M.; Pavlović, D. M.; Kaštelan-Macan, M.; *TrAC, Trends Anal. Chem.* **2007**, *26*, 1043.
3. Qiang, Z.; Adams, C.; *Water Res.* **2004**, *38*, 2874.
4. Fallavena, P. R. B.; Schapoval, E. E. S.; *Int. J. Pharm.* **1997**, *158*, 109.

5. Martínez, V.; Maguregui, M. I.; Jiménez, R. M.; Alonso, R. M.; *J. Pharm. Biomed. Anal.* **2000**, *23*, 459.
6. Wróbel, R.; Chmurzynski, L.; *Anal. Chim. Acta* **2000**, *405*, 303.
7. Ràfols, C.; Rosés, M.; Bosch, E.; *Anal. Chim. Acta* **1997**, *338*, 127.
8. Polster, J.; Lachmann, H.; *Spectrometric Titrations: Analysis of Chemical Equilibria*, VCH Publishers: Weinheim, 1989.
9. Allen, R. I.; Box, K. J.; Comer, J. E. A.; Peake, C.; Tam, K. Y.; *J. Pharm. Biomed. Anal.* **1998**, *17*, 699.
10. Barbosa, J.; Barrón, D.; Jiménez-Lozano, E.; Sanz-Nebot, V.; *Anal. Chim. Acta* **2001**, *437*, 309.
11. Beltrán, J. L.; Şanlı, N.; Fonrodona, G.; Baron, D.; Özkan, G.; Barbosa, J.; *Anal. Chim. Acta* **2003**, *484*, 253.
12. Janos, P.; *J. Chromatogr. A* **2004**, *1037*, 15.
13. Andradi, M.; Buglyo, P.; Zekany, L.; Gaspar, A.; *J. Pharm. Biomed. Anal.* **2007**, *44*, 1040.
14. Foulon, C.; Duhal, N.; Lacroix-Callens, B.; Vaccher, C.; Bonte, J. P.; Goossens, J. F.; *Eur. J. Pharm. Sci.* **2007**, *31*, 165.
15. Nogales, V.; Ruiz, V.; Rosés, M.; Ràfols, C.; Bosch, E.; *J. Chromatogr. A* **2006**, *1123*, 113.
16. Jiménez-Lozano, E.; Marqués, I.; Barrón, D.; Beltrán, J. L.; Barbosa, J.; *Anal. Chim. Acta* **2002**, *464*, 37.
17. Martell, A. E.; Motekaitis, R. J.; *Determination and Use of Stability Constants*, 2<sup>nd</sup> ed., VCH Publishers: New York, 1992.
18. Gans, P.; Sabatini, A.; Vacca, A.; *Talanta* **1996**, *43*, 1739.
19. Erdemgil, F. Z.; Şanlı, S.; Şanlı, N.; Özkan, G.; Barbosa, J.; Guiteras, J.; Beltrán, J. L.; *Talanta* **2007**, *72*, 489.
20. Barbosa, J.; Barrón, D.; Beltrán, J. L.; Sanz-Nebot, V.; *Anal. Chim. Acta* **1995**, *317*, 75.
21. Pérez-Urquiza, M.; Beltrán, J. L.; *J. Chromatogr. A* **2001**, *917*, 331.
22. Jiménez-Lozano, E.; Marqués, I.; Barrón, D.; Beltrán, J. L.; Barbosa, J.; *Anal. Chim. Acta* **2002**, *464*, 37.
23. Aktaş, A. H.; Şanlı, N.; Pekcan, G.; *Acta Chim. Slov.* **2006**, *53*, 214.
24. Hosny, W. M.; El-Medani, S. M.; Shoukry, M. M.; *Talanta* **1999**, *48*, 913.
25. Agrawal, Y. K.; Patel, D. R.; *J. Pharm. Sci.* **1986**, *75*, 190.
26. Pinsuwan, S.; Alvarez-Núñez, A. F.; Tabibi, E. S.; Yalkowsky, S. H.; *J. Pharm. Sci.* **1999**, *181*, 31.
27. Evagelou, V.; Tsantili-Kakoulidou, A.; Koupparis, M.; *J. Pharm. Biomed. Anal.* **2003**, *31*, 1119.
28. <http://libmlc2.chem.uga.edu/sparc/>, accessed in May 2008.
29. Hilal, S. H.; Karicckhoff, S. W.; Carreira, L. A.; *QSAR Comb. Sci.* **2004**, *23*, 709.
30. Barbosa, J.; Beltrán, J. L.; Sanz-Nebot, V.; *Anal. Chim. Acta* **1994**, *288*, 271.
31. Rondinini, S.; Mussini, P. R.; Mussini, T.; *Pure Appl. Chem.* **1987**, *59*, 1549.
32. Mussini, P. R.; Mussini, T.; Rondinini, S.; *Pure Appl. Chem.* **1997**, *69*, 1007.
33. Gran, G.; *Analyst* **1952**, *77*, 661.
34. Serjeant, E. P. In *Potentiometry and Potentiometric Titrations in a Series of Monographs on Analytical Chemistry and Its Applications*; Elving, P. J.; Winefordner J. D., eds.; John Wiley & Sons, Inc.: New York, 1984, vol. 69, ch. 5.
35. <http://www.nlreg.com>, accessed in May 2008.
36. Beltrán, J. L.; Codony, R.; Prat, M. D.; *Anal. Chim. Acta* **1993**, *276*, 441.
37. Barbosa, J.; Barron, D.; Berges, R.; Sanz-Nebot, V.; Toro, I.; *J. Chem. Soc., Faraday Trans.* **1997**, *93*, 1915.
38. Kamlet, M. J.; Abboud, J. L. M.; Taft, R. W.; *Prog. Phys. Org. Chem.* **1981**, *13*, 485.
39. Reichardt, C.; *Solvents and Solvent Effects in Organic Chemistry*, 2<sup>nd</sup> ed., 454 VCH Verlagsgesellschaft: Weinheim, 1988.

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