Technical paper

Spectrophotometric Determination of Acidity Constants of Some Macrolides in Acetonitrile-Water Binary Mixtures

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Received: 07-05-2010

Abstract

The acidity constants of eight macrolides (erythromycin, roxithromycin, oleandomycin, azithromycin, josamycin, tylosin tartrate, tilmicosin and spiramycin) have been determined in acetonitrile-water binary mixtures (30%, 40% and 50% (v/v)) by spectrophotometric method. The pK_a's available in literature determined by various methods are compiled in comparison with the value of this work. These results are expected to essentially facilitate the research on occurrence, fate and effects, analysis method development, and control of antibiotics in various treatment occurrences.

Keywords*:* Macrolides; Acidity constants; Acetonitrile-water mixtures; Spectroscopic method.

1. Introduction

Macrolide antibiotics constitute a very important class of antibacterial compounds which have been widely used in veterinary and medicinal practices to treat a wide range of diseases. Macrolides are characterized by a macrocyclic lactone ring containing 14, 15 or 16 atoms with sugars linked via glycosidic bonds. The macrolides with 16 atoms in the lactone ring represent the most commonly used macrolides in veterinary medicine and examples of these include tylosin, josamycin and spiramycin. Tilmicosin, is a derivative of desmycosin (16-membered-ring macrolide). Erythromycin, roxithromycin and oleandomycin are another example of a macrolide antibiotic; they contain 14 atoms. Azithromycin is one of the world's best-selling antibiotics. It is derived from erythromycin, but it differs chemically from erythromycin in that a methyl-substituted nitrogen atom is incorporated into the lactone ring, thus making the lactone ring 15-membered. Like erythromycin, it appears to bind to the same receptor, 50 S ribosomal subunits of susceptible bacteria and suppresses protein synthesis. A macrolide contains basic dimethylamine [–N(CH_3)$_2$] group which is able to gain a proton.[1]

Acid dissociation constant (pK_a value) is an important parameter to estimate the extent of ionization of drug molecules at different pH values, which is of fundamental importance in the consideration of their interaction with biological membranes.[2,3] Many drug compounds are sparingly soluble in water and a precise determination of their pK_a values poses a challenging problem for potentiometric titration since the accuracy of this method is restricted by its detection limit of about 10^{-4} M. Spectroscopic titration has been utilized as an alternative to determine pK_a values of substances with large molar absorptivities because of its high sensitivity at concentrations of substance as low as 10^{-6} M.[4] However, the compound under investigation must possess chromophore(s) in proximity to the ionization center(s) so that the protonated and deprotonated species exhibit sufficient spectral dissimilarity.

There are some limitations in determining the acidity constants of the molecules such as low solubility in aqueous solutions and the low values of acidity constants. Therefore, to enhance the acidity constants on one hand and to increase the solubility on the other, we forced to choose mixed solvents.[5,6] In addition, the pK_a of the analytes in the mixed solvent is an important parameter for the optimization of separation techniques such as capillary

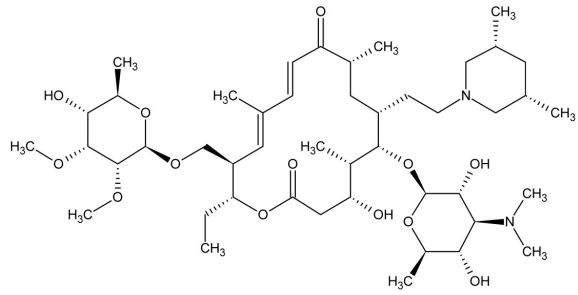
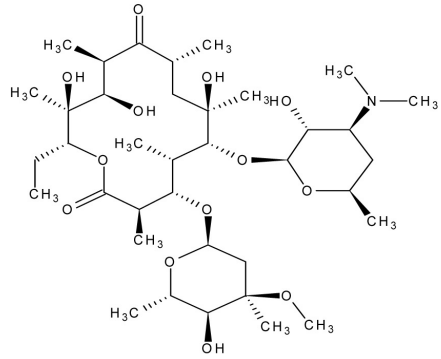
electrophoresis and liquid chromatography of ionisable compounds. An understanding of the factors that determine the acidity or basicity of a solute solved in solvent mixtures, can be decisive to foresee how the aqueous pK_a is modified when the organic modifier is added to the aqueous part of the eluent for separation methods and in order to establish relationships allowing to relate an aqueous pK_a with the pK_a in the organic modifier-water binary mixtures. Moreover, the study of acid-base behavior of analytes in binary organic-water solvent systems could be key in predicting influence of pH on retention and selectivity in LC.[7,8]

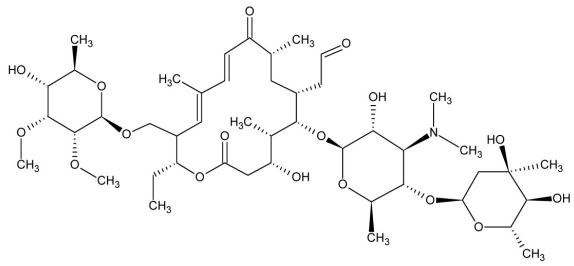
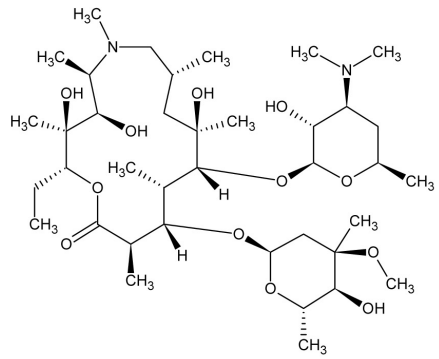
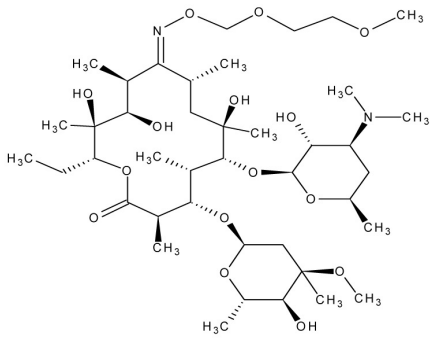
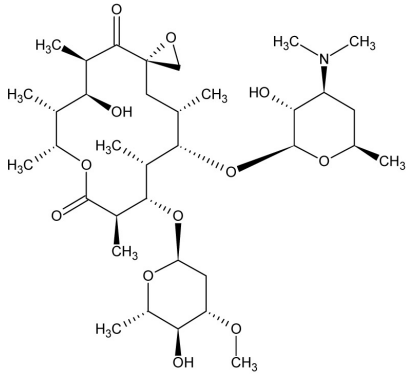
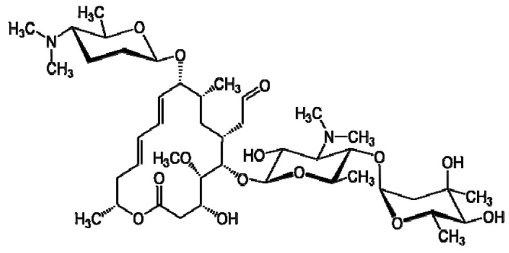
Acetonitrile (MeCN) and its mixtures with water are widely used in separation techniques, due to the excellent characteristics of the pure solvent. MeCN is a very weak base and a very weak acid and therefore it is a good differentiating solvent for both acids and bases. Furthermore, it has low viscosity and ideal good UV transparency. It has a relatively high dielectric constant (ε = 36) and a small autoprotolysis constant (pK_s = 33.6). Although MeCN-water mobile phases have been used in RP-LC separation procedures, the pK_a values of macrolides have not been determined in binary mixtures. Accordingly, MeCN acts as a strongly differentiating solvent with a modest solvating power for many polar ionic solutes.[9]

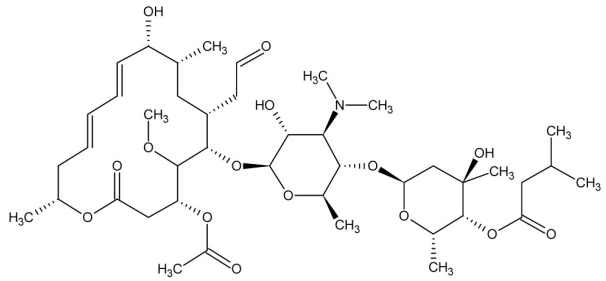
There are lots of pK_a determination techniques for compounds of pharmaceutical or biological interest such as potentiometric titrations,[1,10] UV-Vis,[9,11–13] LC,[10,11,14,15] and CE methodology.[16–17] Among these techniques, the potentiometric titration is a more general method and it does not require 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 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.[14,18] 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.[11,19,20]

In this study, pK_a values of eight macrolides (erythromycin, roxithromycin, spiramycin, oleandomycin, azithromcin, tylosin tartrate, josamycin, tilmicosin; Table1) were determined in 30%, 40%, 50% (v/v) MeCN-water binary mixtures by UV-spectroscopy method. The obtained pK_a values have been compared with those predicted SPARC on-line calculator.[21,22] This program estimates several physico-chemical properties for organic compounds on the basis of their molecular structure.

Table 1. Chemical structure of studied compounds

Compounds name	Structure
Tilmicosin	
Erythromycin	

Compounds name	Structure
Tylosin	
Azitromycin	
Roxithromycin	
Oleandomycin	
Spiramycin	

Compounds name	Structure
Josamycin	

2. Experimental

2. 1. Chemical and Reagents

Analytical reagent grade chemicals were used, unless otherwise indicated. Erythromycin, roxithromycin, tilmicosin, oleandomycin, josamycin, spiramycin, tylosin tartrate, azithromycin (Sigma), acetonitrile (HPLC grade) were used without further purification. Stock solutions of macrolides were prepared in MeCN. Potassium hydroxide (Titrisol), hydrochloride acid (Titrisol), potassium hydrogen phthalate and potassium chloride (ionic strength adjuster; 0.1 mol L^{-1}) were supplied from Merck. All stock solutions of hydrochloride acid, potassium chloride and potassium hydrogen phthalate were prepared by water. Water, with conductivity lower than 0.05 μScm^{-1} was obtained with a Milli-Q water purification system (Milli Pore Corp.).

2. 2. Apparatus

Potentiometric measurements were performed with Mettler-Toledo MA 235 pH/ion (resolution ±0.1 mV) analyzer system. All titrations were carried out under N_2 and at 25.0 ± 0.1 °C, which was maintained by circulating water from a constant-temperature thermostat (Heto CBN 8–30 and temperature control unit Heto HMT 200) through the double-wall *Pyrex* titration cell of 80-mL capacity.

The UV-Vis absorbance spectra were recorded at each pH using Perkin Elmer LAMBDA 25 spectrophotometer, equipped with 1 cm path length cell, controlled by personal computer. A peristaltic pump equipped with the spectrometer was used to circulate the solution from the titration vessel to the spectrophotometer cell, and vice versa, through Teflon or Tygon tubes in a closed loop circuit with continuous flow.

2. 3. Procedure

Before the spectrometric titration, carbonate-free potassium hydroxide solutions were prepared under a nitrogen atmosphere. The ionic strength of KOH solution was adjusted to 0.10 mol L^{-1} by the addition of KCl. The alkali titre and absence of carbonate were periodically checked by pH-metry, using the appropriate Gran function against primary standard oven-dried potassium hydrogen phthalate.[23]

The pK_a values of the macrolides were determined by means of the data obtained from spectrometric titrations in 30%, 40% and 50% (v/v) acetonitrile–water mixtures at 25.0 ± 0.1°C and in 0.1 mol L^{-1} ionic strength (KCl). The spectrophotometric multiple-wavelength pH-titration was carried out as follows: in a first step, the standard emf values, E^o, of the potentiometric cell were evaluated from titrations of a measured amount of an acidic solution, at the same conditions of temperature, ionic strength and solvent composition to be used in later experiments using KOH solutions in the same solvent and ionic strength as the titrant, and checking the calibration parameters from the Gran plots.[23,24] The standard emf of the cell, E^o, is the average of at least 5 standardizations. The standardization of the electrode system was carried out, each time solvent media was changed and the constancy of E^o, values ensured by continual surveillance by means of periodic calibrations.

In a second step, a solution of fully protonated macrolides (50.0 mL containing 5.10^{-5} mol L^{-1} drug) by HCl at the required conditions of temperature, ionic strength and solvent composition were titrated using KOH solutions in the same solvent and ionic strength in the pH range of 2.5–11.0. After each addition, the potential was allowed to stabilize, its value used, in combination with E^o calculated in calibration step, to calculate the pH solution.

In the UV-Vis spectrometric titrations, the test solution was pumped to a spectrometric flow-cell by means of a peristaltic pump. After each addition of titrant, and after waiting for the emf reading to be stable, a spectrum, UV-Vis spectra were recorded with 1 nm resolution at 190–350 nm intervals in order to obtain different spectra around the maximum λ for each macrolides.

2. 4. Data Treatment

Spectrometric titrations data were processed using the program STAR (Stability Constants by Absorbance Readings) which calculates stability constant and molar absorptivities of the pure species by multilinear regres-

UV-Vis spectra and sigmoid curve of pH-absorbance graphic of erythromycin in 50% MeCN-water mixtu-

sion.[25] The program STAR requires a previous model of the chemical equilibria, based upon the existence of certain chemical species, to be postulated. The refinement of equilibrium constants is done using the Gauss-Newton non-linear least squares algorithm by numerical differentiation, until a minimum in the sum of the squares residual (U) is attained. Wavelength (U_{abs}) is obtained:

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

where ns and nw indicate the number of spectra and wavelengths, respectively. $A_{i,j,exp}$ and $A_{i,j,calc}$ 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. The optimization is performed by means of a non-linear least-squares procedure. The minimization process is repeated until the relative change of U between two iterations is ≤ 0.01%.[26]

3. Results and Discussion

Macrolide antibiotics contain a basic dimethylamine [–N(CH_3)$_2$] group (Table. 1), which is able to gain a proton, so, according to their chemical structure, macrolides have usually one pK_a value around 9.[1]

The pK_a value of macrolides are either not known accurately or not available at all. Only limited number of study related with pK_a values of studied macrolides are found in the literature. However some data in literature differ considerably, number of pK_a values and the methods use for their determination are not adequately describe. Table 2 summarizes the literature data and SPARC, available on the pK_a's of the some macrolides.[1,27–35] The Merck Index reports a pK_a of 8.8 for erythromycin, but there is no indication of the method, solvent system, temperature, or ionic strength employed.[27] These parameters are critical because they influence the resulting values. Thus, the pK_a values in Table 2 agree in some instances with values reported here, but in other cases are quite different because of the different methods and different solvents employed. In the present work, we have used a single method, temperature, ionic strength and solvent system, so our data are self consistent and suitable for our purpose.

UV-Vis spectra and sigmoid curve of pH-absorbance graphic of erythromycin in 50% MeCN-water mixtures at different pH values were given in Fig. 1 and Fig. 2, respectively. The dissociation constant values determined by STAR program, for the erythromycin, roxithromycin, tilmicosin, oleandomycin, josamycin, spiramycin studied in 30%, 40%, 50% (v\v) MeCN-water mixtures at 25.0 ± 0.1 °C are collected in Table 3, together with respective standard deviations. These compounds have limited water solubility and need to be dissolved in a co-solvent. Erythromycin, roxithromycin, oleandomycin, josamycin contain only one amine group.

Table 2. Literature and SPARC values for the eight macrolides' pK_a considered in the article.

Macrolidep	K_a	Solvent	Method	Temp.	SPARC
Erythromycin	8.90[1]	aqueous	potentiometric	ambient	7.24
	8.80[27]	not given	not given	not given	
	8.60–8.9[28]	aqueous	titration	not given	
	9.10[28]	90% aqueous D_2O	NMR	30 ^{0}C	
	8.80[29]	D_2O	NMR	not given	
	8.60[30]	50% aqueous DMF	not given	not given	
	8.88[32]	65% aqueous MeCN	potentiometric	25 °C	
Roxithromycin	9.17[1]	Aqueous	potentiometric	ambient	6.64
Azithromycin	8.85[28]	Aqueous	not given	not given	7.44
	8.74–9.45[32]	65% aqueous MeCN	potentiometric	25 °C	
Oleandomycin	8.50[31]	50% aqueous ethanol	not given	not given	7.20
	8.84[32]	65% aqueous MeCN	potentiometric	25 °C	
Tylosin Tartrate	3.31; 7.50[1]	Aqueous	potentiometric	ambient	7.09
	7.73[32]	65% aqueous MeCN	potentiometric	25 °C	
Spiramycin	7.90[33]	not given	not given	not given	7.03 8.30
Tilmicosin	8.18–9.56[32]	65% aqueous MeCN	potentiometric	25 °C	6.47 8.64
Josamycin	7.10[34]	50% aqueous ethanol	not given	not given	6.98
	7.30[35]	50% aqueous MeOH	voltammetric	90 °C	

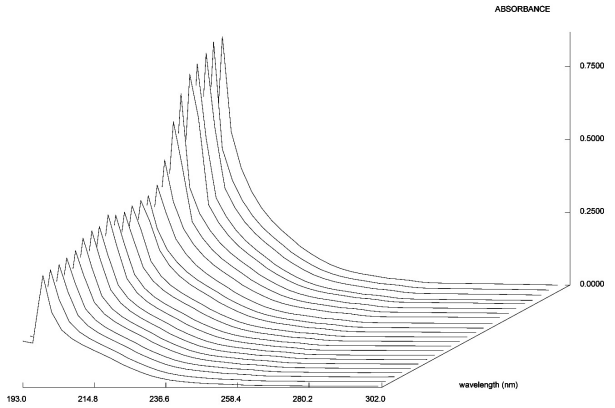


Fig. 1. The wavelength (nm)-absorbance graphic for erythromycin in 50% MeCN-water binary mixture.

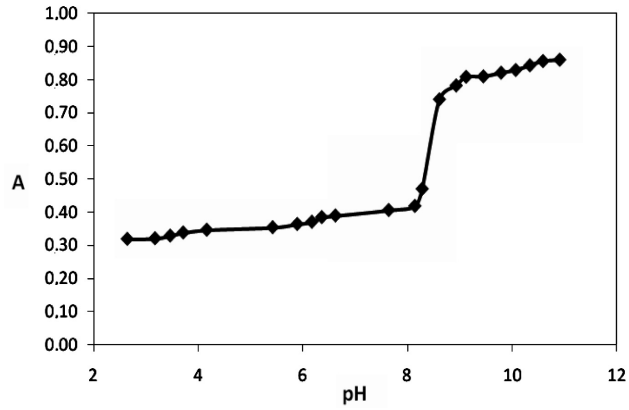


Fig. 2. Sigmoid curve of pH-A graphic for erythromycin in 50% (v/v) MeCN-water binary mixture at 202 nm.

The amine group is able to gain proton under specific pH conditions. Azithromycin, tilmicosin and spiramycin have two nitrogen and they have two pK_a values. But for azithromycin, just one pK_a could be determined. Tylosin is produced as a tylosin tartrate. Tartaric acid has two pK_a values, i.e., 3.03 and 4.37.[4] Thus, the pK_1 of tylosin tartrate may correspond to the tartrate moiety. The pK_2 of tylosin tartrate is believed to correspond to the dimethylamine group.

It is known that one of the most important factors determining the equilibrium constants is the reaction medium. The variation of the pK_a values of macrolides versus the mole fraction of MeCN, X_{MeCN}, in MeCN-water mixtures is presented in Fig. 3. The variation of pK_a values with the mole fraction of MeCN is different for each substance although, in general, pK_a values decrease with the mole fraction of MeCN. The different ways in which pK values change might be explained by the fact

Table 3. The pK_a values determined in this study at 0.10 mol L^{-1} ionic strength and 25 °C.

Compounds	30 % (v/v) MeCN		40 % (v/v) MeCN		50 % (v/v) MeCN	
	pK_1	pK_2	pK_1	pK_2	pK_1	pK_2
Erythromycin	8.62[δ](0.03)*	–	8.54(0.02)	–	8.45(0.03)	–
Roxithromycin	8.55(0.04)	–	8.48(0.08)	–	8.40(0.05)	–
Oleandomycin	8.65(0.03)	–	8.56(0.07)	–	8.46(0.08)	–
Josamycin	7.21(0.01)	–	7.08(0.03)	–	6.94 0.04)	–
Spiramycin	7.26(0.10)	8.39(0.09)	7.13(0.08)	8.20(0.06)	7.02(0.07)	8.00(0.07)
Azithromycin	8.38(0.08)	–	8.31(0.06)	–	8.21(0.07)	–
Tylosin tartrate	3.41(0.09)	7.74(0.06)	3.51(0.09)	7.67(0.07)	3.63(0.09)	7.61(0.06)
Tilmicosin	6.25(0.09)	8.33(0.07)	6.22(0.09)	8.20(0.10)	6.19(0.08)	8.08(0.07)

[δ] The experiments are repeated at least three times
[*] The values between parentheses are the standard deviations

Table 4. The linear equation between experimental pK_a values and the mole fraction of the organic modifier

Compounds	Equation	Regression Coefficient
Erythromycin	$y = -1.30\ x^a\ (0.06)^* + 8.79\ (0.01)$	R = 0.999
Roxithromycin	$y = -1.15\ x\ (0.05) + 8.70\ (0.01)$	R = 0.999
Oleandomycin	$y = -1.46\ x\ (0.07) + 8.84\ (0.01)$	R = 0.999
Josamycin	$y = -2.07\ x\ (0.12) + 7.47\ (0.02)$	R = 0.998
Spiramycin (pK_2)	$y = -2.99\ x\ (0.19) + 8.77\ (0.04)$	R = 0.998
Azithromycin	$y = -1.31\ x\ (0.03) + 8.55\ (0.01)$	R = 0.999
Tylosin tartrate (pK_2)	$y = -0.99\ x\ (0.12) + 7.86\ (0.03)$	R = 0.992
Tilmicosin (pK_2)	$y = -1.91\ x\ (0.20) + 8.57\ (0.04)$	R = 0.995

[a] represents the mole fraction of organic modifier (MeCN).
[*] The values between parentheses are the standard deviations

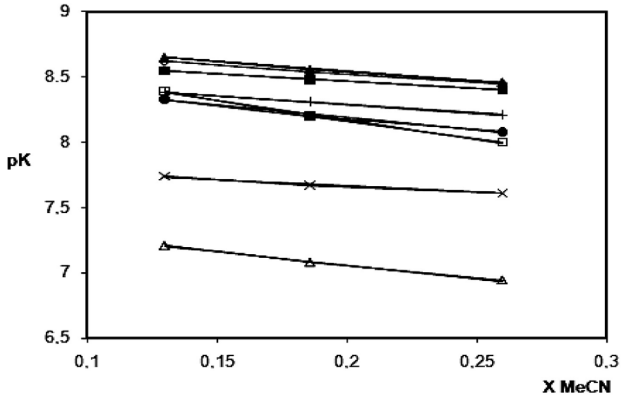


Fig. 3. pK_a values versus mole fraction of MeCN. (□) Spiramycin (pK_2); (△)Josamycin; (●) Tilmicosin (pK_2), (◇)Erythromycin, (▲)Oleandomycin; (■)Roxithromycin; (+)Azithromycin; (x)Tylosin tartrate (pK_2).

that the dissociation process is ruled by electrostatic interaction as well as by specific solute-solvent interactions.

The linear relationships between experimental pK_a values for these analytes and the mole fraction of MeCN are given in Table 4.

4. Conclusions

This paper presents the first study dealing with the determination of pK_a values of macrolides by spectroscopic methods in MeCN-water binary mixtures and gives a possibility for deeper analysis of the processes which take place during LC analysis of macrolides. The determined pK_a values are 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.

5. Acknowledgment

We gratefully acknowledge to Dr. Jose Luis Beltran from University of Barcelona for supporting the program STAR.

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Povzetek

V treh binarnih mešanicah vode in acetonitrila (30 %, 40 % and 50 % (v/v) smo s spektroskopsko metodo določili konstante kislin osmim makrolidom (eritromicin, roksitromicin, oleandomicin, azitromicin, josamicin, tilosin tartrat, tilmocisin, spiramicin).
Določene vrednosti p*K*a se dobro ujemajo s podatki, dostopnimi v literaturi, dobljenimi z različnimi metodami.