Determination of pK_a values of active pharmaceutical ingredients

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The acid dissociation constant is an important physicochemical parameter of a substance, and knowledge of it is of fundamental importance in a wide range of applications and research areas. We present a critical review of published methods and approaches for the determination of acid dissociation constants, with a special emphasis on the pK_a values of active pharmaceutical ingredients.

Keywords: Active pharmaceutical ingredient; Capillary electrophoresis; Acid dissociation constant; Liquid chromatography; Potentiometry; Spectrophotometry

Abbreviations: μ, Electrophoretic mobility; API, Active pharmaceutical ingredient; CE, Capillary electrophoresis; LC, Liquid chromatography; *k*, Retention factor; K_a, Acid dissociation constant; pK_a, Negative decadic logarithm of acid dissociation constant; NMR, Nuclear magnetic resonance; UV-VIS, Ultraviolet-visible spectrometry

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1. Introduction

Active pharmaceutical ingredients (APIs) are among the so-called ''emerging'' contaminants that have caused great concern in recent years. They are continually being introduced in the environment mainly as a result of manufacturing processes, or disposal of unused or expired products and excreta. Many of these substances or their bioactive metabolites end up in waters, soils and sediments, where they can accumulate and induce adverse effects in terrestrial or aquatic organisms [1,2].

Relevant processes regarding pharmaceuticals in the environment include sorption to soils and sediments, complexation with metals and organics, chemical oxidation with natural or water-treatment oxidants, photolysis, volatilization, biodegradation, purification, and analytical isolation. Information on the physical and chemical properties (e.g., the octanol/water partition coefficient, dissociation constants, vapor pressure or Henry's Law constant of an active pharmaceutical ingredient) may help to determine whether a compound is likely to concentrate in the aquatic, terrestrial, or atmospheric environment.

Since most APIs have acidic and/or basic functionalities, their ionization state is controlled by both solution pH and acidic dissociation constants (i.e. K_a values). These different chemical species (cationic, neutral, or anionic) often have vastly different properties with respect to water solubility, volatility, UV absorption, and reactivity with chemical oxidants. The ionized form is usually more water soluble, while the neutral form is more lipophilic and has higher membrane permeability. The extent of ionization is one of several cardinal properties used to estimate the absorption, distribution, metabolism and excretion of compounds in biological systems and the environment. From dissociation constants, the major species of pharmaceuticals present in the environment (usually in neutral pH range) can be estimated [3,4].

Consequently, it is very important to know dissociation constants for environmentally relevant APIs in order to estimate their occurrence, fate and effects.

Knowledge of pK_a values as a function of solvent composition is also useful in applying liquid chromatography (LC) or capillary electrophoresis (CE) for the separation of ionizable compounds. The chromatographic retention and electrophoretic behavior of ionizable compounds

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strongly depend on the pK_a of the compound and the mobile-phase pH [5,6]. Satisfactory knowledge of the acid–base behavior of substances in hydro-organic media is therefore essential to optimize analytical procedures for the separation of ionizable compounds by LC [7,8] and CE [9].

Moreover, the acid–base property of a drug molecule is the key parameter for drug development because it governs solubility, absorption, distribution, metabolism and elimination. Particularly for developing new APIs, the pK_a has become of great importance because the transport of drugs into cells and across other membranes is a function of physicochemical properties, and of the pK_a of the drugs [10].

There are several methods for the determination of dissociation constants. Traditionally, potentiometry [3,11] and UV–VIS absorption spectrometry [5] have been the most useful techniques for the determination of equilibrium constants, due to their accuracy and reproducibility. In the past decade, some alternative techniques for dissociation-constant determination, based on separation methods (e.g., LC [12] and CE [4]) have been developed. Chemical interactions (e.g., acid–base, complex-forming, ion association and other equilibria) are widely exploited to improve separation efficiency and selectivity in LC as well as in electrophoresis. However, these techniques can be used advantageously to study chemical equilibria affecting separations. If an equilibrium is sufficiently fast in comparison with the separation process, then retention characteristics in chromatography (retention factors) or migration characteristics in electrophoresis (effective mobilities) may be expressed as functions of the composition of the mobile phase or background electrolyte, respectively. Using a proper experimental arrangement, the dependencies of retention (migration) characteristics on the mobile phase (background electrolyte) composition can be measured and utilized to calculate equilibrium constants for equilibriums taking place in the mobile phase (background electrolyte) [12].

In most of these methods, a physical property of an analyte is measured as a function of the pH of a solution and resulting data are used for the determination of dissociation constants.

Moreover, pK_a values can also be predicted by computational methods on the basis of molecular structure.

2. pK_a determination

2.1. Potentiometric titration

In the past, potentiometric titration was the standard method for pK_a measurement. In a potentiometric titration, a sample is titrated with acid or base using a pH electrode to monitor the course of titration. The pK_a value is calculated from the change in shape of the titration curve compared with that of blank titration without a sample present. Analysis methods commonly used to derive pK_as from titration curves include Gran's plot [10], second-derivative (Δ^2pH/ΔV^2) [3], and least-squares non-linear regression [3,5].

Potentiometric titration is a high-precision technique for determining the pK_a values of substances. It is commonly used due 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 [10]. Solutions of at least 10^{-4} M are required in order to detect a significant change in shape of the titration curve. To avoid errors, especially for measurements at neutral-to-high pH, carbonate-free solutions must be prepared laboriously [3,5].

2.2. Spectrophotometric methods

An alternative to potentiometric titration is UV–VIS spectrophotometry because it can handle compounds with lower solubility and lower sample concentrations. The main advantage is higher sensitivity (>10^{-6} M) to compounds with favourable molar absorption coefficients. However, in such a case, a compound must contain a UV-active chromophore close enough to the site of the acid–base function in the molecule. Spectral data are recorded continuously during the course of titration by a diode-array spectrometer. The absorption spectra of the sample changes during the course of the titration to reflect the concentration of neutral and ionized species present. The largest change in absorbance occurs at the pH corresponding to a pK_a value. These changes are usually identified from the first derivative of the absorbance against time plot or from overlay plots of the different spectra. The determination of pK_a values by UV–VIS assumes that the solute of interest is pure or that its impurities do not absorb in the UV–VIS range, since the spectra of impurities can overlap with those corresponding to the solutes of interest [5,7,8,10,13].

Spectrophotometric methods offer excellent precision, as in potentiometry, but they require different spectra for different species and reagents must be pure.

Traditionally, spectral data at a single analytical wavelength are acquired from a sample in a series of buffer solutions with known pH values, after which the pK_a is determined. To use this method, the absorption spectra of individual species must be characterized beforehand and the molar absorptivities of protonated and deprotonated species are thus required. These measurements are non-trivial if acid-base equilibria comprise more than two ionization steps or if reacting components are not stable within two pH units of the pK_a value, so a multi-wavelength spectrophotometric approach has been developed to determine acid dissociation. Target-factor analysis has been applied to deduce pK_a values from the multi-wavelength UV absorption data recorded at different pH values [9].

2.3. NMR titration

NMR-pH titration is also an excellent technique for determining pK_a values as the protonation of a basic site leads to electronic deshielding effects on the adjacent NMR-active nuclei, so the average chemical shifts of all the measurable NMR-active nuclei, as a function of pH, are expected to reflect the fractional protonation of each basic group of a molecule. The on-line coupling of a potentiometric titrator with an NMR spectrometer results in a powerful hyphenated technique called NMR-controlled titration [10,14].

To our knowledge and according to available references, NMR titrations have not yet been applied to the pK_a determination of APIs.

2.4. Liquid chromatography (LC)

The determination of pK_a values by means of LC is based on the relationships between capacity factors and the pH of the mobile phase. For each compound and for every mobile phase composition and pH considered, retention time values, t_R, are determined. Retention factors are calculated from $k = (t_R - t_M)/t_M$, where t_M is the hold-up time, which is determined for each mobile-phase composition and pH studied. For monoprotic acid, the observed capacity factors at different pH values can be described as a function of the capacity factors of neutral and anionic species (k_{HA} and k_{A^-}) and their corresponding molar fractions (x_{HA} and x_{A^-}) [6]:

$$k = x_{HA}k_{HA} + x_{A^-}k_{A^-} \text{ or more generally: } k = \sum_i x_i k. \quad (1)$$

The molar fractions can be expressed as a function of the dissociation constants and molar concentration of the hydrogen ion or as a function of pH and pK_a values:

$$x_{HA} = \frac{[H^+]}{[H^+] + K_a} \quad \text{or} \quad x_{HA} = \frac{10^{(pK_a - pH)}}{1 + 10^{(pK_a - pH)}} \quad (2)$$

$$x_{A^-} = \frac{K_a}{[H^+] + K_a} \quad \text{or} \quad x_{A^-} = \frac{1}{1 + 10^{(pK_a - pH)}} \quad (3)$$

Substituting the terms x_i, Eq. (1) can be written as:

$$k = \frac{k_{HA} + k_{A^-}\frac{K_a}{[H^+]}}{1 + \frac{K_a}{[H^+]}} \quad \text{or}$$

$$k = \frac{k_{HA} \cdot 10^{-pH} + k_{A-} \cdot 10^{-pK_a}}{10^{-pH} + 10^{-pK_a}} \quad (4)$$

This equation is related to the chromatographic retention of a monoprotic substance and the pH of the mobile phase, and can be used to determine pK_a values and also to predict the chromatographic behavior of substances. pK_a values are determined by performing a non-linear fit to Eq. (4) [6,12,15]. For simplicity, corrections for activities are not shown. Activity coefficients for ions in a dilute electrolyte solution at 25°C can be estimated according to classical Debye–Hückel theory.

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 pK_a values are higher than those obtained by potentiometric or spectrophotometric methods [5].

One of the most important disadvantages of the LC method is that the pH of the mobile phase and, therefore, the range of 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 pK_a values in water and aqueous–organic mixtures with low contents of organic solvent. Nevertheless, as the main objective of pK_a determination by LC is the optimization of chromatographic separations, this method is perfectly adequate for this purpose.

2.5. Capillary electrophoresis (CE)

The determination of pK_a values by CE is based on observation of the effective mobility of an ionizable compound in a series of electrolyte solutions of constant ionic strength and various pHs. In its uncharged state, a solute has zero mobility while its fully ionized state has maximum mobility. Intermediate mobility is therefore a function of dissociation equilibrium, so, for a monovalent weak acid, the effective mobility is given by $\mu_{eff} = x_i \cdot \mu_{ep,i}$, where x_i is the fraction of the monovalent acid present as the anionic form and $\mu_{ep,i}$ is the electrophoretic mobility of an anion. There is no restriction on the number of ionization equilibria involved, and, for the general case, the effective mobility is given by:

$$\mu_{eff} = \sum_i x_i \mu_{ep,i} \quad (5)$$

where x_i is the mole fraction of species i with electrophoretic mobility $\mu_{ep,i}$.

The fraction of acid present as the anionic form is given by Eq. (3). Using this relation, effective mobility is given by:

$$\mu_{eff} = \frac{\mu_{ep} \cdot 10^{-pK_a}}{10^{-pK_a} + 10^{-pH}} \quad (6)$$

pK_a values are obtained by fitting effective mobility as a function of pH to a suitable model for the number of ionizable groups. Effective mobility is calculated from the experimentally determined migration time of an analyte and the electroosmotic flow at different pH values using the following equation:

$$\mu_{eff} = \frac{LL_{eff}}{V}\left(\frac{1}{t_m} - \frac{1}{t_{eo}}\right) \quad (7)$$

Table 1. Dissociation constants of sulfonamides

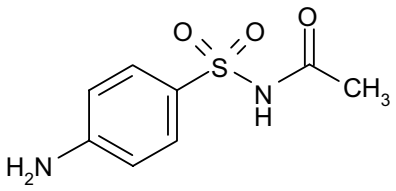
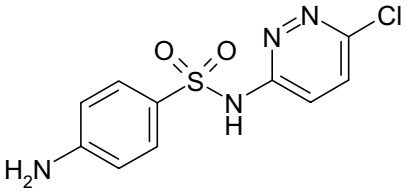
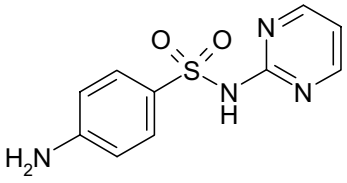
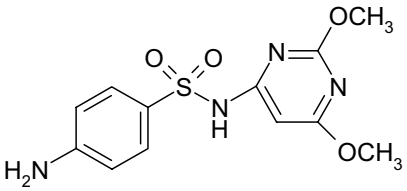
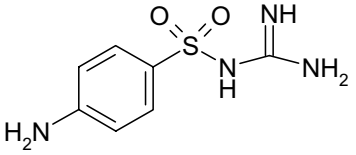
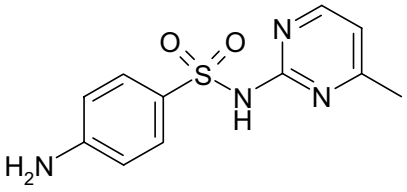
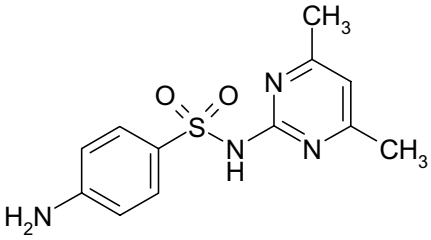
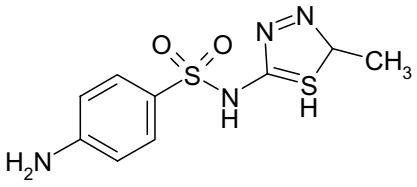
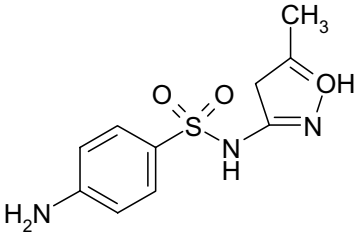
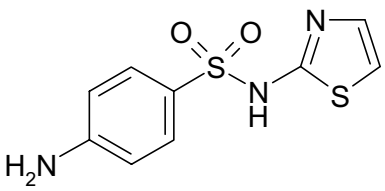
Compound	Chemical structure	pK_a	Method	Ref.
Sulfacetamide		<2.5; 5.27 1.76 ± 0.04; 5.22 ± 0.01 1.95 ± 0.11; 5.30 ± 0.05	CE-MS (positive detection mode) Multi wavelength spectrophotometry UV/pH	[19] [37] [37]
Sulfachloropyridazine		1.87 ± 0.30; 5.45 ± 0.06 1.90; 5.40	Potentiometry CE	[3] [25]
Sulfadiazine		6.43 2.10; 6.28	CE CE	[36] [25]
Sulfadimethoxine		2.13 ± 0.30; 6.08 ± 0.09 1.87; 5.86	Potentiometry CE	[3] [25]
Sulfaguanidine		1.55; 11,24	Calculated (SPARC)	[23]
Sulfamerazine		2.22 ± 0.01; 6.80 ± 0.01 2.17; 6.77 2.06 ± 0.30; 6.90 ± 0.05	UV/pH titration CE Potentiometry	[13] [25] [3]
Sulfamethazine		2.37 ± 0.01; 7.49 ± 0.01 7.65 2.28; 7.42 2.07 ± 0.30; 7.49 ± 0.13	UV/pH titration CE CE Potentiometry	[13] [36] [25] [3]

Table 1 (*continued*)

Compound	Chemical structure	pK_a	Method	Ref.
Sulfamethizole		1.86 ± 0.30; 5.29 ± 0.04	Potentiometry	[3]
Sulfamethoxazole		5.65 1.83; 5.57 1.85 ± 0.30; 5.60 ± 0.04	CE CE Potentiometry	[36] [25] [3]
Sulfathiazole		7.24 2.08; 7.07 2.01 ± 0.30; 7.11 ± 0.09	CE CE Potentiometry	[36] [25] [3]

where L is the total capillary length, L_{eff} is the effective capillary length (to the detector), V is the applied voltage, t_m is the migration time of a solute, and t_{eo} is the migration time of a neutral marker compound that is carried through the column by the electroosmotic flow. Suitable marker compounds for estimating t_{eo} are methanol, mesityl oxide, acetone or dimethyl sulfoxide [8,10,12,16]. The models for weak acids and bases containing up to three ionization centers have been summarized [4].

The effective electrophoretic mobility of weak acids and bases is strongly influenced by environmental factors that affect underlying equilibrium constants (e.g., pH, ionic strength, and temperature) as well as variables that affect ion mobility (e.g., ionic strength, temperature and viscosity) [16]. Electrolyte solutions of a different pH with a low and constant ionic strength as well as effective column thermostatting are therefore required for pK_a measurements. Desirable buffer properties for the measurement of pK_a values by CE include detector compatibility (usually low UV absorbing), reasonable water solubility, acceptable shelf life, and availability in a high-purity form. The most popular buffers for CE are phosphate, acetate, borate and zwitterionic compounds (Good's buffers) [16].

The CE technique is expected to be especially useful if the amount of compound is very limited because, with this technique, only a few nanograms or less are commonly used for analysis. For CE, it is only necessary to determine pH-dependent mobilities, and that has several advantages, e.g.:

- concentration is limited by only the limit of detection of a compound; and,
- the procedure does not require measurement of solute concentration, since CE is a separation technique that can handle impure samples.

pK_a values may also be determined for relatively unstable compounds [17,18]. Instruments are highly automated and require little or no modification for high-throughput applications. The high-throughput screening method (simultaneously determining more than 50 compounds in a single sample) has been successfully developed within an advanced CE technique using mass spectroscopy detection [19,20]. A recent review summarized numerous pK_a determinations by CE [4].

2.6. Combined methods

In recent years, a new procedure has been developed when LC and CE methodologies are used for pK_a determination in combination with a diode-array detector

Table 2. Dissociation constants of tetracyclines

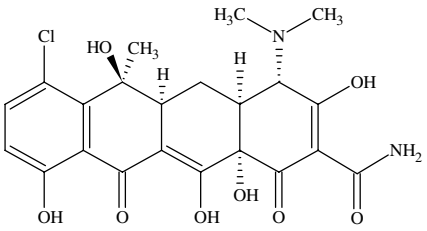
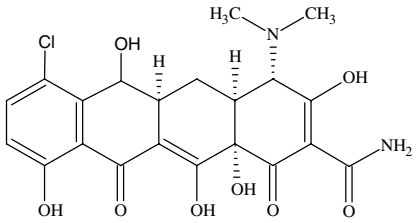
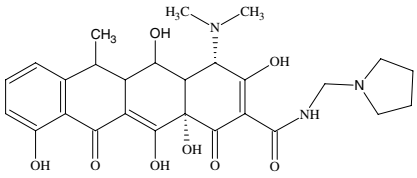
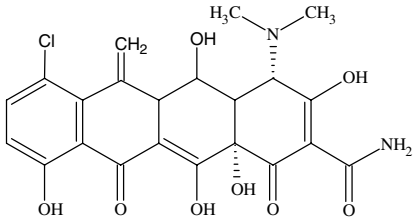
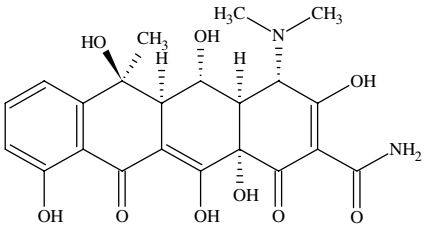
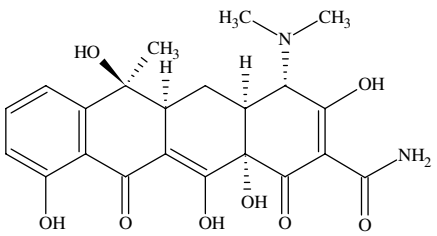
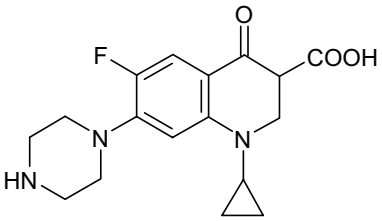
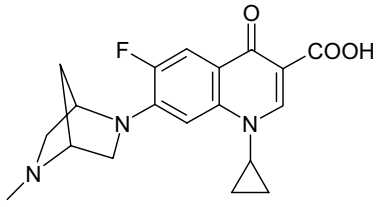
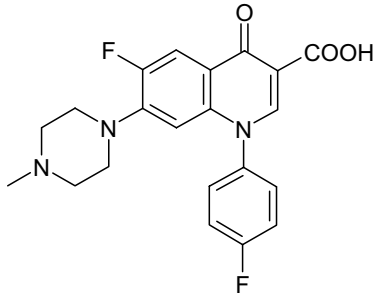
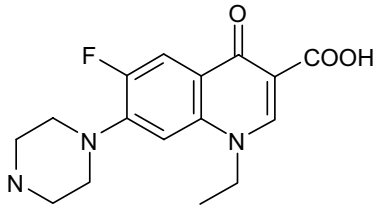
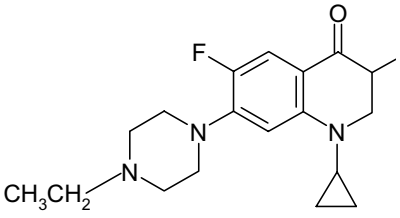
Compound	Chemical structure	pK_a	Method	Ref.
Chlortetracycline		3.33 ± 0.30; 7.55 ± 0.02; 9.33 ± 0.30	Potentiometry	[3]
		3.95; 5.59; 7.94	Calculcated (SPARC)	[22]
Demeclocycline		3.37 ± 0.30; 7.36 ± 0.03; 9.44 ± 0.30	Potentiometry	[3]
		3.99; 5.53; 7.93	Calculated (SPARC)	[22]
Doxycycline		3.02 ± 0.30; 7.97 ± 0.15; 9.15 ± 0.30	Potentiometry	[3]
Meclocycline		4.05 ± 0.30; 6.87 ± 0.39; 9.59 ± 0.30	Potentiometry	[3]
Oxytetracycline		3.04; 8.00	CE-MS (positive detection mode)	[19]
		3.22 ± 0.30; 7.46 ± 0.03; 8.94 ± 0.30	Potentiometry	[3]
		3.23 ± 0.01; 7.22 ± 0.02; 8.82 ± 0.02	Multiwavelength spectrophotometry	[37]
		3.79; 5.41; 8.46	Calculated (SPARC)	[22]
Tetracycline		3.32 ± 0.30; 7.78 ± 0.05; 9.58 ± 0.30	Potentiometry	[3]
		3.01; 5.79; 8.55	Calculated (SPARC)	[22]

Table 3. Dissociation constants of quinolones

Compound	Chemical structure	pK_a	Method	Ref.
Ciprofloxacin		6.42 ± 0.01; 8.29 ± 0.04	UV/pH titration (MDM-water)	[13]
		6.28 ± 0.02; 8.56 ± 0.05	UV/pH titration (MeOH-water)	[13]
		6.09; 8.62	Potentiometry	[31]
		5.86 ± 0.05; 8.24 ± 0.07	CE	[8]
		6.33 ± 0.01; 8.84 ± 0.04	CE-DAD	[8]
		3.01 ±0.30; 6.14 ± 0.13; 8.70 ± 0.09; 10.58 ± 0.30	Potentiometry	[3]
		5.05 ± 0.15; 6.35 ± 0.07; 8.95 ± 0.04	CE	[30]
		5.90 ± 0.15; 8.89 ± 0.11	Potentiometry/fluorimetry	[39]
		6.68, 8.63	Calculated (SPARC)	[22]
Danofloxacin		6.07 ± 0.06; 8.56 ± 0.07	CE	[8]
		6.32 ± 0.02; 8.73 ± 0.05	CE-DAD	[8]
Difloxacin		5.66 ± 0.04; 7.24 ± 0.06	CE	[8]
		5.80 ± 0.01; 8.26 ± 0.11	CE-DAD	[8]
		6.49; 7.74	Calculated (SPARC)	[22]
Enoxacin		6.00; 8.50	Potentiomtry	[31]
		5.05 ± 0.08; 6.25 ± 0.05; 8.80 ± 0.02	CE	[30]
		6.43; 6.75	Calculated (SPARC)	[22]
Enrofloxacin		5.88 ± 0.03; 7.74 ± 0.03	CE	[8]
		6.09 ± 0.01; 7.91 ±0.05	CE-DAD	[8]
		3.85 ± 0.30; 6.19 ± 0.18; 7.59 ± 0.25; 9.86 ± 0.30	Potentiometry	[3]
		5.86 ± 0.05; 8.24 ± 0.07	CE	[41]
		6.12 ± 0.05; 7.68 ± 0.42	UV/pH	[42]
		6.68; 7.87	Calculated (SPARC)	[22]

(*continued on next page*)

Table 3 (*continued*)

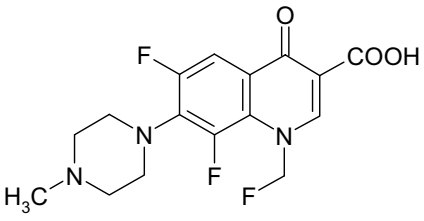
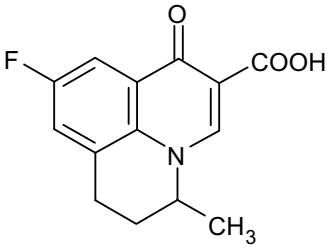
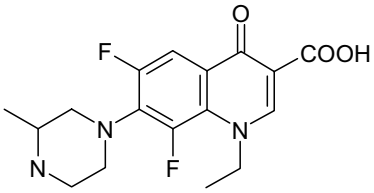
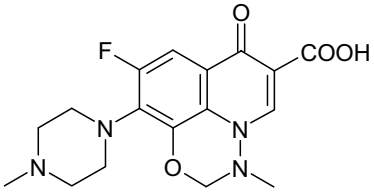
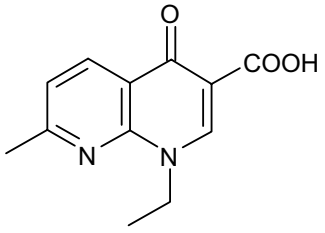
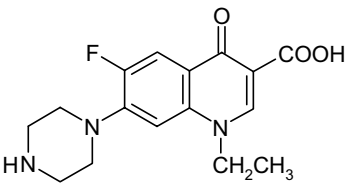
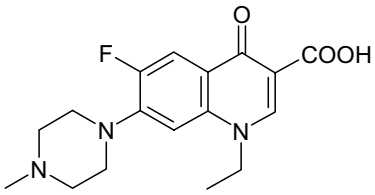
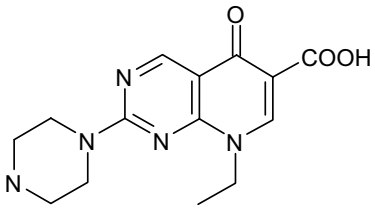
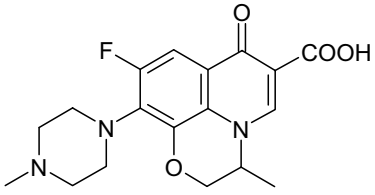
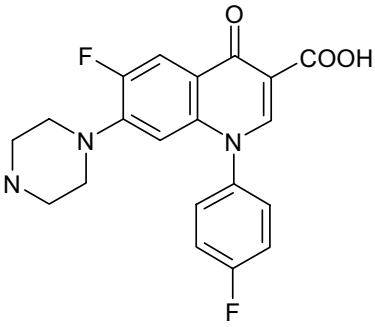
Compound	Chemical structure	pK_a	Method	Ref.
Fleroxacin		5.46; 8.00	Potentiometry	[31]
		5.46; 8.00	Potentiometry/fluorimetry	[40]
		6.42; 7.57	Calculated (SPARC)	[22]
Flumequine		6.35 ± 0.01	CE	[30]
		6.42	CE/MS (negative detection mode)	[19]
		6.65	Calculated (SPARC)	[22]
Lomefloxacin		5.00 ± 0.10; 6.25 ± 0.05; 9.00 ± 0.03	CE	[30]
		6.56; 8.47	Calculated (SPARC)	[22]
Marbofloxacin		5.69 ± 0.10; 8.02 ± 0.20	CE	[8]
		5.51 ± 0.03; 8.38 ± 0.13	CE-DAD	[8]
Nalidixic acid		5.95	Potentiometry	[31]
		6.01 ± 0.05	CE	[8]
		6.04 ± 0.05	CE-DAD	[8]
		6.00 ± 0.01	CE	[30]
		6.50	Calculated (SPARC)	[22]
Norfloxacine		6.22; 8.38	Potentiometry	[31]
		5.94 ± 0.05; 8.22 ± 0.07	CE	[8]
		6.32 ± 0.01; 8.42 ± 0.05	CE-DAD	[8]
		3.11 ± 0.30; 6.10 ± 0.19; 8.60 ± 0.10; 10.56 ± 0.30	Potentiometry	[3]
		5.00 ± 0.10; 6.25 ± 0.04; 8.45 ± 0.03	CE	[30]
		6.36 ± 0.12; 8.62 ± 0.08	Potentiometry/fluorimetry	[39]
		6.22; 8.38	Potentiometry (ACN-water)	[40]
		8.63	Calculated (SPARC)	[22]

Table 3 (*continued*)

Compound	Chemical structure	pK_a	Method	Ref.
Pefloxacin		6.21 ± 0.12; 7.87 ± 0.10 6.68; 7.83	Potentiometry/fluorimetry Calculated (SPARC)	[39] [22]
Pipemidic acid		5.42 ± 0.05; 8.18 ± 0.09 5.59 ± 0.02; 8.33 ± 0.01 5.25 ± 0.12; 6.15 ± 0.06; 8.90 ± 0.04 6.16; 6.30	CE CE-DAD CE Calculated (SPARC)	[8] [8] [29] [22]
Ofloxacin		6.22 ± 0.05; 7.81 ±0.08 6.15 ± 0.04; 8.19 ± 0.06 6.05; 8.11 5.20 ± 0.06, 6.20 ± 0.03; 8.20 ± 0.02 5.97 ± 0.08; 8.28 ± 0.02 6.67, 7.92	UV/pH titration (MDM-water) UV/pH titration (MeOH-water) Potentiomtery CE Potentiometry/fluorimetry Calculated (SPARC)	[13] [13] [31] [30] [39] [22]
Sarafloxacin		5.62 ± 0.08; 8.18 ± 0.09 5.87 ± 0.01; 8.88 ± 0.08 6.49; 8.54	CE CE-DAD Calculated (SPARC)	[8] [8] [22]

(DAD) for absorbance measurements. In these cases, pK_a values can be determined from the absorbance spectra obtained at the maxima of chromatographic or electrophoretic peaks [21]. That way, pK_a values may be obtained from two independent methods:

- mobility/pH data and spectra/pH data in CE procedures (CE-DAD); and,
- capacity factor/pH data and spectra/pH data in LC procedures (LC-DAD) [6].

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 [5]. Dissociation constants are also easy to determine by the LC-MS technique. The use of CE-MS provides high sensitivity as well as high selectivity, thereby ensuring that impurities that might interfere with compounds are not detected [19].

2.7. Computational methods

Theoretical pK_a values can be calculated by computational methods (e.g., SPARC [22] and ACD/Lab [23]).

Table 4. Dissociation constants of macrolides

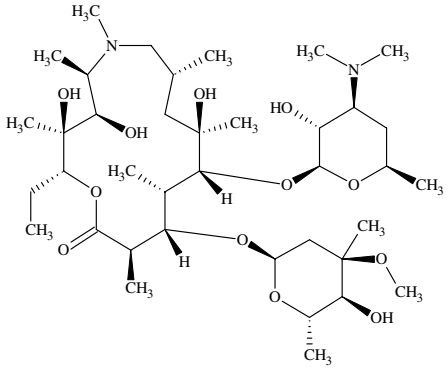
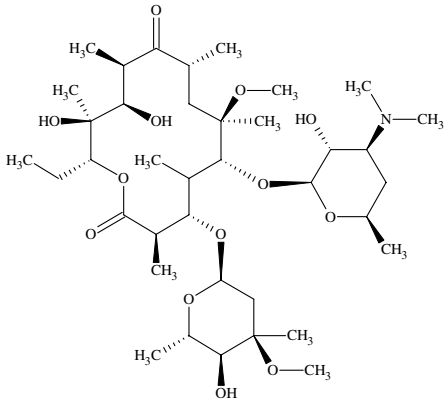
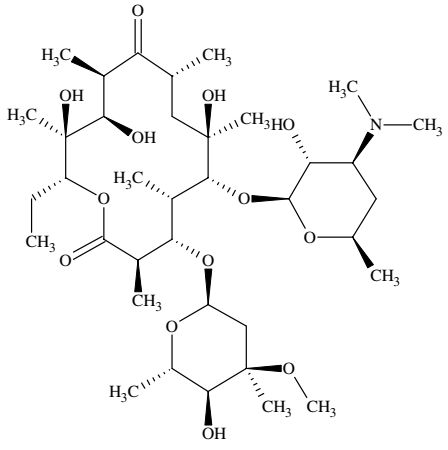
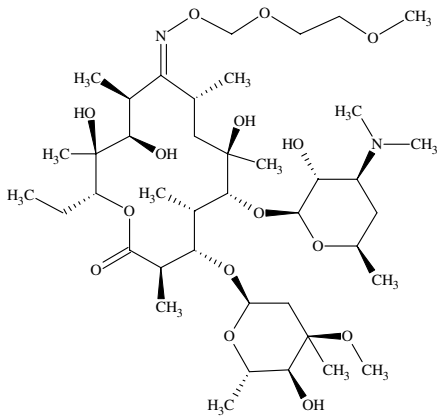
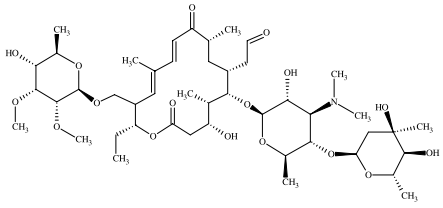
Compound	Chemical structure	pK_a	Method	Ref.
Azithromycin		7.34	Calculated (SPARC)	[22]
Clarithromycin		7.25	Calculated (SPARC)	[22]
Erythromycin		8.90 ± 0.15 7.24	Potentiometry Calculated (SPARC)	[3] [22]

Table 4 (*continued*)

Compound	Chemical structure	pK_a	Method	Ref.
Roxithromycin		9.17 ± 0.30	Potentiometry	[3]
Tylosin		3.31 ± 0.30; 7.50 ± 0.13	Potentiometry	[3]

SPARC is an on-line calculator that estimates the macroscopic and microscopic pK_a of any organic compound solely from its chemical structure.

ACD/pK_a DB is a software program that calculates accurate acid-base ionization constants (pK_a values) under 25°C and zero ionic strength in aqueous solutions for almost any organic structure.

3. pK_a determination in non-aqueous media

For substances sparingly soluble in water, aqueous-organic solvent mixtures are used to estimate their pK_a values [13,24]. The dissociation of uncharged substances in aqueous-organic mixtures is ruled by electrostatic interactions, as well as specific solute-solvent interactions (solvation effects). In order to estimate aqueous pK_a values, several experiments at different organic solvent concentrations should be performed. Aqueous pK_a values that estimate by an extrapolation procedure (e.g., the Yasuda-Shedolovsky method) can be used to deduce the pK_a values at zero organic solvent composition [6,13]. The selected organic solvents are usually alcohols because they are amphiprotic and neutral solvents, and they show great ability to dissolve ionizable compounds. Then, the specific solute-solvent interactions should not be too different from those established in water.

However, not all compounds dissolve in a single-component organic solvent-water mixture, so recently a new, multi-component, co-solvent mixture, comprising equal volumes of methanol, dioxane and acetonitrile, and referred to as MDM, has been found to be efficient for pK_a measurement of various APIs. The application of MDM-water mixtures improves the solubility of poorly water-soluble APIs, so their pK_a values can be measured in a lower proportion of organic solvent [13]. The use of water-organic mixtures requires the correct measurement of pH in these media. Measurements are performed using IUPAC standardization rules.

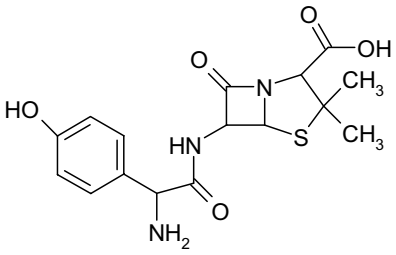
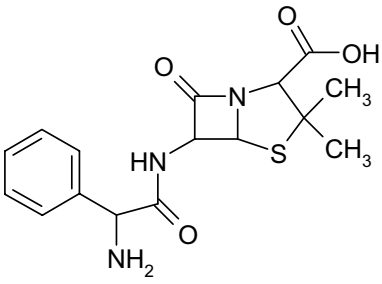
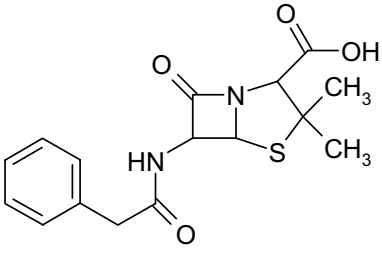
4. pK_a values of active pharmaceutical ingredients

The aqueous pK_a values of APIs determined in water or extrapolated from dissociation constants determined in water-organic media are summarized in Tables 1–8.

4.1. Antibiotics

4.1.1. Sulfonamides. A sulfonamide contains one basic amine group (–NH_2) and one acidic amide group (–NH–).

Table 5. Dissociation constants of β-lactams

Compound	Chemical structure	pK_a	Method	Ref.
Amoxicillin		3.39, 6.71; 9.41	Calculated (SPARC)	[22]
Ampicillin		2.55 ± 0.01; 7.14 ± 0.01	Potentiometric	[13]
		2.50; 7.04	CE-MS (positive detection mode)	[19]
		2.41; 6.94	CE-MS (negative detection mode)	[19]
		3.39; 6.61	Calculated (SPARC)	[22]
Penicillin G (benzylpenicillin)		3.42	Calculated (SPARC)	[22]

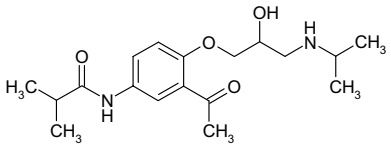
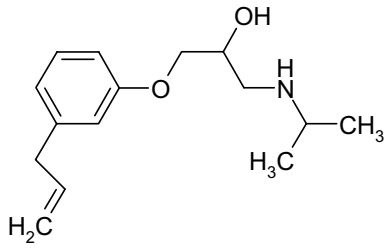
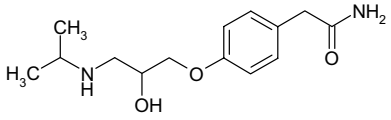
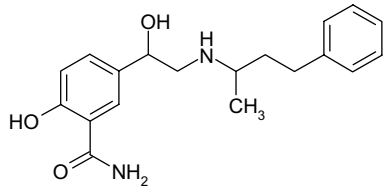
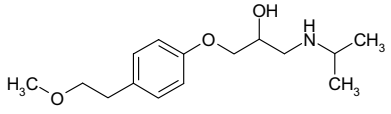
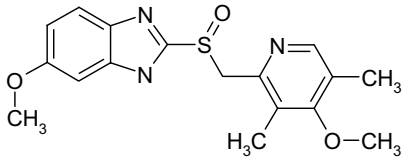
The amine group is able to gain a proton, while the amide group is able to release proton under specific pH conditions. As shown in Fig. 1, K_{a1} is the dissociation constant for equilibrium between the positively charged, protonated amino group of sulfonamide and its electrically neutral conjugate base, whereas K_{a2} refers to an equilibrium involving the loss of the sulfonamide proton to yield its negatively charged conjugate [3,25,26].

4.1.2. Tetracyclines. Tetracyclines show three pK_a values of approximately 3, 7 and 9. The first dissociation constant is associated with the deprotonation of C3 hydroxyl. The loss of protons from O12 and dimethylammonium constitutes K_{a2} and K_{a3}, although the exact assignment of these dissociation constants remains controversial [3]. As indicated by their acid-dissociation constants, tetracyclines contain localized charges across all pH values and only achieve an overall neutral state as zwitterions in the approximate range of pH 3–9. Under alkaline conditions, tetracyclines assume a conformation that allows hydrogen bonding between N4 and OH12a. Under neutral and acidic conditions, the N4 position becomes protonated, disrupting the previous conformation, and a hydrogen-bond interaction occurs with O3 [27] (see Fig. 2).

4.1.3. Quinolones. The basic structure of quinolones is shown in Fig. 3.

Quinolones are nitrogen-containing, eight-membered heterocyclic aromatic compounds with a ketone group at position 4 and a carboxylic group at position 3. The main nucleus usually contains one nitrogen atom (quinolines), but analogues have additional nitrogens at position 2 (cinnolines), position 8 (naphthyridines) or positions 6 and 8 (pyridopyrimidines). Since the discovery of nalidixic acid (the prototype antibacterial quinolone) in 1962, several structural modifications have enhanced their biological and pharmacological activities. These modifications include the introduction of alkyl or aryl groups at position 1 and fluoro and piperazinyl

Table 6. Dissociation constants of β-blockers

Compound	Chemical structure	pK_a	p$K_{a,MeOH}$ [43]	Method	Ref.
Acebutolol		9.21	11.1 ± 0.09	Calculated (SPARC)	[22]
Alprenolol		9.38 ± 0.1 9.35	11.1 ± 0.06	CE Calculated (SPARC)	[18] [22]
Atenolol		9.48 ± 0.01 9.61 9.42 ± 0.1 9.64 9.40 9.41	11.1 ± 0.07	Potentiometry (MDM-water) CE CE CE-MS (positive detection mode) Potentiometry Calculated (SPARC)	[13] [17] [18] [19] [34] [22]
Labetalol		7.35 8.87	10.5 ± 0.06	Potentiometry Calculated (SPARC)	[30] [22]
Metoprolol		9.44 ± 0.1 9.48	11.3 ± 0.07	CE Calculated (SPARC)	[18] [22]
Omeprazole		4.14; 8.90		CE/MS (positive detection mode)	[19]

(*continued on next page*)

Table 6 (*continued*)

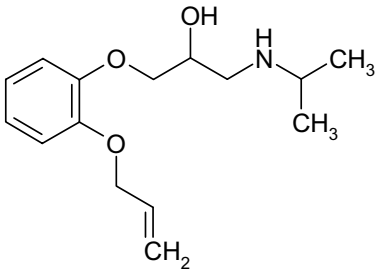
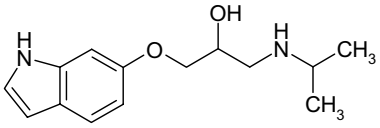
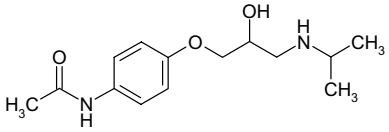
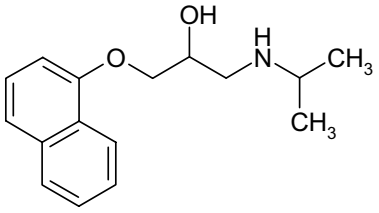
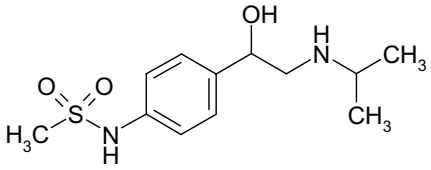
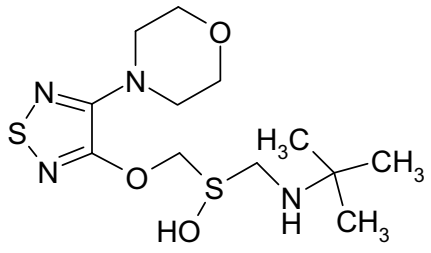
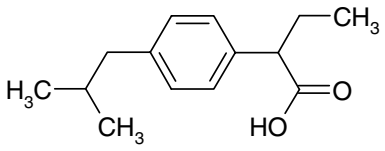
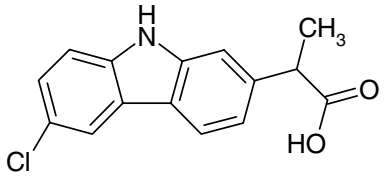
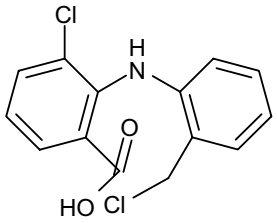
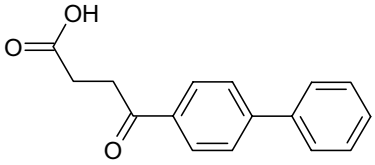
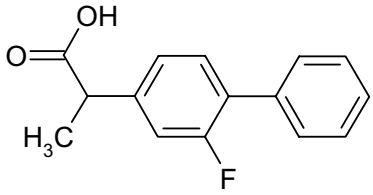
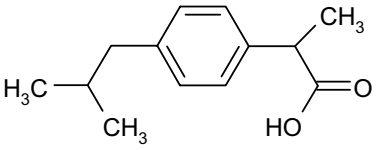
Compound	Chemical structure	pK_a	p$K_{a,MeOH}$ [43]	Method	Ref.
Oxprenolol		9.62 9.16	11.4 ± 0.02	Potentiometry Calculated (SPARC)	[34] [22]
Pindolol		9.16	11.3 ± 0.04	Calculated (SPARC)	[22]
Practolol		9.41	11.3 ± 0.07	Calculated (SPARC)	[22]
Propranolol		9.49 ± 0.2 9.57 9.48	11.2 ± 0.06	CE CE-MS (positive detection mode) Calculated (SPARC)	[17,18] [19] [22]
Sotalol		9.01	11.2 ± 0.06	Calculated (SPARC)	[22]
Timolol		9.34 8.89	11.1 ± 0.07	Potentiometry Calculated (SPARC)	[34] [22]

Table 7. Dissociation constants of anti-anflammatories

Compound	Chemical structure	pK_a	Method	Ref.
Butibufen		4.64	Potentiometry (isopropyl alcohol-water)	[24]
		4.41	LC	[44]
		4.45	Calculated (SPARC)	[22]
Carprofen		4.63	Potentiometry (isopropyl alcohol-water)	[24]
		4.25	Calculated (SPARC)	[22]
Diclofenac		4.16	Potentiometry (isopropyl alcohol-water)	[24]
		4.21	CE-MS (negative detection mode)	[19]
		4.03	LC	[44]
		4.06	Calculated (SPARC)	[22]
Fenbufen		4.56	Potentiometry (isopropyl alcohol-water)	[24]
		4.40	LC	[44]
		4.61	Calculated (SPARC)	[22]
Flurbiprofen		4.35	Potentiometry (isopropyl alcohol-water)	[24]
		4.21	LC	[44]
		4.20	Calculated (SPARC)	[22]
Ibuprofen		4.42 ± 0.08	Potentiometry (MDM-water)	[13]
		4.45 ± 0.04	Potentiometry (methanol-water)	[13]
		4.51	CE	[17]
		4.52	Potentiometry (isopropyl alcohol-water)	[24]
		4.30	LC	[44]
		4.24 ± 0.03	Multiwavelength spectrophotometry	[37]
		4.45 ± 0.05	UV/pH	[37]
		4.43	CE-MS (negative detection mode)	[19]
		4.45	Calculated (SPARC)	[22]

(*continued on next page*)

Table 7 (*continued*)

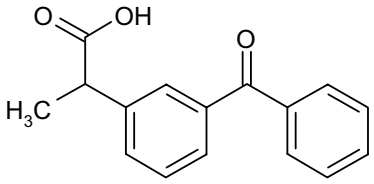
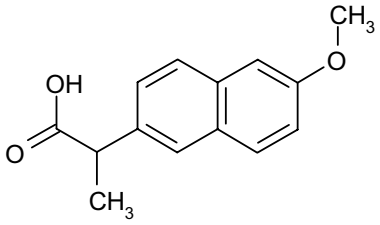
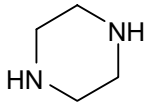
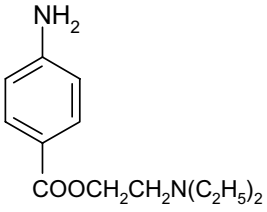
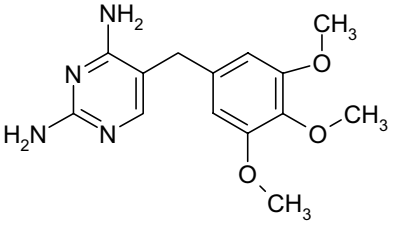
Compound	Chemical structure	pK_a	Method	Ref.
Ketoprofen		4.36 4.25	Potentiometry (isopropyl alcohol-water) Calculated (SPARC)	[24] [22]
Naproxen		4.38 ± 0.03 4.16 ± 0.02 4.57 4.37 4.38	Potentiometry (MDM-water) Potentiometry (methanol-water) Potentiometry (isopropyl alcohol-water) LC Calculated (SPARC)	[13] [13] [24] [44] [22]

Table 8. Dissociation constants of other active pharmaceutical ingredients

Compound	Chemical structure	pK_a	Method	Ref.
Piperazine		5.33; 9.73 9.71 3.23 ± 0.30; 6.76 ± 0.12 9.70	CE Potentiometry (acetonitrile-water) Potentiometry Calculated (SPARC)	[30] [40] [3] [22]
Procaine		2.28 ± 0.08; 9.02 ± 0.01 8.84 2.24; 8.84	UV/pH titration CE Calculated (SPARC)	[13] [38] [22]
Trimethoprim		3.23 ± 0.30; 6.76 ± 0.12 4.09; 6.32	Potentiometry Calculated (SPARC)	[3] [22]

substitutions at positions 6 and 7, respectively. The fluoro group at position 6 originates fluoroquinolones [5].

The carboxylic group at position 3 makes these compounds acidic. In addition, the 7-piperazinyl quinolones include additional amine groups, which are basic, so, in

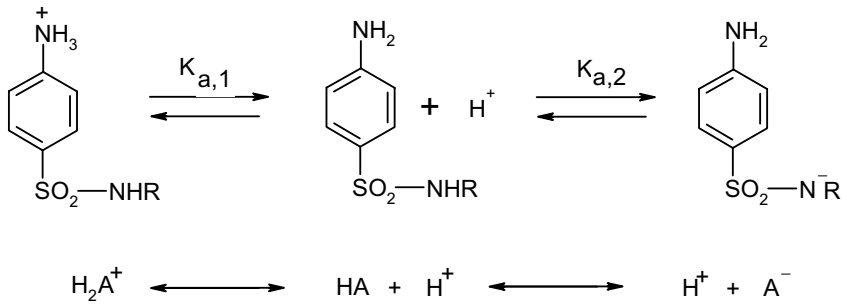


Figure 1. Dissociation equilibrium of sulfonamides.

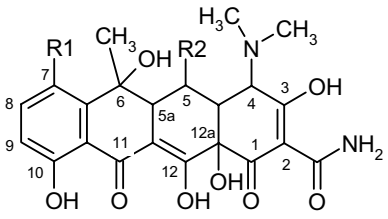


Figure 2. Structure of tetracyclines.

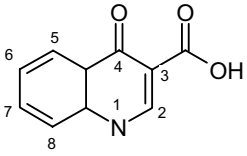


Figure 3. Basic structure of quinolones.

an aqueous solution, 7-piperazinyl quinolones show three different species, which are cationic, zwitterionic and anionic, while other quinolones can only be neutral or anionic. The existing equilibria for these two types of quinolones are shown in Fig. 4.

Quinolones with only one pK_a value are referred to as acidic quinolones and those with two pK_a values (due to the presence of a piperazinyl ring) are called piperazinyl quinolones. The reported values of pK_a for acidic quinolones (flumequin and naldixic acid) range from 5.95 to 6.35 [28,29](See Fig. 5).

In reference papers, there is no agreement on the assignment of pK_a values obtained. Some authors have suggested that three protonation/deprotonation equilibria, instead of two, are involved in quinolones with a piperazinyl substituent [30] while others have reported four pK_a values because fluoroquinolones contain one carboxylic group and three basic nitrogen sites [3].

The dissociation constant of quinolones have also been determined in water-acetonitrile mixtures using different methods [31,32]. In order to enable LC separation of

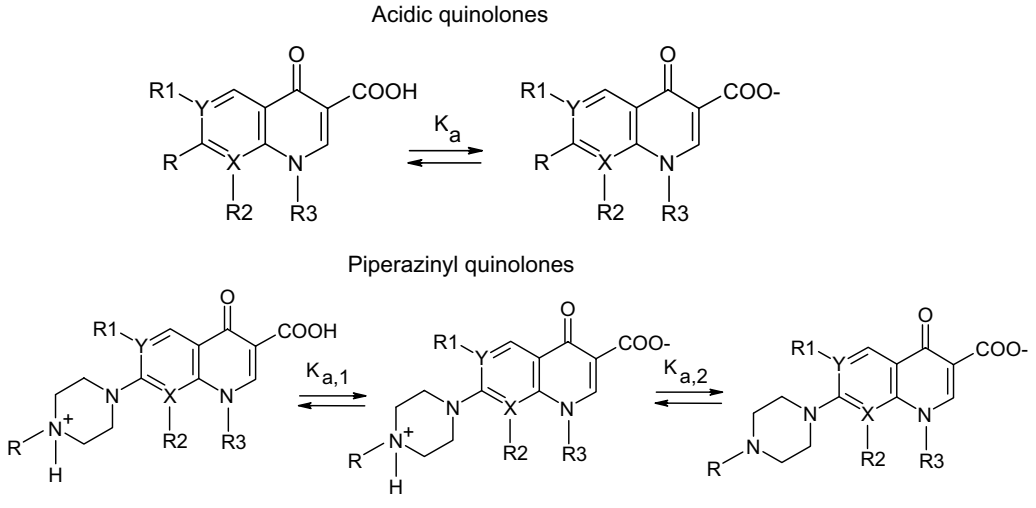


Figure 4. Acid-base equlibria for piperazinyl quinolones and acidic quinolones.

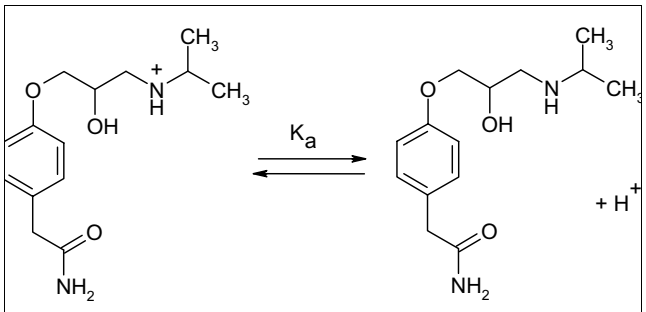


Figure 5. Acid-base equilibrium of atenolol.

quinolones, their pK_a values have been determined in water-organic media [15].

4.1.4. Macrolides. A macrolide contains a basic dimethylamine [–N(CH_3)$_2$] group, which is able to gain a proton, so, according to their chemical structure, macrolides have only one pK_a value around 9. Only tylosin has a pK_{a2} value (about 7.50), which corresponds to the dimethylamine group. Tylosin is produced as tartarate, so the pK_{a1} value of tylosin (3.31) may correspond to the tartarate moiety [3].

4.1.5. β-lactams. Penicillins in aqueous solution are present as neutral, anionic or, in the case of amoxicillin and ampicillin, intermediate forms (zwitterions), due to their presence in the molecule of carboxylic and amino groups [33].

4.2. β-blockers

A β-blocker contains one basic amine group (–NH_2) able to gain a proton, so it has only one pK_a value (in the range 8.6–9.7). The major difficulty in obtaining reliable values for the dissociation constants of β-blockers by potentiometry is their low solubility in aqueous solutions. A high concentration of the drug is required ($\geqslant 10^{-4}$ M) in order to obtain a worthwhile increase in pH at the equivalence point, so the pK_a values of β-blockers have been determined in methanol [34,43]. To overcome problems of low water solubility, the pK_a values of β-blockers have been determined by CE [18] combined with a short-end injection [17]. Table 6 summarizes the aqueous pK_a values of β-blockers as well as p$K_{a,MeOH}$. The average difference in aqueous and methanolic dissociation constants is about 2 pK_a units.

4.3. Anti-inflammatories

Anti-inflammatories are sparingly soluble in water. pK_a values in reference papers have been determined in mixtures of water and an organic solvent in order to get suitable solubility. Organic solvents used are mainly alcohols [13,35] or MDM [13].

5. Concluding remarks

Several advanced analytical methods have been used for the determination of dissociation constants of APIs.

The potentiometric methodology gives the best accuracy and better precision than any of the methods mentioned. This is mainly due to the fact that the electrode system is calibrated before each potentiometric titration, and the electrode remains in the solution during entire titration, so that calibration parameters are the same for all the titration data corresponding to the same experimental run.

By comparison, in the 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 a solute and the hold-up time, giving lower precision.

The LC-DAD procedure does not use retention factors. Instead, it uses spectra measured by a DAD, but the procedure will be affected by errors in the pH measurement.

CE permits pK_a determination in aqueous solutions without difficulties, but that is not the case for LC, in which retention can be very significant without addition of an organic modifier.

In LC, the pK_a values of drugs can be determined in media only where suitable retention is obtained. For poorly soluble compounds, these methods as well as CE-DAD cannot offer sufficient sensitivity to measure compounds with aqueous solubilities below 10 μM. This problem can be overcome by using a mass spectrometer as a detector.

Acknowledgement

This work has been supported by the EU EMCO Project (INCO CT 2004-509188 – Reduction of Environmental Risks, Posed by Emerging Contaminants through Advanced Treatment of Municipal and Industrial wastes) and Croatian Ministry of Science, Education and Sports Projects (125-1253008-1350 – Advanced analytical methods for pharmaceuticals determination in the environment and 125-2120898-3148 – Croatian nomenclature in analytical chemistry).

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