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Review

Determination of acid dissociation constants by capillary electrophoresis☆

Salwa K. Poole[a,*], Sneha Patel[a], Karen Dehring[a], Heather Workman[a], Colin F. Poole[b]

[a] *Discovery Technologies, Pfizer Global Research and Development, Ann Arbor Laboratories, 2800 Plymouth Road, Ann Arbor, MI 48105, USA*
[b] *Department of Chemistry, Wayne State University, Detroit, MI 48202, USA*

Abstract

Capillary electrophoresis affords a simple, automated approach for the measurement of pK_a values in the range 2–11 at a throughput of less than 1 h per sample per instrument. Agreement with literature values is usually within 0.20 log units with a precision better than 0.07 log units. The attractive features of capillary electrophoresis for pK_a measurements are: (1) conventional instrumentation with a high level of automation are suitable for all measurements; (2) because it is a separation method samples need not be of high purity; (3) samples of low water solubility with suitable chromophores are easily handled (detection limits in the μM range); (4) sample consumption per measurement is in the microgram range; and (5) since only mobilities are measured, exact knowledge of concentrations is not needed. The general approach can be extended to pK_a measurements in aqueous–organic solvent mixtures and non-aqueous solvents with suitable calibration. The widespread use of absorbance detection in capillary electrophoresis means that the sample must have a suitable chromophore for detection. The main source of controllable error is the accuracy of buffer standardization and their stability in use, and uncontrollable error, the retentive interactions of the sample with the column wall. The latter seems to be a rare problem in practice for typical operating conditions.

Keywords: Reviews; Dissociation constants

Contents

1. Introduction

The acid dissociation constant, usually indicated as its pK_a value, is a fundamental property of weak acids and bases. For compounds with a single ionizable group, it is defined as the pH at which a compound is 50% ionized. Once the

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* Corresponding author. Tel.: +1-734-622-3077; fax: +1-734-622-2716.
E-mail address: salwa.poole@pfizer.com (S.K. Poole).

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pK_a value is established, the extent of ionization at any pH for that compound is easily calculated. This is an important property for weak acids and bases since the physicochemical properties of the neutral and ionized forms are generally different. 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. These properties are critical to the development of new human and veterinary drugs, crop protecting agents, etc.

Advances in the approach by which new biologically active compounds are identified and optimized for particular applications using high throughput activity screens and combinatorial chemical synthesis has dramatically increased the demand for physicochemical property measurements in the life science industries. The availability of physicochemical property data at an early stage of compound selection allows a reduction of attrition rates and shorter development times in discovery programs. Thus, laboratories that measured a few pK_a values per month less than a decade ago have had to adapt or develop new procedures to handle requests for several hundred pK_a measurements per week. In addition, the typical sample presented for measurement has changed from a high-purity, reference material available in relatively large amounts, to solutions of 1 mg or less of samples of variable purity. Over the last few years the 96-well microtitre plate holding 10 mM solutions in dimethyl sulfoxide has become widely adopted for handling and storage of drug discovery samples in the pharmaceutical industry. Methods that can handle samples presented in this format are desirable for integrated compound screening and physical property measurements in the pharmaceutical industry. This paradigm shift has had a considerable impact on the selection of methods for pK_a measurements and in some measure has contributed to the rapid growth and acceptance of capillary electrophoresis for this purpose.

2. Traditional methods

Historically, potentiometric titration was the standard method for pK_a measurements. For high-purity, water-soluble compounds available in relatively large quantities, this is still largely the case today. In a potentiometric titration, the sample is titrated with acid or base using a pH electrode to monitor the course of the titration. The pK_a value(s), is calculated from the change in shape of the titration curve compared with that of a blank titration without sample present [1–3]. Several milligrams of sample are usually required in 5 ml or more of solvent. In concentration terms, solutions of at least 5×10^{-4} M are required in order for a significant change in shape of the titration curve to be detected. Titrations typically take from 20–40 min per compound. To avoid errors, especially for measurements at neutral to high pH, carbonate free solutions must be laboriously prepared. Mixtures of aqueous and organic solvents can be used to circumvent solubility problems at the expense of low sample throughput. Aqueous-solution pK_a values are obtained by extrapolation of the observed pK_a values for a series of mixed solvents to zero organic solvent composition [4,5].

In recent years, dual-phase potentiometric titrations that afford both pK_a and log K_{OW} (octanol–water partition coefficient) values have gained in popularity [4]. Log K_{OW} is used to model lipophilicity and is used together with pK_a values to estimate biophysical and environmental properties [6]. Dual-phase potentiometric titrations use a direct titration with base in the absence of octanol followed by backtitration with acid in the presence of octanol. Conventional methods are subject to the same limitations as potentiometric titrations with respect to sample amounts and solution volumes. Sample throughput is fairly slow with a single pK_a measurement requiring about 20–25 min. Microscale titrations utilizing less than 10–20 μl of sample solution and 10–100 μl of octanol have been described but little used [7].

Spectrophotometric titrations are generally considered the main alternative to potentiometric titrations for measuring pK_a values of water-soluble compounds of high purity (or at least containing impurities that do not interfere in the spectroscopic measurements) [8–10]. The main advantage is higher sensitivity ($>10^{-6}$ M) for compounds with favorable molar absorption coefficients. In this case, however, the sample must possess chromophore(s) close to the ionizable groups such that the neutral and ionized forms exhibit sufficiently different spectral properties for their identification in mixtures of the two forms. Spectrophotometric methods are therefore selective, since some samples in a general screen will be unable to meet this requirement. Spectral data are recorded continuously during the course of the 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 the pK_a values. These changes are usually identified from the first derivative of the absorbance against time plot or from overlay plots of the different spectra. Target factor analysis is also used to improve the accuracy of pK_a assignments from the absorbance against time data.

The spectral gradient analysis method provides a dramatic increase in the sample throughput of spectrophotometric titrations [11,12]. In this method, two specially formulated buffer systems are dispensed to create a linear pH gradient from 3 to 11 over time. The buffers are selected to minimize changes in ionic strength and buffer capacity during the titration. Calibration of the pH gradient is achieved using standards with known pK_a values. To determine pK_a values a constant flow of sample is infused into the pH gradient and spectral changes recorded as a function of pH with a diode array spectrometer. For typical operating conditions, a gradient time of 90s is used and 11 or more absorbance

spectra are recorded over each pH unit. The method is easily automated and is compatible with samples stored in a 96-well microtitre plate. The main advantages are reduced sample consumption and high sample throughput (4 min per sample) compared with conventional spectrophotometric titrations. The fundamental limitation of conventional spectrophotometric titrations, however, the need for an identifiable chromophore shift associated with changes in ionization, is retained. Sample impurities and degradants with similar absorption properties to the target compound may interfere in the measurements.

3. Capillary electrophoresis

The determination of pK_a values by capillary electrophoresis is based on the observation of the effective mobility of an ionizable compound in a series of electrolyte solutions of constant ionic strength and different pH. The pK_a values are obtained by fitting the effective mobility as a function of pH to a suitable model for the number of ionizable groups. The attractive features of capillary electrophoresis for pK_a measurements are:

(1) Since capillary electrophoresis is a separation technique it can handle impure samples.
(2) Instruments are highly automated and require little or no modification for high throughput applications.
(3) Precise information of sample concentration is unnecessary. Only mobilities are used in calculations.
(4) Sample consumption is minute.
(5) Sparingly soluble compounds with suitable chromophore(s) are easily handled (UV absorbance is commonly used for detection).
(6) No special demands are placed on the purity of electrolyte solutions.

3.1. Theory

The thermodynamic acid dissociation constant of a monoprotic weak acid, HA, is defined as:

$$HA \Leftrightarrow H^+ + A^- \tag{1}$$

$$K_a^T = \frac{\gamma_{A^-}\gamma_{H^+}[H^+][A^-]}{[HA]} \tag{2}$$

where K_a^T is the thermodynamic acid dissociation constant, all γ terms are activity coefficients and all terms in brackets are molar concentrations. The activity coefficient for the neutral form of the acid is assumed to be unity. Given that the pH of the solution is $\gamma_{H^+}[H^+]$, Eq. (2) can be rewritten in the more convenient form:

$$pK_a^T = pH - \log\gamma_{A^-} - \frac{\log[A^-]}{[HA]} \tag{3}$$

Activity coefficients for ions in dilute electrolyte solution at 25 °C can be estimated from classical Debye–Huckel theory [13,14]:

$$-\log\gamma = \frac{0.5085z^2\sqrt{I}}{1 + 0.3281a\sqrt{I}} \tag{4}$$

where I is the ionic strength of the solution, z the charge on the ion, and a the diameter of the hydrated ion in Angstroms. In general, exact values for the ion diameter will be unknown, and for small ions a value of 5 Å is assumed (typical values from 1 to 11). Substituting Eq. (4) into Eq. (3) gives:

$$pK_a^T = pH - \frac{\log[A^-]}{[HA]} + \left[\frac{0.5085z^2\sqrt{I}}{1 + 0.3281a\sqrt{I}}\right] \tag{5}$$

To calculate pK_a^T a method is required to determine the ratio $[A^-]/[HA]$ at some value of pH, or better, to determine $[A^-]/[HA]$ as a function of pH and by curve fitting determine the $pH = pK_a^T$ at which $[A^-] = [HA]$.

In capillary electrophoresis the effective mobility of an ion, m_{eff}, is used to describe the overall electrophoretic mobility of the ionic forms of a compound resulting from any number of equilibrated species, where equilibration is fast compared with the separation time. In such cases, a single peak is observed for all interconverting species that depends on the properties of the electrolyte solution. Thus, for a monovalent weak acid the effective mobility is given by $m_{eff} = \alpha m_{ep}$, where α is the fraction of the monovalent acid present as the anionic form and m_{ep} is the electrophoretic mobility of the anion. The fraction of acid present as the anionic form is given by:

$$\alpha = \frac{K_a}{[H^+] + K_a} \tag{6}$$

or

$$\alpha = \frac{1}{1 + 10^{(pK_a - pH)}} \tag{7}$$

Using this relation, it is possible to rewrite $[A^-]/[HA]$ in terms of mobility:

$$m_{eff} = \left[\frac{10^{-pK_a}}{10^{-pK_a} + 10^{-pH}}\right] m_{ep} \tag{8}$$

There is no restriction on the number of ionization equilibria involved, and for the general case, the effective mobility is given by:

$$m_{eff} = \sum_i x_i m_{ep,i} \tag{9}$$

where x_i is the mole fraction of species i with an electrophoretic mobility $m_{ep,i}$. The models for weak acids and bases containing up to three ionization centers are summarized in Table 1 [15–21]. For simplicity corrections for activities are not shown and apparent pK_a values are converted to the thermodynamic values through Eq. (5). For a buffer with an ionic strength of 50 mM at 25 °C the correction term

Table 1
Model equations for pK_a determination by capillary electrophoresis

Compound type	Model equation[a]
Mono base	$m_{\text{eff}} = \dfrac{\alpha \times 10^{-\text{pH}}}{10^{-\text{p}K_a} + 10^{-\text{pH}}}$
Mono acid	$m_{\text{eff}} = \dfrac{\alpha \times 10^{-\text{p}K_a}}{10^{-\text{p}K_a} + 10^{-\text{pH}}}$
Di base	$m_{\text{eff}} = \dfrac{\alpha_1 \times [10^{-\text{pH}}]^2 + \alpha_2 \times 10^{-\text{p}K_{a1}} \times 0^{-\text{pH}}}{[10^{-\text{pH}}]^2 + 10^{-\text{p}K_{a1}} \times 10^{-\text{pH}} + 10^{-\text{p}K_{a1}} \times 10^{-\text{p}K_{a2}}}$
Di acid	$m_{\text{eff}} = \dfrac{\alpha_1 \times 10^{-\text{p}K_{a1}} \times 10^{-\text{pH}} + \alpha_2 \times 10^{-\text{p}K_{a1}} \times 10^{-\text{p}K_{a2}}}{[10^{-\text{pH}}]^2 + 10^{-\text{p}K_{a1}} \times 10^{-\text{pH}} + 10^{-\text{p}K_{a1}} \times 10^{-\text{p}K_{a2}}}$
Mono acid/mono base	$m_{\text{eff}} = \dfrac{\alpha_1 \times [10^{-\text{pH}}]^2 + \alpha_2 \times 10^{-\text{p}K_{a1}} \times 10^{-\text{p}K_{a2}}}{[10^{-\text{pH}}]^2 + 10^{-\text{p}K_{a1}} \times 10^{-\text{pH}} + 10^{-\text{p}K_{a1}} \times 10^{-\text{p}K_{a2}}}$
Tri base	$m_{\text{eff}} = \dfrac{\alpha_1 \times [10^{-\text{pH}}]^3 + \alpha_2 \times 10^{-\text{p}K_{a1}} \times [10^{-\text{pH}}]^2 + \alpha_3 \times 10^{-\text{p}K_{a1}} \times 10^{-\text{p}K_{a2}} \times 10^{-\text{pH}}}{[10^{-\text{pH}}]^3 + 10^{-\text{p}K_{a1}} \times [10^{-\text{pH}}]^2 + 10^{-\text{p}K_{a1}} \times 10^{-\text{p}K_{a2}} \times 10^{-\text{pH}} + 10^{-\text{p}K_{a1}} \times 10^{-\text{p}K_{a2}} \times 10^{-\text{p}K_{a3}}}$
Tri acid	$m_{\text{eff}} = \dfrac{\alpha_1 \times 10^{-\text{p}K_{a1}}[10^{-\text{pH}}]^2 + \alpha_2 \times 10^{-\text{p}K_{a1}} \times 10^{-\text{p}K_{a2}} \times [10^{-\text{pH}}] + \alpha_3 \times 10^{-\text{p}K_{a1}} \times 10^{-\text{p}K_{a2}} \times 10^{-\text{p}K_{a3}}}{[10^{-\text{pH}}]^3 + 10^{-\text{p}K_{a1}} \times [10^{-\text{pH}}]^2 + 10^{-\text{p}K_{a1}} \times 10^{-\text{p}K_{a2}} \times 10^{-\text{pH}} + 10^{-\text{p}K_{a1}} \times 10^{-\text{p}K_{a2}} \times 10^{-\text{p}K_{a3}}}$
Di acid/mono base	$m_{\text{eff}} = \dfrac{\alpha_1 \times [10^{-\text{pH}}]^3 + \alpha_2 \times 10^{-\text{p}K_{a1}} \times 10^{-\text{p}K_{a2}} \times [10^{-\text{pH}}] + \alpha_3 \times 10^{-\text{p}K_{a1}} \times 10^{-\text{p}K_{a2}} \times 10^{-\text{p}K_{a3}}}{[10^{-\text{pH}}]^3 + 10^{-\text{p}K_{a1}} \times [10^{-\text{pH}}]^2 + 10^{-\text{p}K_{a1}} \times 10^{-\text{p}K_{a2}} \times 10^{-\text{pH}} + 10^{-\text{p}K_{a1}} \times 10^{-\text{p}K_{a2}} \times 10^{-\text{p}K_{a3}}}$
Mono acid/di base	$m_{\text{eff}} = \dfrac{\alpha_1 \times [10^{-\text{pH}}]^3 + \alpha_2 \times 10^{-\text{p}K_{a1}} \times [10^{-\text{pH}}]^2 + \alpha_3 \times 10^{-\text{p}K_{a1}} \times 10^{-\text{p}K_{a2}} \times 10^{-\text{p}K_{a3}}}{[10^{-\text{pH}}]^3 + 10^{-\text{p}K_{a1}} \times [10^{-\text{pH}}]^2 + 10^{-\text{p}K_{a1}} \times 10^{-\text{p}K_{a2}} \times 10^{-\text{pH}} + 10^{-\text{p}K_{a1}} \times 10^{-\text{p}K_{a2}} \times 10^{-\text{p}K_{a3}}}$

[a] α values are fitting constants equal to the electrophoretic mobility of the ionized form of the compound with subscript 1, 2 and 3 equal to the order of ionization.

is about 0.08 for a monovalent ion. For the weaker pK_a of a diprotic acid ($z = 2$) the correction term is about 0.25 for the same electrolyte conditions. For zwitterionic compounds the correction is not straightforward and requires further experiments.

The effective mobility is calculated from the experimental conditions by [22]:

$$m_{\text{eff}} = \frac{LL_{\text{D}}}{V}\left[\frac{1}{t_{\text{s}}} - \frac{1}{t_{\text{eo}}}\right] \tag{10}$$

where L_{D} is the distance from the injection end of the capillary to the detector and for on-column detection will be shorter than the column length, L, over which the voltage V is applied, t_{s} is the migration time for the sample, and t_{eo} 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. The widespread use of dimethyl sulfoxide as a solvent in drug discovery explains why it has become the most frequently used marker compound for estimating t_{eo} for pK_a measurements. Favorable properties of dimethyl sulfoxide are its strong UV absorption below 230 nm and low volatility.

A typical plot of the variation of the effective mobility of a monovalent acid as a function of electrolyte pH is shown in Fig. 1. At low pH the effective mobility of the acid is zero. All forms of the acid are present in the neutral form and migrate through the column with the electroosmotic mobility marker. At higher pH the effective mobility of the acid exhibits the characteristic sigmoidal shape of the acid titration curve. The effective mobility of the ion at any pH is proportional to the equilibrium mole fraction of the acid present as the anionic form. At a sufficiently high pH, all the acid is dissociated, and the effective mobility is identical to the electrophoretic mobility of the anion.

3.2. Instrumental aspects

There are no special instrument requirements for the determination of pK_a values. Most commercial instruments are fully automated and suitable for unattended operation. Compatibility with samples presented in a 96-well format may be important for some laboratories. Since equilibrium constants depend on temperature effective thermostating of the column is required. Because electrolyte solutions of

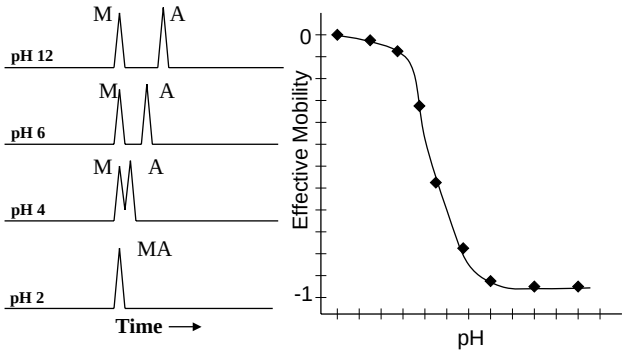

Fig. 1. Influence of the pH of the electrolyte solution on the effective electrophoretic mobility of a monovalent weak acid in capillary electrophoresis. The separations show the migration order of the anion (A) and a neutral compound (M), used as an electroosmotic flow marker.

low ionic strength and normal operating voltages are used both air-cooled and liquid-cooled column thermostats should prove adequate.

The photodiode array detector is the most common detector used in capillary electrophoresis and virtually the only detector used for routine pK_a measurements. Since typical electrolyte solutions are transparent at low UV wavelengths there are few samples for which satisfactory results cannot be obtained. Inevitably, however, there will be some samples that fail to respond to the detector and cannot be measured, and others that may be misidentified because low concentrations of an impurity with a large molar absorption coefficient are mistaken for a sample with a weak detector response. For samples of low water solubility, detection may depend on the sample having a reasonable molar absorption coefficient at a suitable observation wavelength. Except as noted, it is usually possible to measure the effective mobility of samples at low μM concentrations.

Indirect detection methods have been employed for non-UV-absorbing samples [23,24]. The selection of the buffer is critical to success. The buffer must have reasonable UV-absorption at a convenient observation wavelength, the buffer ion must have similar electrophoretic mobility to the ionized form of the sample and the buffer must have sufficient buffering capacity in the pH region close to the sample pK_a. For example, Hagberg et al. [23] used 1,2,4-benzenetricarboxylic acid as a buffer for measurement of pK_a values of low-molecular-mass aliphatic carboxylic acids and Mercier et al. [24] used phenylphosphonic acid as a buffer for the measurement of pK_a values of alkylphosphonic acids. In both cases the separation capillary was dynamically coated with a layer of polybrene to obtain convenient migration times by reversing the direction of the electroosmotic flow.

Photodiode array detection also allows the UV spectra to be recorded at the sample peak maximum for each electrolyte solution used to determine the relationship between the effective mobility and pH. Independent of the fit of the effective mobility to the model for analysis, it is possible to estimate pK_a values in favorable cases from changes in the observed absorption data alone [25–27]. This approach is analogous to the spectrophotometric titration method (Section 2) and was used to confirm the results obtained from effective mobility measurements. Since no additional experiments are required, sample throughput is not affected.

A capillary electrophoresis instrument with a 96-capillary array separation cassette and diode array absorbance detector was recently introduced by CombiSep (Ames, IA, USA) [28]. A conference report indicates the possibility of using this instrument for high throughput measurement of pK_a values by multiplexed, absorbance-based vacuum modulated capillary electrophoresis [29]. By separating samples in parallel pK_a values for eight compounds could be determined simultaneously in twelve electrolyte solutions of different pH with a throughput of 16 compounds per hour. For laboratories that require a high sample throughput this is a promising approach.

3.3. Electrolyte solutions

The effective electrophoretic mobility of weak acids and bases is strongly influenced by environmental factors that affect the underlying equilibrium constants, such as pH, ionic strength, and temperature, as well as variables that affect the ion mobility, such as ionic strength, temperature and viscosity [18,22]. Thus, electrolyte solutions of different pH with a low and constant ionic strength as well as effective column thermostating are required for pK_a measurements. These features minimize temperature and viscosity differences for the electrolyte solutions and stabilize the apparent acid dissociation constants. In addition, the electroosmotic velocity is less variable for electrolyte solutions of constant ionic strength. It is difficult to determine the exact temperature within a capillary and different instruments may afford more efficient temperature control than others. As a minimum assessment that temperature effects are not responsible for unexpected changes in ion mobilities, measurements should be made under conditions that obey Ohm's law. This will generally be the case if the ion current is minimized while meeting the general requirements for adequate buffer capacity and electrochemical stability.

Desirable buffer properties for the measurement of pK_a values by capillary electrophoresis 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 capillary electrophoresis are phosphate, acetate, borate and zwitterionic compounds (Good's buffers) [22]. The buffer recipes used in our group to cover the pH range 2 to 11.4 with an ionic strength of 50 mM are summarized in Table 2. Sodium chloride is added to adjust the ionic strength and hydrochloric acid or sodium hydroxide the pH values. Alternative buffer recipes are easily found in the references indicated in this review. Electrolyte solutions covering a wide pH range are desirable for samples with unknown or variable pK_a values, typical of samples received in laboratories supporting drug discovery. A narrower pH range is suitable for samples with a small range of pK_a values.

The electroosmotic velocity of electrolyte solutions in fused-silica capillary columns is low at pH < 4 and reaches a maximum at about pH 7–8. The low electroosmotic velocity at low pH results in long migration times. The electrophoretic mobility of anions is in the opposite direction to the electroosmotic flow and may exceed the electroosmotic velocity and, thus, never reach the detector located at the cathode end of the column. Early attempts to overcome this problem used reversed polarity for measurements in capillary columns coated with a layer of positively charged polymer to reverse the electroosmotic flow direction [23,24,30]. These strategies are inconvenient and unsuitable for high throughput measurements where the acid–base character of samples is either unknown or variable. Pressure-assisted capillary electrophoresis affords a more convenient solution and is now widely used to minimize run times with low pH

Table 2
Buffer recipes for pK_a measurements by capillary electrophoresis

Run order	Buffer pH	Inlet pressure (p.s.i.)[a]	Buffer composition (ionic strength = 50 mM)	Current (μA)
1	11.35	0.9	0.672 ml 1 M HCl + 0.618 ml butylamine + 658.5 mg NaCl in 0.25 l water	110
2	10.50	0.9	7.855 ml 1 M HCl + 1.235 ml butylamine + 266.8 mg NaCl in 0.25 l water	100
3	2.00	1.6	2.093 ml 85% H_3PO_4 + 2759.8 mg $NaH_2PO_4{\cdot}H_2O$ in 0.5 l water	125
4	9.65	0.9	772.9 mg H_3BO_3 + 9.466 ml 1 M NaOH + 907.8 mg NaCl in 0.5 l water	90
5	2.85	1.6	0.156 ml 85% H_3PO_4 + 1409.6 mg NaH_2PO_4 + 822.8 mg NaCl in 0.5 l water	100
6	8.80	0.9	772.9 mg H_3BO_3 + 3.802 ml 1 M NaOH + 1238.9 mg NaCl in 0.5 l water	100
7	3.70	1.5	0.882 ml HCOOH + 1710.6 mg NaOOCH in 0.5 l water	90
8	7.95	1.0	618.3 mg H_3BO_3 + 0.581 ml 1 M NaOH + 696.6 ml NaCl in 0.25 H_2O	100
9	4.55	1.5	0.487 ml CH_3COOH + 532.3 mg $NaCOOCH_3$ + 352.4 mg NaCl in 0.25 l water	90
10	7.10	1.2	317.2 mg $NaH_2PO_4{\cdot}H_2O$ + 482.7 mg Na_2HPO_4 in 0.25 l water	55
11	6.25	1.3	713.4 mg $NaH_2PO_4{\cdot}H_2O$ + 153.3 mg Na_2HPO_4 + 239.0 mg NaCl in 0.25 l water	75
12	5.40	1.4	0.0896 ml CH_3COOH + 694.3 mg $NaCOOCH_3$ 237.85 mg NaCl in 0.25 l water	85

[a] Fused-silica capillary 50 cm × 75 μm internal diameter.

electrolyte solutions [20–22,27,31,32]. By optimizing the pressure applied to the buffer vial at the capillary inlet it is possible to maintain a (nearly) constant electroosmotic velocity for all electrolyte solutions and a fixed run time for all measurements. This is convenient for automated operation. The pressure sequence employed with the different buffers used for pK_a measurements in our group is indicated in Table 2. If the pressure is set too high, the separation between the neutral marker and sample may be inadequate and the pressure-induced parabolic flow may degrade peak shapes. With pressure-assisted capillary electrophoresis run times are typically 2–3 min at each pH and 40–60 min to measure the pK_a values for each sample, including conditioning and rinse steps.

To increase sample throughput, Wan et al. [31] used pressure-assisted capillary electrophoresis and short-end capillary injection. The distance between the injector and detector was 8.5 cm and the polarity was reversed so that the electroosmotic flow was in the detector direction. The sample throughput achieved, however, was not much greater than for more typical conditions described above. Ishihama et al. [27] used pressure-assisted capillary electrophoresis and two-dimensional photodiode array detection to determine migration times. The sample throughput was reduced to about 14 min per sample, sufficient to allow pK_a measurements for some hydrolytically unstable compounds that could not be obtained by conventional measurements. Eventually, the limit to sample throughput is dictated by two factors. The need to obtain a reasonable separation range for the neutral marker and ionized form of the sample and the number of pH buffers used to fit the effective mobility data to the appropriate model equation for interpretation. For this reason, parallel separations in a capillary-array is probably the best option for increasing sample throughput [29].

Dilute electrolyte solutions are desirable to minimize activity corrections, temperature gradients and viscosity differences. However, it is important that solutions have sufficient buffer capacity to maintain a fixed pH in the presence of the sample. Inadequate buffer capacity may result in pH drift and errors in mobility and pK_a values. This requires a compromise in which electrolyte solutions of a low and constant ionic strength but adequate buffer capacity are employed for all measurements. For typical sample concentrations, electrolyte solutions with an ionic strength in the range 10–100 mM are usually adequate.

Buffer properties can change in use over time because of electrochemical reactions at the electrodes and the absorption of carbon dioxide from the air. The most general electrochemical reaction is the hydrolysis of water, which leads to an accumulation of hydrogen ions at the cathode and hydroxide ions at the anode [15,19,32–35]. Representative data for buffer depletion during the high throughput measurement of pK_a values is provided by Jia et al. [32], summarized in Table 3. The initial and final pH values refer to the start and end of a sequence to measure pK_a values for 20 compounds over 20 h of automated operation without buffer replenishment. Small changes (ΔpH = 0.01–0.07) were observed for buffers below pH 10 due to electrolysis. These changes are tolerable for all but the most accurate measurements. For the pH 10 and 11 buffers, the pH dropped from 0.1 to 0.24 pH

Table 3
Stability of electrolyte solutions after 20 h of automated operation to determine pK_a values for 20 compounds (ionic strength = 10 mM, voltage = 30 kV)

pH_i (initial)	pH_a (anode)	pH_c (cathode)	ΔpH	
			Anode	Cathode
2.42	2.45	2.47	0.03	0.05
2.95	2.90	3.00	−0.05	0.05
3.90	3.85	3.86	−0.05	−0.04
4.76	4.69	4.72	−0.07	−0.04
5.85	5.79	5.89	−0.06	0.04
6.64	6.57	6.65	−0.07	0.01
7.42	7.37	7.45	−0.05	0.03
8.71	8.66	8.70	−0.05	−0.01
9.33	9.27	9.32	−0.06	−0.01
10.0	9.90	9.94	−0.10	−0.06
11.0	10.76	10.83	−0.24	−0.17

units over the course of the measurements. This change in pH was due mainly to the absorption of carbon dioxide from the air. These changes are outside the acceptable error for measuring pK_a values. Purging the buffer reservoirs with a nitrogen atmosphere is a poor solution because of the open design of electrolyte reservoirs to accommodate automation of capillary electrophoresis instruments. The programmed replenishment of electrolyte solutions is a possible solution but is not supported by all automated instruments. A practical solution for samples in batches of 20 is to run each sample in the high-pH electrolyte solutions first, after which each sample is run in the remaining buffers in an alternating high-to-low pH fashion [20,21].

To obtain stable migration times new capillary columns are flushed with 1 M sodium hydroxide for 15 min (inlet pressure 20 p.s.i.; 1 p.s.i. = 6894.76 Pa) followed by deionized water for 15 min (inlet pressure 50 p.s.i.). These or similar conditions are commonly used for any new fused-silica column for use in capillary electrophoresis. For each measurement of effective electrophoretic mobility a sequence made up of column conditioning steps followed by the actual mobility measurement is required. The time and number of the conditioning steps adversely affect sample throughput. There is no consensus among individually developed methods for the need and number of sequential conditioning steps between mobility measurements in different electrolyte solutions. In our group we find it adequate to flush the column from the detector end with water for 0.5 min (50 p.s.i.) followed by the electrolyte solution to be used for the measurement for 0.75 min (50 p.s.i.). We then alternate the buffer solutions (Table 2) from high to low after running the high pH electrolyte solutions first (see buffer stability above). Other authors add a rinse step with sodium hydroxide before the water rinse and for high pH buffers a rinse with hydrochloric acid. Since there is no definitive recommendation that can be made for the column conditioning steps, the reader will have to be guided by his/her practical experience and the importance of sample throughput in establishing the method.

3.4. Data analysis

Almost all pK_a values are determined by fitting the appropriate model from Table 1 by non-linear regression to a series of effective mobility measurements collected at different pH values. A typical fit for a sample with two basic and one acidic ionization center is shown in Fig. 2. An appropriate experimental design for non-linear regression is to space the electrolyte pH values at roughly equal intervals (e.g. 0.85 pH units) over the pH operating range of 2–12 [20,21,36]. For monoprotic acids and bases, it is possible to linearize the general model for the fit of the effective mobility to the pH of the electrolyte solutions. Linear regression, however, is adversely affected by the wider variance in the mobility values determined in the low pH electrolyte solutions [36]. Weighted linear regression and non-linear

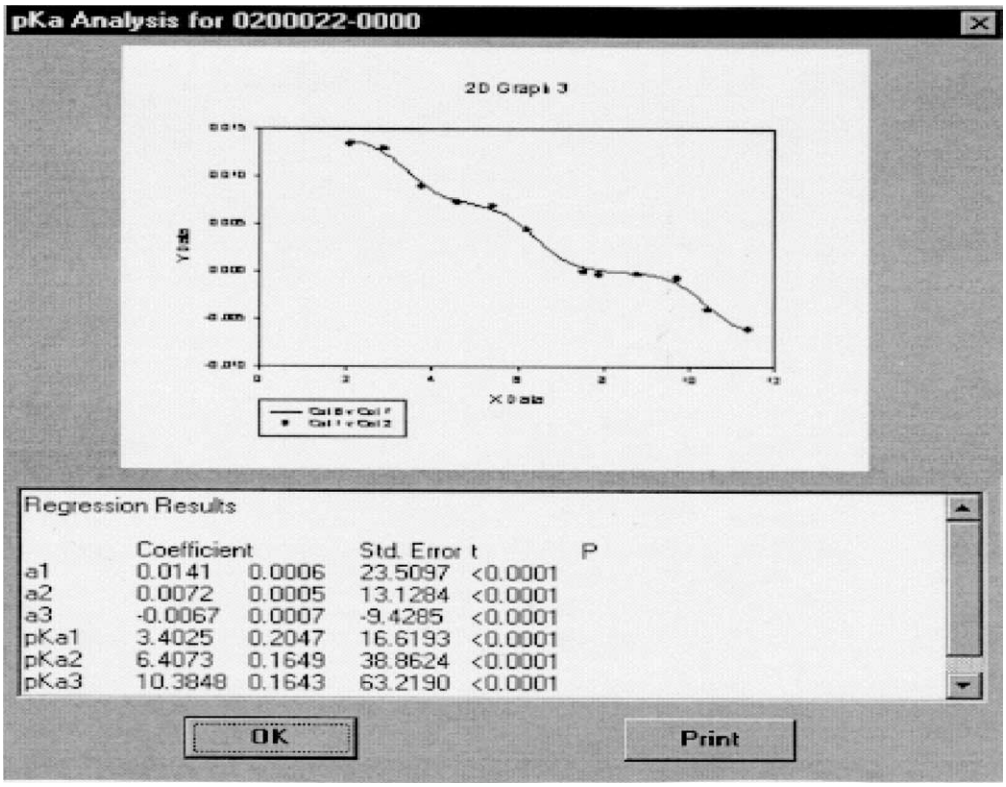

Fig. 2. Curve fit for the change in effective electrophoretic mobility of a diacid monobase compound as a function of the pH of the electrolyte solution.

regression afforded similar results that were much closer to literature values than observed for linear regression. These results tend to confirm that non-linear regression is the preferred fitting technique for all models, in broad agreement with the consensus among literature reports.

If an acceptable estimate of the pK_a value is available, a wide pH scale for measurements is unnecessary. A group of equally spaced pH buffers arranged symmetrically around the estimated pK_a value for the range pH = pK_a ± 2 is generally suitable.

An experienced analyst is required to identify the appropriate model equation from Table 1 based on the appearance of the effective mobility against pH plot and the expected pK_a value from the structure of the molecule. This may not always be obvious for compounds with an unexpected ionization center or with closely spaced pK_a values. Miller et al. [21] have proposed a novel method to automate the identification of the appropriate model equation using the empirical relationship between the effective mobility, molecular mass (M_r) and calculated charge (z_c):

$$m_{\text{eff}} = \frac{a\, z_c}{(M_r)^b} \tag{11}$$

to calculate z_c for anions, where $a = 0.185$ and $b = 0.492$, and for cations, where $a = 0.389$ and $b = 0.633$. The most positive and negative experimental effective mobility values are entered into Eq. (11) to estimate z_c. After rounding to the nearest whole number, the z_c values are used to assign the appropriate model (e.g. monobasic, dibasic, dibasic monoacid, etc.)

4. Non-aqueous and partially-aqueous electrolytes

There are only a few reports of the measurement of pK_a values for other than completely aqueous electrolyte solutions. Aqueous–organic solvent mixtures were used to estimate pK_a values for samples sparingly soluble in water [26,37,38], and in one case, to estimate pK_a values of weak bases to model their interactions in reversed-phase liquid chromatography [39]. Measurement of pK_a values in non-aqueous solvents were made primarily to explain migration properties and to optimize separations of ionizable compounds [40–43]. Samples of low water solubility may be more soluble in organic solvents, which in addition, have a significant effect on the ionization properties of weak acids and bases. On the other hand, solvents of low dielectric constant are of limited practical interest, since virtually no solvent separated ions exist in solution. Most studies so far use either water–organic solvent mixtures or pure alcohols, acetonitrile and their mixtures.

For partially aqueous electrolyte solutions, aqueous pK_a values are estimated by extrapolation of the apparent pK_a values for several mixed solvent systems to zero organic solvent composition [26,37,38]. This approach is not a substitute for direct measurements since a large number of additional experiments are required, typically multiples of the number of solvent compositions employed for the extrapolation. In addition, the electroosmotic velocity and ion mobility values are smaller for aqueous–organic solvent mixtures than for purely aqueous solutions, extending the time for measurements.

The standard pH scale defined for water has limited applicability to organic solvents. A new pH* scale for the solvated proton in the organic solvent is required. When pK_a^* values for suitable acids in the selected solvent are available, these can be used as a reference scale without further calibration (each acid is mixed with an equimolar portion of a suitable salt) [41]. Using this approach it was shown that for a series of bases the pK_a^* values in methanol were about 2.7 units higher than in aqueous solution but much smaller than the difference for acids, which increased by about 5 units. For aqueous–alcohol mixtures containing up to 80% (v/v) propan-1-ol [32], ethanol [35] or methanol [36] the pK_a^* values for 26 benzoic acids increased by up to 2–2.5 units compared with their values in water. The significant increase in pK_a values for the aqueous–organic solvent mixtures was explained by the lesser ability of the alcohols to solvate the anion leading to an additional loss of stabilization of the ionized particle. Although the number of studies and variety

Table 4
Typical pK_a values for some weak acids and bases determined by capillary electrophoresis using the electrolyte solutions indicated in Table 2 under high-throughput conditions

Compounds	pK_a values		
	Measured	Lit.	Difference
Benzoic acid	4.16	4.21	0.05
2-Chloro-5-nitrobenzoic acid	2.01	2.17	0.16
4-Chlorophenol	9.21	9.43	0.22
Flufenomic acid	3.63	3.65	0.02
Ibuprofen	4.14	4.41	0.27
Ketoprofen	4.02	4.23	0.21
1-Naphthol	9.38	9.30	−0.08
Phenylacetic acid	4.27	4.32	0.05
o-Phthalic acid	2.97	2.95	−0.02
	5.11	5.41	0.30
Resorcinol	9.05	9.30	0.25
	11.36	11.06	−0.30
Salicylic acid	2.93	2.98	0.05
2-Aminopyridine	6.71	6.65	−0.06
4-Aminopyridine	9.29	9.13	−0.16
Benzocaine	2.52	2.51	−0.01
2-Bromoaniline	2.33	2.53	0.20
4-Chloroaniline	3.97	3.99	0.02
Impiramine	9.58	9.49	−0.09
Nicotine	3.24	3.12	−0.12
	8.06	8.02	−0.04
Pyrilamine	9.10	9.02	−0.08
2,4,6-Trimethylpyridine	6.65	6.45	−0.20
3-Aminobenzoic acid	2.86	3.07	0.21
3-Amino-2-naphthoic acid	4.76	5.01	0.25

of solvents evaluated is not great, they suggest that pK_a^* values can be measured in non-aqueous and partially aqueous electrolyte solutions by capillary electrophoresis with the same facility, and by similar methods, to aqueous solutions.

5. Applications

Compounds of interest to the pharmaceutical industry dominate applications of capillary electrophoresis for the measurement of pK_a values [15,16,20,21,27,30–33]. High throughput methods are now commonplace in support of drug discovery. Sample throughput is typically about 20–35 samples per day per instrument for compounds with pK_a values between 2 and 11. Some representative examples using the buffer systems described in Table 2 are shown in Table 4. The agreement with other methods (literature values in Table 4) is acceptable. The consensus value for the difference between pK_a values measured by electrophoresis and literature values is about 0.2 log units for pK_a values between 3 and 10. The agreement is generally not as good for weak bases with pK_a values between 2 and 3 and for weak acids with pK_a values between 10 and 11. When considering differences with literature methods, it is important to note that there is error in both the literature pK_a values and those measured by capillary electrophoresis. The absolute accuracy of pK_a values determined by capillary electrophoresis is unknown. The results obtained by capillary electrophoresis, however, show good agreement with other common measurement methods, which often are more demanding from the point of view of sample size and purity, automation, sample throughput and expense. The repeatability of pK_a values measured by capillary electrophoresis is typically reported to be in the range 0.02–0.07 log units.

Table 5
Measurement of pK_a values for miscellaneous compounds by capillary electrophoresis

Compound	Buffer range	Ref.
Ten alkaloids from Chinese herbs	3.81–9.29	[46]
Fifteen alkylphosphonic acids (used indirect detection)	2.0–4.0	[24]
Eight 2-amino-2-oxazolines (anti-hypertensive agents)	4.77–9.69	[47]
Five anthraquinones from Chinese herbs		[38]
Three anthrocyclines (antibiotics)	4.20–8.20	[52]
Nine cephalosporins (antibiotics)	2.0–9.0	[50]
Ten cytokinins (phytohormones)	1.5–6.0	[17]
Eight dihydrofolate reductase inhibitors		[49]
Eight heterocyclic aromatic amines	2.99–9.03	[48]
Twelve hydroxy-*s*-triazine degradation products	2.05–12.7	[51]
Four low-molecular-mass organic acids (used indirect detection)	2.54–5.10	[23]
Twelve quinolines (antibiotics)	2.0–11.0	[26]
Ropinirole (anti-Parkinson's disease) and five impurities	2.20–11.42	[45]
Ten sulfonated azo dyes	5.6–12	[25]
^{99m}Technetium radiopharmaceuticals (used γ-ray sensitive detector)	2.71–6.60	[44]

Reports of pK_a measurements by capillary electrophoresis for specific compound types are summarized in Table 5. Most applications were reported in the last few years reflecting the growing awareness of the value of capillary electrophoresis for pK_a measurements and the increasing availability of capillary electrophoresis instruments in analytical laboratories.

6. Conclusions

Capillary electrophoresis is now the method of choice for the high-throughput determination of pK_a values in industry. Results are in good agreement with traditional methods. Only compounds lacking a suitable chromophore, due to the widespread use of absorbance detection, and compounds with pK_a values outside of the range 2–11, are difficult to handle. Already a number of groups are looking at ways to further increase sample throughput in response to an increasing workload. The most promising solution is represented by instruments employing capillary arrays operated in parallel for the measurement of effective electrophoretic mobilities. In a general sense, this retains all working aspects of current methods for pK_a measurements combined with the advantages of parallel sample processing for a higher throughput. If these instruments prove to be as rugged and reliable as conventional-single column instruments then there is little reason why they should not be accepted in laboratories with high throughput requirements. Another area likely to receive attention is the automation of data analysis. This process is quite time consuming and to some extent depends on the skill of the analyst to select the appropriate model for curve fitting. Automation of this process would free-up skilled laboratory staff for other purposes.

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