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Automated pK_a determination at low solute concentrations by capillary electrophoresis

J.A. Cleveland, Jr. and M.H. Benko

Discovery Research, DowElanco, 9410 Zionsville Road, Indianapolis, IN 46268-1053 (USA)

S.J. Gluck* and Y.M. Walbroehl

Analytical Sciences, 1897B Building, Dow Chemical Co., Midland, MI 48667 (USA)

ABSTRACT

Capillary electrophoresis is investigated for the determination of thermodynamic pK_a measurements at low solute concentrations, a current limitation of potentiometric titrations. It is not necessary to accurately know the concentration of a titrant or solute. The method relies on measuring the ionic mobility of the solute as a function of pH. Mobility and pH data are fitted to an equilibrium expression with a non-linear regression. The detection limit for benzoic acid is 2 μM. Equations are introduced to remove the need to measure buffer pH outside of the capillary and to handle potential discontinuities in solute mobility between different buffer solutions caused by changes in the shape of the solute molecule and the buffer viscosity.

INTRODUCTION

Knowledge of dissociation constants (*i.e.*, pK_a) is fundamental for understanding and quantifying chemical phenomenon, biological activity and environmental fate [1–3]. The determination of dissociation constants of weakly acidic or basic compounds is routine using established techniques if the compound has amenable physical properties [4–7]. However, the low solubility of many pharmaceutical and agricultural compounds in water precludes convenient pK_a determinations. Recently [8,9] capillary electrophoresis (CE) has been introduced as a method for convenient and precise aqueous pK_a determination. Our principal reason for investigating this approach lies in the high sensitivity and selectivity of CE relative to potentiometry. In this paper we, therefore, explore the benefits of CE for pK_a measurements.

* Corresponding author.

CE offers several advantages over the two most commonly used methods for pK_a determination: potentiometric titration and ultraviolet spectroscopy [4–6]. Precise potentiometric titration at low concentrations requires time-consuming solvent preparation for carbonate-free solutions and the availability of fully automated, commercial instrumentation for multiple sample determinations is limited. Furthermore, compounds must be soluble at a concentration $\geq$1 mM, although indirect determination of pK_a below this limit is possible using computer-assisted techniques [10]. Solubility limitations may also be circumvented by working in mixed solvents. The obtained pK_a values may provide information for ranking compounds within a family; however, extrapolation of the data to an aqueous state is dubious because of different solvation mechanisms and uncertainty in defining the standard state [4–6]. Another common alternative for determining the pK_a of low solubility compounds is UV–Vis spectrophotometric titration [4–6,11]. Determination of pK_a values by

UV–Vis spectrometry hinges on the neutral and ionic species having different spectra. When this criterion is met, excellent precision is obtained; however, these measurements are time-consuming and no automated instrumentation is available commercially. Other, less common but not all inclusive, methods include conductivity [5], calorimetry [12] and isotachophoresis (ITP) [13–15] for determining pK_a values. Conductivity was largely replaced by potentiometry, calorimetry remains a rather specialized approach and, as outlined by Beckers *et al.* [8], the calculation of mobilities and pK_a values in ITP can be laborious as compared to the CE methodology.

The advantages of using CE to determine accurate thermodynamic pK_a values of compounds with diverse solubilities are numerous. CE requires small amounts of sample at low solute concentrations. Indeed, the procedure does not require measurement of solute or titrant concentrations, only migration times. Commercial CE instruments are automated and as a result, ionic species distribution curves of the solute can be generated in a timely manner, thereby minimizing potential solute decomposition. Furthermore, calculations are straightforward and independent of solute purity. This procedure has the potential to become a universal technique for determining aqueous pK_a values in the 1 to 12 pH range.

THEORETICAL

Definition of pK_a^{th}

The thermodynamic equilibrium constant associated with the dissociation of a weak acid, *e.g.*,

$$HZ \rightleftharpoons H^+ + Z^- \quad (1)$$

is defined as

$$K_a^{th} = \gamma_{Z^-} \gamma_{H^+} \cdot \frac{[H^+][Z^-]}{[HZ]} \quad (2)$$

where γ_{HZ}, the activity coefficient of the undissociated acid, is assumed to be 1. Eqn. 2 can be rewritten in the form

$$pK_a^{th} = pH - \log \gamma_{Z^-} - \log \frac{[Z^-]}{[HZ]};$$

$$pH = -\log [H^+] \quad (3)$$

Activities can be calculated from Debye–Hückel theory at 25°C according to the relationship

$$-\log \gamma = \frac{0.5085 z^2 \sqrt{\mu}}{1 + 0.3281 a \sqrt{\mu}}; \quad \mu = \frac{1}{2} \sum_{i=1} C_i z_i^2 \quad (4)$$

where a is the hydrated diameter of an ion in Å, C is the molarity of the ion, z is the valency of the ion, and μ is the ionic strength of the solution. In general the exact value of the parameter a, which can range from 1–11 Å, will not be known. Throughout this study, the value 5 Å was assumed.

Substituting eqn. 4 into eqn. 3 gives

$$pK_a^{th} = pH - \log \frac{[Z^-]}{[HZ]} \pm \frac{0.5085 z^2 \sqrt{\mu}}{1 + 0.3281 a \sqrt{\mu}} \quad (5)$$

where the activity correction is positive for acids and negative for bases.

Mobility and pK_a^{th}

In CE, a voltage, V, is applied across a capillary of length, L_c, resulting in an electric field strength E given by

$$E = V / L_c \, (kV/cm) \quad (6)$$

The electrophoretic mobility of an ion is generally expressed as

$$m_e = v_e / E \quad (7)$$

where v_e is the local electrophoretic velocity and E is the local electric field strength. If the distance from the injection point to the detector is L_d, and the migration time of an analyte is t_{app}, the apparent mobility, m_{app}, is given by

$$m_{app} = \frac{v_{app}}{E} = \frac{L_c L_d}{t_{app} V} \quad (8)$$

In general, m_{app} does not equal m_e because the observed velocity is the sum of electrophoretic and electroosmotic flow (EOF)

$$m_{app} = (v_e + v_{EOF}) / E \quad (9)$$

Hydrated cations in the vicinity of the capillary wall result in electroosmotic flow of the bulk liquid through the detector towards the cathode (ground). EOF is calculated by spiking the analyte solution with a marker which remains neutral throughout the entire sequence of buffers

used in a determination. Hence, electrophoretic mobility is determined according to the relation

$$m_e = (m_{app} - m_{EOF}) = \frac{L_c L_d}{V}\left(\frac{1}{t_{app}} - \frac{1}{t_{EOF}}\right) \quad (10)$$

When an acid, HZ, is deprotonated, the net electrophoretic mobility, m_e, in a given buffer of a given concentration is given by $m_e = \alpha m_a$, where m_a is the electrophoretic mobility of the fully deprotonated species Z^- and α is the fraction of analyte ionized. Using this relation, it is possible to rewrite $[Z^-]/[HZ]$ in terms of mobility:

$$\frac{[Z^-]}{[HZ]} = \frac{\alpha}{1-\alpha} = \frac{m_e}{m_a - m_e} \quad (11)$$

For acids, this relation can be substituted into eqn. 5 to give

$$pK_a^{th} = pH - \log\left(\frac{m_e}{m_a - m_e}\right) + \frac{0.5085z^2\sqrt{\mu}}{1 + 0.3281a\sqrt{\mu}} \quad \text{(acids)} \quad (12)$$

It is also possible to derive an analogous expression for bases, B:

$$pK_a^{th} = pH + \log\left(\frac{m_e}{m_b - m_e}\right) - \frac{0.5085z^2\sqrt{\mu}}{1 + 0.3281a\sqrt{\mu}} \quad \text{(bases)} \quad (13)$$

where m_b is the electrophoretic mobility of the fully protonated species, BH^+.

EXPERIMENTAL

Buffer considerations

A number of factors must be considered in forming a standard buffer series for automated pK_a determination. Electroosmotic flow can be expressed as

$$m_{EOF} = \epsilon\zeta/4\pi\eta \quad (14)$$

where ϵ is the dielectric constant of the solution, η is the viscosity, and ζ is the zeta potential measured at the shear plane close to the liquid–solid interface. The zeta potential is inversely proportional to the charge per unit surface area, the valency, and the square root of the buffer concentration. Hence, the run time is directly proportional to the square root of the buffer concentration. In cases of very low EOF such that $m_{EOF} < m_e$, anions will not be detected.

While dilute buffers are desirable from considerations of the run time, Joule heating and activity, concentrated buffers also offer certain advantages, such as buffering capacity. Concentrated buffers are less likely to exhibit pH shifts due to CO_2 dissolution. Sample stacking, which can lead to sensitivity enhancements of 10–100×, also increases as the ionic strength of the run buffer is increased relative to the sample.

The buffer series given in Table I represents a reasonable compromise of the considerations given above and is by no means the only possible series.

TABLE I

STANDARD BUFFER SERIES

Component	pH	[HZ]	$[Z^-]$	μ	$\log\gamma$[a]
(1) citrate	2.64	0.009	0.002	0.002	0.020
(2) citrate	3.14	0.002	0.002	0.002	0.020
(3) acetate	3.75	0.020	0.002	0.002	0.020
(4) acetate	4.25	0.006	0.002	0.002	0.020
(5) acetate	4.75	0.002	0.002	0.002	0.020
(6) acetate	5.25	0.001	0.005	0.005	0.028
(7) acetate	5.75	0.001	0.014	0.014	0.045

[a] Sign of correction is (+) for acids and (−) for bases.

Instrument parameters

A SpectraPHORESIS 1000 (Spectra-Physics Analytical, Fremont, CA, USA) was used for all experiments. A 2-s hydrodynamic injection was performed. Since the hydrodynamic injection rate is 6 nl/s for a 70 cm × 75 μm untreated fused-silica capillary (Polymicro Technology, Phoenix, AZ, USA), 12 nl was loaded onto the column. The separation distance, L_d, was 68 cm. The temperature was set at 25°C. Absorption was monitored at 220 nm. With the instrument operating at 25 kV, currents of <20 μA were observed.

In order to equilibrate the column and thereby minimize hysteresis effects, the following wash cycle was performed prior to each run in a sequence: (1) 2.5 min with 0.1 *M* NaOH, (2) 2.5 min with water, and (3) 5.0 min with running buffer.

Because the SpectraPHORESIS 1000 is equipped with a single reservoir for the buffer near the detector, it is not possible to match running and trailing buffers in a sequence. This feature can lead to significant back-migration through the column if the reservoir is filled with an alkaline (highly mobile) buffer while the running buffer is acidic (weakly mobile). No back-migration was observed when the reservoir was filled with the pH 4.25 acetate buffer in Table I.

Method

All solutions were prepared using distilled, deionized, and filtered water (ASTM type I specification). Stock citrate buffers were prepared by titrating citric acid solutions with 0.1 *M* NaOH until the desired pH was reached. Stock acetate buffers were prepared by combining appropriate amounts of sodium acetate and glacial acetic acid to achieve the proper pH and concentration. Prior to an experiment, stock solutions were diluted by a factor of 10.

Equal volumes of the neutral marker (200 μM mesityl oxide) in water and analyte solutions were combined and filtered into a 2-ml sample vial. Mesityl oxide was preferred as a marker over toluene and benzyl alcohol because of its larger absorption coefficient; peak shapes for the three molecules were nearly identical when compared on normalized amplitude and time scales.

Because the buffers are relatively dilute, CO_2 dissolution or absorption can change pH significantly over time. Prior to each analysis, an Orion pH meter was calibrated using NIST (National Institutes of Standards and Technologies)-traceable buffers having pH 2.00 ± 0.02, 4.00 ± 0.01 and 6.00 ± 0.01. The autosampler in the CE apparatus was purged with nitrogen during runs as an added precaution against CO_2 absorption.

Unless otherwise noted, the seven buffers given in Table I were used in order from high to low pH. Since each mobility determination was 20 min in length, the total analysis time per sample (including wash cycles) was 3.5 h. No attempt was made to optimize total analysis time which could probably be cut in half by optimizing the column length, field strength, run time and wash cycle time.

Mobilities were calculated according to eqn. 10. Data pairs of pH and m_e were imported into MathCad (MathSoft, Cambridge, MA, USA) where m_a and pK_a were determined by performing a non-linear fit to eqns. 12 and 13.

RESULTS

It has been observed that at low pH silica capillaries can exhibit hysteresis over many days [16]. Table II shows calculated m_{app}, m_{EOF} and m_e values for benzoic acid in ascending and descending pH sequence modes. In ascending mode, m_{EOF} is significantly lower in every instance due to hysteresis; however, m_e is remarkably reproducible. As a result, the order in which the buffers are run does not appreciably affect the outcome of the pK_a determination.

Plots of m_e *vs*. pH are given in Fig. 1 for acids and in Fig. 2 for bases. Simulated curves using the parameters obtained from the non-linear fit are superimposed on the data. In Table III, CE-determined pK_a values are compared with literature values ($\mu = 0$) [12]. Agreement is to within 0.07 pH units in all cases. The largest discrepancy exists for pyridine, which because of its weak UV chromophore, necessitated preparation at the 1 m*M* level. Because buffer concentrations range from 4–22 m*M*, an analyte at the 1 m*M* level changes the pH. As a result, the pH of the local environment of the analyte will not be

TABLE II

STABILITY OF EFFECTIVE MOBILITY

Buffer pH	Sequence direction	m_{app} ($\times 10^5$ cm^2/V s)	m_{EOF} ($\times 10^5$ cm^2/V s)	m_e ($\times 10^5$ cm^2/V s)
6.59	Descending	53.905	90.741	−36.836
	Ascending	42.813	79.870	−37.057
6.06	Descending	48.756	85.515	−36.759
	Ascending	38.477	75.269	−36.792
5.71	Descending	50.725	87.578	−36.853
	Ascending	39.692	75.969	−36.277
4.68	Descending	36.870	65.203	−28.333
	Ascending	24.691	52.688	−27.997
4.01	Descending	36.995	51.770	−14.775
	Ascending	41.142	44.384	−14.456
3.19	Descending	41.142	44.384	−3.242
	Ascending	36.787	40.033	−3.246

TABLE III

CE-DETERMINED pK_a VALUES *VS.* LITERATURE VALUES AT 25°C [12]

Molecule	Acid/Base	pK_a (lit.)	pK_a (CE)	Solute concentration (CE)
Pyridine	Base	5.19	5.26	1 m*M*
Aniline	Base	4.60	4.66	150 μM
Cinnamic acid	Acid	4.40	4.40	50 μM
Benzoic acid	Acid	4.20	4.18	50 μM
p-Bromoaniline	Base	3.88	3.85	50 μM
Salicylic acid	Acid	2.98	2.96	50 μM
o-Bromoaniline	Base	2.53	2.55	50 μM

equivalent to that measured for the buffer alone which is a reasonable explanation for the error in the pyridine measurement. For the buffer series used here, accuracy to within 0.03 pH units is possible for analyte concentrations of <100 μM.

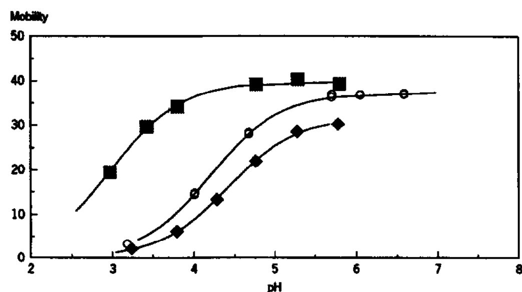

Fig. 1. Plots of mobility ($m_e \times 10^5$ cm^2/V s) *vs.* pH for acids with superimposed curve fits. ■ = Salicylic acid; ○ = benzoic acid; ◆ = cinnamic acid.

Very few data are required to attain an accurate pK_a by this technique. In fact, it is not even necessary to bracket the pK_a. For example, only

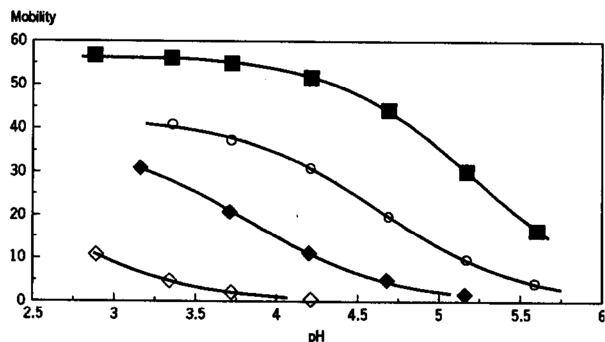

Fig. 2. Plots of mobility ($m_e \times 10^5$ cm^2/V s) *vs.* pH for bases with superimposed curve fits. ■ = Pyridine; ○ = aniline; ◆ = *p*-bromoaniline; ◇ = *o*-bromoaniline.

four points were used for *o*-bromoaniline, all of which were above the pK_a. Similarly, six points were used for salicylic acid, all above the pK_a of the acid.

DISCUSSION

Limitations/factors impacting precision

The practical range for buffer pH in CE is between 2 and 12. For pH values greater than 12, the concentration of highly mobile OH^- results in excessive conductivity. For pH values less than 2, the same effect can be attributed to the presence of H^+. It is reasonable, then, that the pK_a range for this technique is between 1 and 12 since it is not necessary to cross the pK_a in the experiment.

The upper sample concentration limit depends on the accuracy required for the measurement and the concentrations of the running buffers. For the buffers used in this study, sample concentrations of <100 μM were necessary in order to obtain pK_a values to within 0.03 of the reported values.

Detection limit in CE is a function of a number of parameters, the most significant for this application being the optical absorption coefficient. Using increasingly dilute solutions of benzoic acid, $S/N = 2$ was observed at a concentration of 2 μM. Therefore, the detection limit under the conditions of this study can be expressed as $\varepsilon^{220}bc = 1 \cdot 10^{-4}$, where ε^{220} is the molar extinction coefficient at 220 nm, b is the path length and c is the solute concentration. Alternate detection schemes must be sought for those cases where the analyte is a very weak UV chromophore and/or has sparingly low solubility in water.

Sample stacking is a means of increasing sensitivity by a factor of 10–100 by preconcentrating the analyte [17]. While some sample stacking does occur, as manifest by the narrow widths of analyte peaks relative to marker peaks, the effect is not as great as it might be because of the low ionic strengths ($0.002 < \mu < 0.014$) of the running buffers. Another limitation to sample stacking is the inability to further preconcentrate at the solubility limit of the solute. If the sample is injected from a saturated solution, stacking is limited according to the solubility relations

$$S = S_0 + \frac{S_0}{\gamma_{\pm}^2} \text{antilog}(\text{pH} - \text{p}K_a) \quad \text{(acids)} \qquad (15a)$$

$$S = S_0 + \frac{S_0}{\gamma_{\pm}^2} \text{antilog}(\text{p}K_a - \text{pH}) \quad \text{(bases)} \qquad (15b)$$

where S_0 is the molecular or intrinsic solubility.

A potential source of error is solute adsorption to the capillary wall. Though not observed in our work, it is most likely to occur for bases and larger molecules. Even if the analyte were not totally bound to the capillary wall, severely skewed peak shapes could lead to errors in the mobility measurement.

For the mobility calculations, it was assumed that the electric field strength was carried entirely by the running buffer. Because of the small injection size, coupled with the diluteness of the buffers, this assumption is reasonably valid. The 12-nl injection volume corresponds to 2.7 mm, or <0.5% of the total column length. In this study, because of the inherent resolution of CE and the ideal behavior of the solutes studied, the largest factor impacting precision appears to be measurement of buffer pH.

Potential improvements

Electrophoretic mobility is usually expressed by the following equation

$$m_e = q/6\pi a\eta \qquad (16)$$

where q is the charge of the species' ionic cloud, a is its Stokes radius and η is the buffer viscosity. However, a more accurate treatment would include a numerical factor, f, accounting for the shape of the ion [18]. Since the parameters η, q and a are all sensitive to the buffer used, m_a will also be sensitive to these parameters. Discontinuities are most likely to occur when buffer composition is changed, such as when making the transition from acetate to citrate.

While η, a property of the buffer, can easily be measured experimentally, the parameters q, a, and f cannot . It is possible to calculate m_a from the ionic equivalent conductance, λ_c, and Faraday's constant, F [8]. However, an empirical

approach might prove more practical for routine analysis. The samples could be spiked with both neutral and ionized markers; the ionized marker being a species which is totally ionized for every buffer in the series. The mobility of the ionized marker, m_+, could then be used as an additional parameter and included in modified versions of eqns. 12 and 14:

$$pK_a^{th} = pH - \log\left[\frac{m_e}{(m_a m_+ / m_{max}) - m_e}\right] + \frac{0.5085 z^2 \sqrt{\mu}}{1 + 0.3281 a\sqrt{\mu}} \quad (17)$$

$$pK_a^{th} = pH + \log\left[\frac{m_e}{(m_b m_+ / m_{max}) - m_e}\right] - \frac{0.5085 z^2 \sqrt{\mu}}{1 + 0.3281 a\sqrt{\mu}} \quad (18)$$

where m_{max} is a normalization constant corresponding to the maximum absolute value of m_+ measured.

A second potential improvement lies in the measurement of buffer pH which is the largest source of error in these measurements and also one of the most labor intensive operations. A solute with a well established pK_a (pK'_a) is added to the sample injection mixture to serve as an internal pH and activity correction reference. Eqn. 17 becomes

$$pK_a^{th} = pK'_a + \log\left[\frac{m'_e}{(m'_a m_+ / m_{max}) - m'_e}\right] - \log\left[\frac{m_e}{(m_a m_+ / m_{max}) - m_e}\right] \quad (19)$$

where the reference compound is an acid and the unknown is an acid and

$$pK_a^{th} = pK'_a - \log\left[\frac{m'_e}{(m'_a m_+ / m_{max}) - m'_e}\right] - \log\left[\frac{m_e}{(m_a m_+ / m_{max}) - m_e}\right] + \frac{1.017 z^2 \sqrt{\mu}}{1 + 0.3281 a\sqrt{\mu}} \quad (20)$$

where the reference compound is a base and the unknown is and acid. Eqn. 18 becomes

$$pK_a^{th} = pK'_a - \log\left[\frac{m'_e}{(m'_a m_+ / m_{max}) - m'_e}\right] + \log\left[\frac{m_e}{(m_b m_+ / m_{max}) - m_e}\right] \quad (21)$$

where the reference compound is a base and the unknown is a base, and

$$pK_a^{th} = pK'_a + \log\left[\frac{m'_e}{(m'_b m_+ / m_{max}) - m'_e}\right] + \log\left[\frac{m_e}{(m_a m_+ / m_{max}) - m_e}\right] - \frac{1.017 z^2 \sqrt{\mu}}{1 + 0.3281 a\sqrt{\mu}} \quad (22)$$

where the reference compound is an acid and the unknown is a base.

CONCLUSIONS

An automated method for obtaining pK_a values for acids and bases using CE was described and investigated. The method has several advantages for determining the pK_a values of compounds with low water solubility. For example, compounds of limited water solubility need not be prepared in a co-solvent and it is not necessary to accurately know the concentration of a titrant or solute. There is no time-consuming preparation of carbonate-free buffers. The detection limit using the parameters, instrumentation and electrolytes of this study was $\varepsilon^{220}bc = 1 \cdot 10^{-4}$ which, for benzoic acid, was 2 μM, 500 times lower than a typical detection limit via potentiometric titration. The accuracy was within 0.03 pH units for sample concentrations below 100 μM. The potential pK_a range which may be achieved is between 2 and 12. The analysis time was 3.5 h/sample, which could be reduced by a factor of two by optimizing experimental parameters.

Equations were introduced for handling potential discontinuities between buffer solutions in a, η and f. These equations were expanded remove the need to measure pH values of the running buffers by using an *in situ* probe of known pK_a to determine the pH in the CE column. Implement-

ing both of these procedures would be expected to improve the accuracy and decrease the labor for a determination.

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