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Estimation of pK_a values using microchip capillary electrophoresis and indirect fluorescence detection

Christa A. Currie [a], William R. Heineman [a,*], H. Brian Halsall [a], Carl J. Seliskar [a], Patrick A. Limbach [a], Francisco Arias [b], Kenneth R. Wehmeyer [b]

[a] *Department of Chemistry, University of Cincinnati, P.O. Box 210172, Cincinnati, OH 45221-0172, USA*
[b] *Procter and Gamble Pharmaceuticals, 8700 Mason-Montgomery Rd., Mason, OH 45040, USA*

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Abstract

Microchip capillary electrophoresis (CE), coupled with indirect fluorescence detection was investigated for estimating the pK_a values of non-fluorescent compounds. The CE method is based on the differences in electrophoretic mobility of the analyte as a function of the pH of the running buffer. Nine compounds were tested, including several of pharmaceutical importance, with pK_a values from 10.3 to 4.6. All buffers contained 5-TAMRA as the fluorescent probe for indirect detection. Calculated pK_a values agreed well with literature values obtained by traditional methods, differing not more than 0.2 from the literature value. The current work on single lane chips demonstrates the principle of microchip CE with indirect detection as a viable method for estimating pK_a values. However, increased throughput will be required using a multilane chip to enable the approach to be used practically.

Keywords: Microchip capillary electrophoresis; pK_a; Indirect fluorescence detection

1. Introduction

The last few years have seen many advances in drug research, design, and discovery. A result of this is that modern combinatorial synthetic methods yield a large number of compounds. A key property of these compounds of physiological import is the pK_a value, which can affect the pharmacokinetics of a compound, absorption and partitioning, and the affinity for drug metabolizing enzymes and drug targets [1]. Traditional ways to measure pK_a values include potentiometry and spectroscopy, both of which have a low sample throughput, high materials consumption, and require a high purity sample [2]. More recently, high performance liquid chromatography (HPLC) has been used to measure pK_a [3–6], but although these methods can be used for samples of lower purity, they often require large amounts of solvents and long analysis times [2].

Capillary electrophoresis (CE), however, has minimal solvent and sample requirements, faster analysis times, and generates little waste and has been investigated as an attractive alternative technique [7–9]. UV detection is perhaps the most popular [10–20] of the CE-based approaches for the estimation of pK_a values, although conductometric [18], amperometric [21], and indirect UV detection [22] have also been used. In an important study, Barbosa et al. compared CE with traditional methods and concluded that values determined by CE are in good agreement with both literature and calculated values determined by traditional methods [2].

The migration of CE to a microchip platform has gained increasing attention during the past decade [23–25]. Since the introduction of the micro-total analysis systems in 1989, numerous reports have been published concerning the successful transition of CE systems to microchips [26–29]. While UV detection is convenient in conventional CE systems, it is not commonly used for microchips because the short optical path lengths offer limited sensitivity. Therefore, fluorescence detection has been the method of choice

* Corresponding author. Tel.: +1 513 556 9210; fax: +1 513 556 9239.
E-mail address: william.heineman@uc.edu (W.R. Heineman).

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for most microchip applications [30], even though most analyte species do not fluoresce intrinsically. Such species can be detected without using chemical derivatization, by indirect fluorescence detection, where a highly fluorescent compound is added to the buffer system to provide a stable fluorescent background signal. As analytes migrate through the detection area they dilute, or displace, the absorbing fluorophore and reduce the background signal [31–33]. The displacement is due to the requirement of charge neutrality when the charged analyte is in the presence of an absorbing fluorophore of the same charge [34]. Indirect fluorescence detection has been used on a microchip to determine phenolic compounds [35], explosives [36], photographic developing agents [37], biogenic amines [38], and sugars [39].

Here, we present, for the first time, the use of microchip capillary electrophoresis and indirect fluorescence detection to measure the pK_a values of a variety of pharmaceuticals.

2. Experimental

2.1. Chemicals

Sodium tetraborate, boric acid, sodium phosphate (dibasic), sodium phosphate (monobasic), acetic acid, ethosuximide, procaine hydrochloride, ranitidine, prilocaine hydrochloride, cimetidine, aniline and dimethyl sulfoxide (DMSO) were from Sigma (St. Louis, MO, USA). Sulfanilamide, uracil, and 2,4,6-collidine were from Aldrich (Milwaukee, WI, USA) and 5-carboxytetramethylrhodamine (TAMRA) was from Molecular Probes Inc. (Eugene, OR, USA). Reagent grade sodium hydroxide (0.1 M) and hydrochloric acid (0.1 M) and sodium acetate were from J.T. Baker (Phillipsburg, NJ, USA).

2.2. Microchip CE equipment

All microchip electrophoresis experiments were done on a Micralyne Microfluidic Tool Kit (μTK) instrument (Micralyne Inc., Edmonton, AB, Canada). The system consists of a high voltage (HV) power supply and a laser-induced fluorescence (LIF) detection system containing a 532 nm frequency-doubled Nd-YAG laser (4 mW), a dichroic beam splitter, a 550 nm long-pass filter, a 568.2 nm bandpass filter and a PMT detector. The μTK instrument was controlled by LabView software (National Instruments, Austin, TX, USA), and the data were recorded and analyzed by Turbochrome 4.0 (Perkin-Elmer Corp., Cupertino, CA, USA). All the microchip experiments were completed on a Micralyne (Micralyne Inc., Edmonton, AB, Canada) simple cross glass chip (Fig. 1). The detection distance was set at 74 mm from the intersection of the injection and separation channels.

2.3. Preparation of Buffers and Samples

Acetate, phosphate and tetraborate stock buffers were prepared to cover pH's from 3.4 to 5.4, 5.7 to 8.0 and 7.5 to 12.1, respectively. Working buffer stock solutions were prepared by mixing the two appropriate buffer stock solutions in equivalent amounts as shown in Table 1, diluting to an ionic strength of 0.01 M, and adjusting the pH to the appropriate value with 0.1 M NaOH or 0.1 M HCl measured with a Beckman 50 series pH meter (Beckman Coulter Inc., Fullerton, CA, USA) which was calibrated daily. All working buffer solutions were prepared fresh daily and stock buffer solutions were stable for at least one month. Working buffer solutions were prepared for indirect detection by adding an aliquot of a TAMRA stock solution to the appropriate run buffer to give final TAMRA concentrations of 0.1, 1, 2, 5, and 10 μM.

Fig. 1. Diagram of a simple T Micralyne microchip. B: buffer, BW: buffer waste, S: sample, and SW: sample waste.

Stock solutions (10 mM) of all analytes were prepared in water, and DMSO was added to each sample at a final concentration of 5% (v/v), to be used as a neutral marker. Analyte samples were prepared by diluting the 10 mM analyte stock solutions to 1 mM using the appropriate stock run buffer.

2.4. Microchip separation conditions

The microchip was conditioned by washing sequentially with 0.1 M NaOH (5 min), dd-H_2O (10 min), and working buffer (10 min). This procedure was also repeated between working buffers of different pH's. The conditioning was done by applying a vacuum to one reservoir and filling the other three reservoirs with the appropriate solution. At the end of each day the microchip was cleaned with 0.1 M NaOH (5 min) and dd-H_2O (10 min). The channels were dried under vacuum and the microchip was stored dry.

All solutions were loaded manually into the microchip using a micropipet. The working buffer was loaded into the buffer reservoir (50 μL), buffer waste (50 μL) and sample waste (45 μL). Analytes were loaded into the sample reser-

Table 1
Buffer system for pK_a determination

pH range	Stock solution
3.4–5.4	0.5 M sodium acetate 0.5 M acetic acid
5.7–8.0	0.1 M sodium phosphate (dibasic) 0.5 M sodium phosphate (monobasic)
7.5–9.2	0.05 M sodium tetraborate 0.2 M boric acid
9.2–12.1	0.05 M sodium tetraborate 0.1 M sodium hydroxide

Stock solutions were prepared monthly. All run buffers were prepared fresh daily by mixing equivalent amounts of the stock solutions and diluting to the desired ionic strength.

Table 2
Voltage program for pK_a determination[a]

	Conditioning	Injection	Separation
Buffer	2.50 kV	3.50 kV	2.50 kV
Buffer waste	grnd	3.95 kV	grnd
Sample	2.25 kV	3.50 kV	2.25 kV
Sample waste	2.25 kV	grnd	2.25 kV
Time	240 s	15 s	90 s

[a] Three step voltage program used for pK_a determination with indirect fluorescence detection.

voir (50 μL). A 240 s conditioning step was done online, prior to the first sample injection, followed by four repeat on-chip injections using the voltage protocol in Table 2. A "pinched sample plug" was generated at the intersection with voltage applied to the sample, sample waste, and buffer reservoirs while holding the sample waste at ground. During the separation, voltages were applied to the sample and sample waste to prevent sample leakage and pull back any remaining sample at the intersection, and to the buffer reservoirs, while the buffer waste was held at ground.

2.5. Calculation of pK_a values

The relationship between the electrophoretic mobility (μ_e) of small analytes and the ionization constant of acids and bases has long been developed [40]. The method relies on the principle that the analyte has an electrophoretic mobility that depends on the pH of the surrounding solution. If the analyte is fully charged, it has maximum mobility; and if the analyte is neutral, it has no electrophoretic mobility. Intermediate mobilities are a function of the ionization equilibrium and can be obtained by regression analysis. The μ_e of an analyte can be found by measuring the migration time of the analyte (t_{app}) and the migration time of a neutral marker (t_{eof}) according to Eq. (1), where V is the applied voltage; L_d, the length from the injection intersection to the detection point; and L_c, the total channel length.

$$\mu_e = \frac{L_c L_d}{V}\left(\frac{1}{t_{app}} - \frac{1}{t_{eof}}\right) \quad (1)$$

The pK_a value for an analyte is determined by calculating the μ_e of the analyte for a series of buffers bracketing the pK_a value. Two methods of calculation, non-linear and linear regression, have been used. In the case of the linear regression model the inverse of the μ_e is directly proportional to the inverse of the hydrogen ion activity (a_{H+}) according to equation 2, where μ_{HB} (the mobility of the fully ionized form of the analyte) and K_a are regression parameters that can be solved for the pK_a using Microsoft Excel [15].

$$\frac{1}{\mu_e} = \frac{K_a}{\mu_{HB}}\left(\frac{1}{a_{H+}}\right) + \frac{1}{\mu_{HB}} \quad (2)$$

Alternatively, non-linear regression analysis can be used for a compound having a single ionizable group by plotting the electrophoretic mobility versus the pH according to Eq. (3). Non-linear regression analysis can be used to calculate the pK_a value where μ_a (the mobility of the fully ionized form of the analyte) and pK_a are regression parameters [41].

$$\mu_e = \frac{\mu_a}{10^{(pH-pKa)} + 1} \quad (3)$$

For this work, the pK_a values were calculated by linear regression since it applies to pK_a measurements where data points are collected in a narrow range of pH values [15].

3. Results and discussion

3.1. Investigation of separation conditions

The pK_a value of the analyte is estimated by measuring the migration of the analyte in relation to the migration of a neutral marker over a specific pH range. In this range, the charge, or the percentage of analyte that is charged, is changing, thus changing its separation. Also, since the only parameter needed from the electropherograms is migration time, baseline resolution is not a requirement. Therefore, when determining the system parameters, the goal is not to find the optimum separation conditions at a particular pH (as is normally done with a separation), but to find universal conditions that will yield some level of separation (rather than complete resolution) of the analyte, neutral marker, and system peaks at all pH's. In addition, the use of indirect fluorescence detection, and the effect the system parameters have on the sensitivity of the detection, must also be considered. Uracil was used as a standard to adjust the system parameters for the pK_a determinations.

An initial concentration of 4 μM TAMRA was used as the background electrolyte for indirect fluorescence detection. The buffer ionic strength was evaluated by measuring the detection limits of uracil at different ionic strengths. Better detection limits are obtained for indirect detection when the ionic strength is minimized; however, an increase in ionic strength improves separation performance. Since complete baseline resolution was not required for this work, the ionic strength was adjusted to the lowest ionic strength that still provided a buffering effect. It was determined that an ionic strength of 0.01 M met these requirements.

Injection and separation voltages were also examined. As previously stated the goal when adjusting the separation conditions (i.e. voltage, detection length) is to find conditions that will result in a separation between the analyte, the neutral marker, and any system peaks at all pH values. For this reason, long separation times and a long detection length were chosen to ensure that, under all conditions, the analyte was sufficiently separated from the several system peaks often associated with indirect detection, and the neutral marker [42,43]. This was investigated by examining the separation of uracil at pH 10, where the analyte is fully charged and most easily separated from the neutral peaks, and at pH 8.5, where complete separation is quite a challenge. At higher

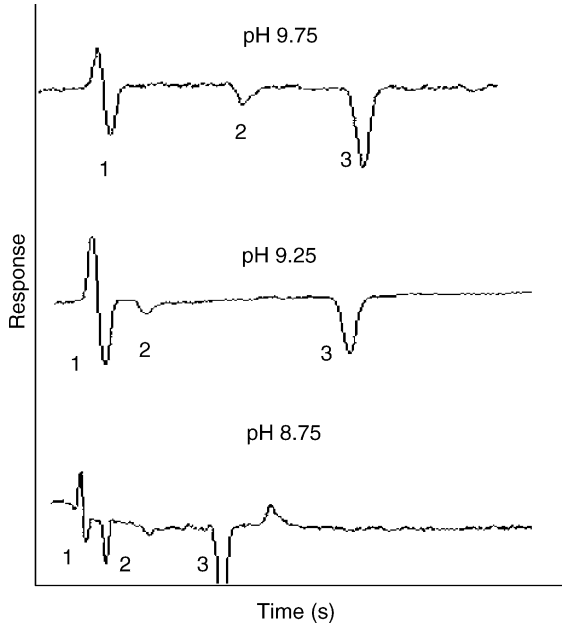

Fig. 2. Electropherograms showing the migration of (1) DMSO, (2) uracil, and (3) system peak as a function of pH. pK_a assay conditions: 74 mm detection length, voltage program as shown in Table 2 with a run time of 90 s, 2 μM TAMRA as the background electrolyte, 0.01 M ionic strength buffers as shown in Table 1.

voltages it was found that the analyte could not be adequately resolved from the system peaks, even at the higher pH. It was also noted that higher voltages resulted in a higher detection limit. This is supported by Morin et al. [44], who observed a three- to four-fold improvement in the detection limits as the separation voltage decreased. At very low voltages the separation time became quite long, and distorted peaks were observed; therefore, a midrange voltage program was used. The separation voltage conditions are given in Table 2. Representative electropherograms obtained for uracil at three pH's under these ionic strength and voltage conditions are shown in Fig. 2. The neutral DMSO peak migrates with the EOF, and, relative to DMSO, uracil migration varies with pH, increasing in μ_e as the working buffer pH passes through uracil's pK_a. The later migrating peak is a system peak observed with the working buffer in the indirect detection mode.

3.2. Optimization of indirect detection

Critical steps in developing indirect detection are optimizing the choice of fluorophore and its working concentration. The ideal fluorophore should have a high extinction coefficient to provide adequate fluorescence at low concentrations. The fluorophore should have mobility similar to the analytes so that the fluorophore is present in the sample band for displacement, and it should have a fairly consistent fluorescence response at the working buffer pH's. The concentration of the fluorophore must also be optimized, since if it is too high, the difference between the background signal and the displacement by the analyte will be decreased, leading to lower sensitivity. If it is too low, the baseline noise increases and the sensitivity decreases [45]. Here, TAMRA was used as the fluorophore. An important feature of the TAMRA dye is that although its fluorescence intensity is independent of the pH of the working buffer [46], it has ionizable groups (pK_a < 4.0) that will affect the required charge displacement phenomenon for indirect detection below pH 4, and therefore the suitability of TAMRA. However, between pH's 4 and 12 TAMRA is charged and can be used for indirect fluorescence detection.

The optimum concentration of fluorophore was determined by analyzing a standard solution of sulfanilamide (1 mM) in sodium tetraborate buffer (pH 11, 0.01 M ionic strength). In theory, compounds of similar charge will have similar detection limits due to the charge displacement nature of the detection system. Therefore, optimization of the fluorophore with one analyte is applicable to all analytes of similar charge. Concentrations of TAMRA between 0.1 and 10 μM were evaluated. The signal-to-noise (S/N) ratio for each TAMRA concentration was found by measuring the peak height of the analyte versus the peak height of a blank sample at the same migration time. It was concluded, based on the S/N ratio, that 2 μM was the optimum concentration of TAMRA in this system (data not shown). In addition, it was found that an on-line conditioning step helped to prevent instability in the baseline during the analysis by allowing the system to reach a stable baseline and providing stabilization time for the laser. This conditioning step consisted of applying for 4 min the same voltages used during separation.

3.3. pK_a estimation of test compounds

Nine compounds with pK_a values between 4.6 and 10.1 were chosen as test analytes for the effectiveness of the method. Buffers were prepared between 0.75 pH units above and below the literature pK_a value. Analytes were tested using the optimized indirect fluorescence detection and separation conditions. The μ_e for each analyte and the mobility of the neutral marker were determined, and the pK_a values were calculated using linear regression. There was good agreement between the literature values and those determined by the microchip method (Table 3). The between-day reproducibility over 3 days was measured for uracil and found to be less than 0.22%.

The feasibility of estimating pK_a values by indirect detection microchip CE was demonstrated, however the time required for the measurements in a single-lane chip was limiting. Although the separation for any given working buffer was completed in no more than a few minutes, the total time to complete a pK_a determination for a single compound was about 3–4 h. Therefore, the output of the method was limited to two samples a day. The major time consuming factors for the single-lane chip were the time required to fill and condition the chip between buffers. Clearly, a chip with sufficient lanes would allow pK_a values to be determined in just a few

Table 3
pK_a values determined by microchip CE

Compound	Literature pK_a	Microchip pK_a	Difference
Sulfanilamide	10.31[a]	10.48	0.17
Ethosuximide	9.50[b]	9.52	0.02
Uracil	9.20[c]	9.19	−0.01
Procaine HCl	8.80[b]	8.84	0.04
Ranitidine	8.40[c]	8.54	0.14
Prilocaine HCl	7.90[b]	8.12	0.22
2,4,6-Collidine	7.33[c]	7.36	0.03
Cimetidine	6.73[c]	6.73	0.00
Aniline	4.61[a]	4.54	−0.07

Microchip conditions: voltage conditions as shown in Table 2. Detection length 74 mm. Full conditioning used before the first run and between different buffers. Each pK_a value above is calculated from six data points where each data point is the average of three injections.

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[b] C. Hansch et al., Comprehensive Medicinal Chemistry, vol. 6, New York, Pergamon Press, 1990.

[c] Data from ACD database (http://www.acdlabs.com).

minutes. We are currently developing an appropriate 12-lane chip and apparatus.

4. Conclusions

Microchip CE with indirect fluorescence detection was shown to be an accurate method to estimate pK_a values for a wide variety of compounds. Current requirements for an adequate screening method require accuracy within 0.5 pH units and the calculated accuracy of our method was well within this range. While the current method does not provide any advantage over traditional CE in terms of sample throughput, it does demonstrate the applicability of microchip CE to determine pK_a values. The development of a multilane chip approach is expected to allow pK_a values to be obtained in a few minutes.

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