

Rapid pK_a Estimation Using Vacuum-Assisted Multiplexed Capillary Electrophoresis (VAMCE) with Ultraviolet Detection

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ABSTRACT: A rapid approach for estimating the pK_a value of small organic molecules was developed using vacuum-assisted multiplexed capillary electrophoresis (VAMCE) with ultraviolet detection. The VAMCE method employed a 96-capillary array, arranged in a standard 8 × 12 microtiter plate configuration, with each row of capillaries filled with 12 individual running buffers of equal ionic strength (I = 50 mM) covering a pH range from 2.2 to 10.7. A separate compound was injected hydrodynamically into each row of capillaries allowing the estimation of pK_a values for eight compounds in a single run. The application of a vacuum during the separation generated a bulk fluid flow and allowed the electrophoretic separation to be completed within 5 min. The complete VAMCE method, conditioning, and electrophoretic separation was optimized to allow the pK_a estimation for between 128 to 168 compounds in an 8-h period. The VAMCE method provided a reliable approach for estimating pK_a values both within- and between-day. The pK_a values for a series of 96 compounds estimated by VAMCE agreed well with some of literature pK_a values with an average absolute difference of 0.22 log units.

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INTRODUCTION

Many drug compounds contain at least one acidic and/or basic functionality, and the ionization state of these groups play a large role in determining the physicochemical properties of a compound.¹ Additionally, the ionization state will impact a number of other parameters such as membrane permeability, cell–drug interactions, plasma protein binding, tissue distribution, and drug metabolism.^{1–4} Also, knowledge of a compound's dissociation constant (pK_a) value plays an important role in the development of drug

delivery formulations.⁴ The pK_a for a compound provides a means of determining the extent of ionization of the compound at any solution pH. Determining the pK_a value for new chemical entities (NCEs) provides a means of calculating the solution ionization state of the compound and provides information for the drug discovery and development processes.^{1–5} The ability to generate large numbers of NCEs by combinatorial chemistry coupled with the hundreds of thousands to millions of compounds existing in various pharmaceutical company repositories has increased the need for rapid pK_a determinations.

A number of approaches have been used to determine the pK_a value of small organic molecules including titrations based on potentiometry, spectrophotometry, solubility, and liquid–liquid

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partitioning.^{1-3,6-10} However, traditional potentiometric and spectrophotometric titrations have been the two most widely used techniques.^{1,3,7} In the traditional mode both methods can provide accurate pK_a values with a few mg of material in a reasonable time frame. More recent work in these two areas has centered around the development of microscale techniques that reduce the amount of compound required to the microgram level.^{1,11} However, both traditional and microscale versions of these methods require relatively pure compound, an accurate weighing of the compound and compound stability under the assay conditions.^{1-3,6,7,12} Additionally, traditional potentiometric titrations are typically limited to the analysis of compounds with a water solubility in the 10⁻⁴ M range and spectrophotometric methods require the chromophore be in proximity to the ionization center to ensure a sufficient spectral change between the unionized and ionized species.^{1-3,6,7,10,12}

In the past decade a number of groups have demonstrated capillary electrophoresis (CE) with UV detection as an alternative approach for pK_a determinations.^{1-6,9,12-29} The advantages of CE for pK_a determinations include a microgram sample requirement due to the small injection volume; no need for high compound purity because CE provides a separation, and no weighing of the sample because only migration times are needed to calculate the pK_a.^{1-3,5,6,12}

In CE, a high voltage (10 to 30 kV) is applied across the ends of a narrow bore (20 to 100 μm i.d.) fused silica capillary that is immersed in and filled with a conductive buffer. In the normal polarity mode, the injection end of the capillary (anode) is held at a positive potential relative to the detection end (cathode). The applied voltage induces an electrophoretic migration of charged compounds toward the oppositely charged electrode, and the electrophoretic mobility of a compound is dependent on its size and charge. In addition to the electrophoretic movement, the applied voltage also causes a bulk fluid flow, referred to as the electroosmotic flow (EOF). In simple aqueous buffers, without the addition of modifiers, the EOF is always in the direction of the cathode. The combination of electrophoretic migration and EOF allows positive and negatively charged species to be separated during the same run, while all neutral compounds move unresolved with the EOF.

The electrophoretic mobility of an acidic or basic compound is a function of the ionization state of the compound, and is therefore determined by the pH of the running buffer. The effective electrophoretic

mobility (μ_{eff}) of an acidic or basic compound is given by eq. 1,

$$\mu_{\text{eff}} = (\alpha)(\mu_a) \quad (1)$$

where α is the fraction of the compound in the ionized state and μ_a is the electrophoretic mobility of the fully ionized compound.^{9,14} The μ_{eff} of a compound can be determined from the electropherogram using eq. 2,

$$\mu_{\text{eff}} = (L_d L_t / V)(1/t_m - 1/t_{nm}) \quad (2)$$

where L_d is the capillary length from the injector to the detector, L_t is the total capillary length, V is the applied voltage, t_m is the migration time of the compound, and t_{nm} is the migration time of a neutral compound that moves with the EOF.^{1,3,4,13,16} A plot of the μ_{eff} for a monofunctional basic or acidic compound gives a sigmoidal curve that is described by eqs. 3 and 4, respectively, where μ_{eff} is the effective electrophoretic mobility and μ_a is the electrophoretic mobility of the fully ionized compound.^{4,5,9,14-17}

$$\mu_{\text{eff}} = \frac{(\mu_a)(10^{-\text{pH}})}{(10^{-\text{pK}_a} + 10^{-\text{pH}})} \quad (3)$$

$$\mu_{\text{eff}} = \frac{(\mu_a)(10^{-\text{pK}_a})}{(10^{-\text{pK}_a} + 10^{-\text{pH}})} \quad (4)$$

A nonlinear regression fit of the data using the appropriate equation allows the calculation of the apparent pK_a (pK_a') value of the compound.^{1,4,5,9,14-17} The thermodynamic pK_a value can then be obtained, if desired, for an acidic or basic compound by correcting for ionic strength (I) effects using eqs. 5 and 6, respectively.^{3,4,13,17} The A and B terms are Debye-Huckel parameters, a_o , is the hydrated diameter of the ion, z is the charge number of the ion, and I is the ionic strength.^{1,9,13,17}

$$\text{pK}_a = \text{pK}_a' + \frac{Az^2 I^{1/2}}{1 + Ba_o I^{1/2}} \quad (5)$$

$$\text{pK}_a = \text{pK}_a' - \frac{Az^2 I^{1/2}}{1 + Ba_o I^{1/2}} \quad (6)$$

In water, A has a value of 0.5085, B has a value 0.3281, a_o can range from 1–11 Å, and I is calculated using eq. 7

$$I = 0.5 \sum C_i z_i^2 \quad (7)$$

where C_i is the concentration of the running buffer and z_i is the charge on the corresponding

buffer ions.^{1,3,9,13,17} It should be noted the correction in eq. 7 is valid up to I values of ~ 0.5 M.

One issue in using CE for rapid pK_a estimations is acidic running buffers, generally pH 4 and below, result in a negligible EOF, and hence, long run times. Several groups have demonstrated the use of pressure-assisted CE (PACE) to increase the throughput of pK_a estimations.^{1-4,15,16} In PACE, a pressure is applied to the injection end of the capillary to induce a bulk fluid flow in the capillary thus reducing the separation time. Coupling PACE with UV detection, several groups have reported medium throughput assays capable of providing pK_a data on one compound in 40 min,¹⁵ 20 compounds/day,¹ 100 compounds/week,⁴ and 96 compounds/day.² The use of PACE with mass spectrometry has been reported to provide a throughput of 50 compounds in 150 min when pooling samples together and deconvoluting each compound by its mass.³

Parallel or multiplexed capillary electrophoresis (MCE) was pioneered by the Mathies,^{30,31} Yeung,^{32,33} and Kambara³⁴ groups. The use of multiplexed capillary arrays can increase sample throughput without a tremendous increase in instrumentation complexity or cost. Multiplexed capillary gel electrophoresis has been broadly applied for DNA sequencing and helped drive the completion of the Human Genome Project.³⁵⁻⁴⁵ A few reports have addressed the development, characterization, and application of MCE for enzyme analysis, analysis of endogenous enzyme levels from *in vitro* cell systems, combinatorial screening of catalysis reaction conditions, analysis of small organic molecules, metabolite analysis, and peptide mapping.⁴⁶⁻⁵¹ Recently, the application of MCE for the rapid determination of octanol-water partition coefficients of neutral and basic compounds was reported.^{52,53} We report on the development and application of vacuum-assisted multiplexed capillary electrophoresis (VAMCE) with UV detection for the rapid estimation of pK_a values of monofunctional acidic and basic organic compounds. A 96-capillary array for VAMCE allows the estimation of the pK_a value for 40 compounds in roughly a 2-h period.

EXPERIMENTAL

Chemicals

Phenylbutazone, chlorambucil, diflunisal, and tolmetrin sodium dihydrate were purchased from ICN Biomedicals, Inc. (Aurora, OH). 2-Phenyl-1,

3-indandione was obtained from TCI-EP (Tokyo, Japan). Sulindac was bought from Biosciences, Inc. (La Jolla, CA). Probenecid was from Avocado Research Chemicals, Ltd. (Heysham, Lancs, England). Warfarin was from Ultra Scientific (North Kingstown, RI). Phenolphthalein was purchased from J.T. Baker, Inc. (Phillipsburg, NJ). Sodium hydroxide (0.1 N) was from EMD Chemicals Inc (Gibbstown, NJ). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO). All chemicals were reagent grade or higher. The distilled deionized water (dd-H₂O) was filtered using a Mini-Q filter system (Millipore, Bedford, MA).

Reagents and Solutions

Twelve CE running buffers covering a pH range from 2.2 to 10.7 (leveled ionic strength of $I=50$ mM) and a $10\times$ outlet reservoir buffer concentrate (100 mM sodium tetraborate) were from CombiSep (Ames, IA). The pH values of the running buffers were measured with a calibrated pH meter at 25°C by the manufacturer and are accurate to ± 0.02 pH units. The pH of the buffers from 2.2 to 9.2 were stable for 3 months (within ± 0.04 pH units) when kept tightly sealed between usage. However, the pH 10.7 running buffer dropped by 0.06 pH units over the same time period. Testing of the buffer pH prior to use in the pK_a determinations confirmed the values specified by the manufacturer. The CE running buffers were used as received and the $10\times$ outlet buffer was diluted 10-fold with dd-H₂O prior to use to give a 10 mM sodium tetraborate (pH 9.3) outlet reservoir buffer. The pH of the running buffers were checked prior to and during the course of the runs and were found to remain constant for at least five replicate runs.

Samples

All acidic compounds were dissolved in the pH 2.2 running buffer, and all basic compounds were dissolved in pH 10.7 running buffer. Approximately 1 mg of each compound was added to series of individual vials along with 4 μ L of DMSO and 2 mL of the appropriate buffer. Samples that did not readily dissolve were placed in a sonicator for 10 min, and undissolved material was removed from the solution by centrifugation. An aliquot (0.1 mL) of each sample was added to a single row of a 96-well microtitre tray. Eight sample compounds were analyzed on a plate (see Fig. 1).

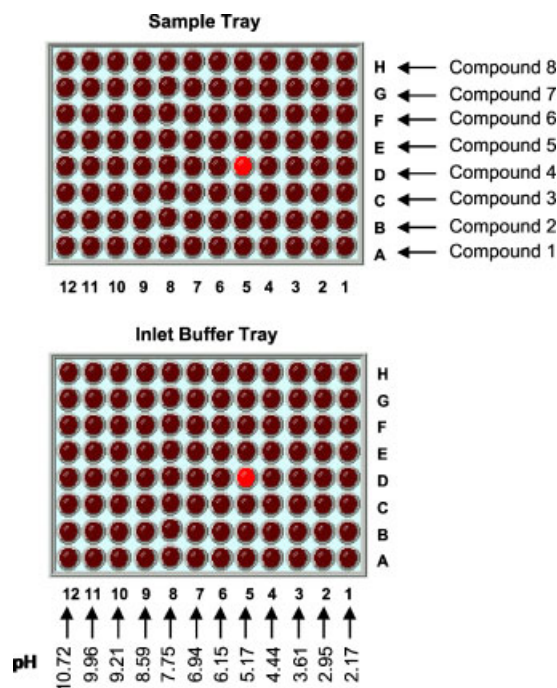


Figure 1. Typical layout of a 96-well sample tray (top) and inlet buffer tray (bottom) for use with 96-capillary VAMCE. Up to eight compounds are loaded in rows of 12 in the sample tray (100 μ L per well). Twelve different pH buffers are loaded orthogonally to the sample tray in a deep well plate (1.2 mL/well). The highlighted well corresponds to the electropherogram obtained for compound 4 at pH 5.17. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

pK_a Estimation

A cePRO 9600TM multiplexed capillary electrophoresis system (CombiSep) equipped with 96 uncoated fused-silica capillaries (28 cm effective length, 54 cm total length, 75 μ m i.d., 150 μ m o.d.), a zinc lamp for UV detection at 214 nm and a photodiode detector was used for pK_a estimations. A running buffer microtitre plate was prepared by adding the 12 CE running buffers to each row of the plate in ascending pH order (see Fig. 1). Thus each row of the plate contained a complete array of the 12 running buffers ranging from pH 2.2 to pH 10.7. The outlet reservoir was filled with the 10-mM borate outlet buffer. Before the first run, the capillaries were rinsed sequentially for 5 min with 0.1 N sodium hydroxide, dd-H₂O, and 10 mM borate buffer. Following the rinsing step, the running buffer plate was placed at the injection end of the capillaries and the buffers were pulled through the capillaries using a vacuum level of

−2.0 psi for 6 min. Samples were then injected hydrodynamically by applying a vacuum of −0.2 psi and various time intervals (2 to 20 s) were evaluated to determine the effect on the calculated pK_a values. Following the injection, the ends of the capillaries were returned to the running buffer plate and a 4.5 kV voltage was applied together with a vacuum to achieve the separation. A range of vacuum levels, −0.1 to −1.2 psi, were evaluated for the separation. Additional sample plates were then processed in a similar manner but with only conditioning with 10 mM borate buffer unless indicated otherwise. The use of a relatively low field strength, 83 V/cm, combined with forced air flow across the capillaries serves to reduce effects of Joule heating during the pK_a analysis.

After the separation, the effective mobility of each compound was determined for each running buffer. The pK_a value for each compound was then calculated by fitting the effective mobility of the compound and the pH values of the buffer using a nonlinear regression using pK_a EstimatorTM software from CombiSep. The apparent pK_a values are reported in the remainder of the manuscript; no effort was made to convert these values to the thermodynamic pK_a values.

RESULTS

Optimization of the Separation Parameters

Benzoic acid and 4-aminopyridine were chosen as model acidic and basic compounds, respectively, for the evaluation and optimization of the various separation parameters for the pK_a estimations. The electropherograms obtained for the two model compounds with the 12 CE running buffers are shown in Figure 2. The effective mobility was calculated for each compound using eq. 2 and the resulting plot of the electrophoretic mobilities versus pH was used to calculate the pK_a values for the two compounds (Fig. 3). The two model compounds were used to optimize a variety of separation conditions including the separation vacuum level, the sample injection time, the capillary conditioning step, and the running buffer replenishment requirements.

VAMCE Vacuum Level

The separation time is an important variable in determining the overall assay throughput for the VAMCE estimation of pK_a values. In CZE, as the

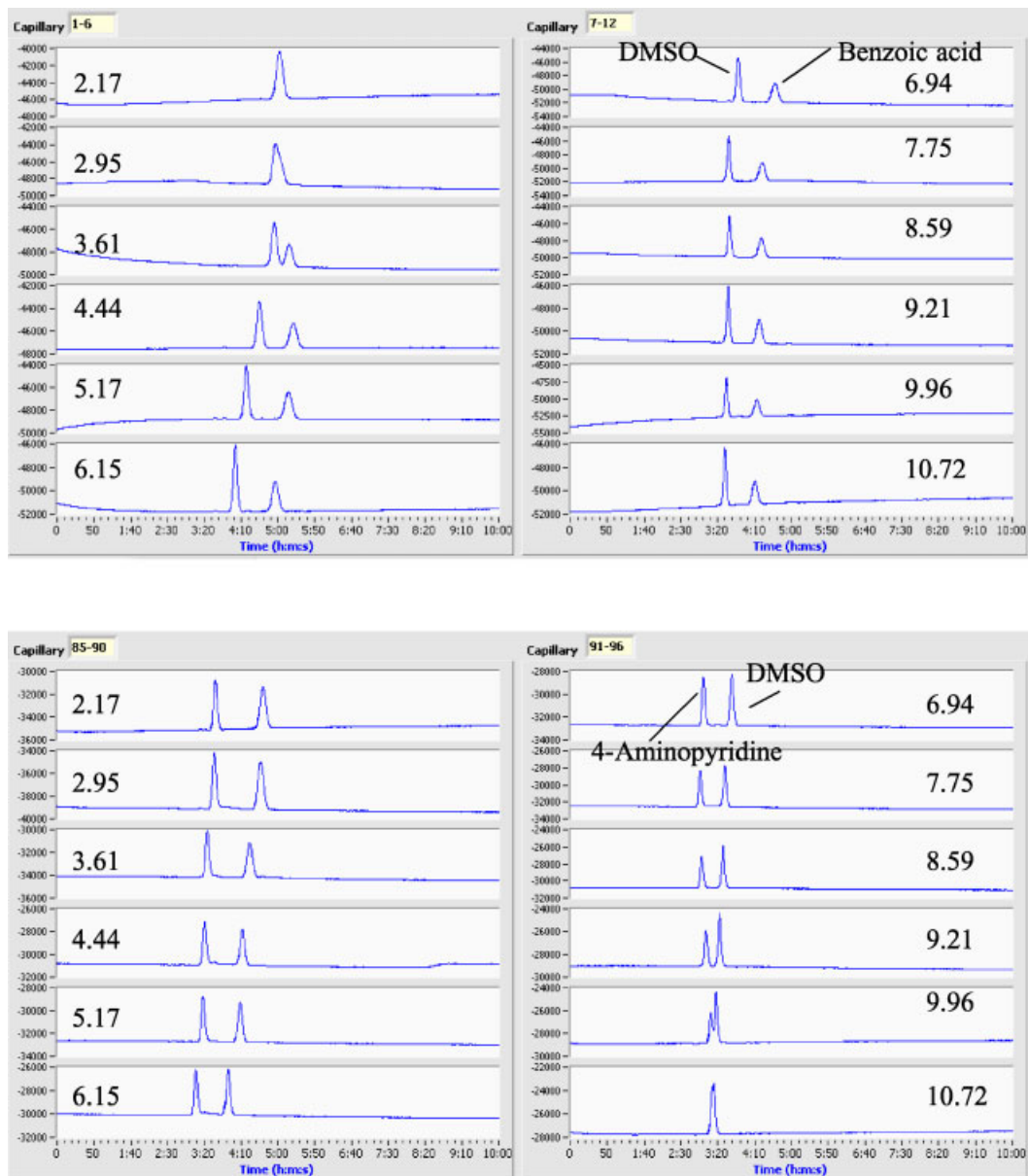


Figure 2. Electropherograms of benzoic acid (top trace) and 4-aminopyridine (bottom trace), each spiked with DMSO, obtained by VAMCE with various running buffers (pH values of running buffer indicated on the electropherograms). VAMCE assay conditions: injection = -0.2 psi for 5 s; separation voltage = 4.5 kV, separation vacuum level = -0.4 psi. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

pH of the running buffer is lowered, there is a corresponding decrease in the EOF, and therefore an increase in the time required for the migration of the neutral marker and the analyte.^{51,54} Through the application of a vacuum, VAMCE provides a means of shortening the analysis time by superimposing a controlled bulk fluid flow in addition to the EOF. The bulk fluid flow results in

a parabolic flow profile and decreases the efficiency achieved from the plug flow profile generated by the EOF.^{51,54} Therefore, the optimization of the separation vacuum level required a compromise between achieving a rapid analysis time and maintaining sufficient separation performance. The separation of benzoic acid in pH 5.17 running buffer under a variety of VAMCE

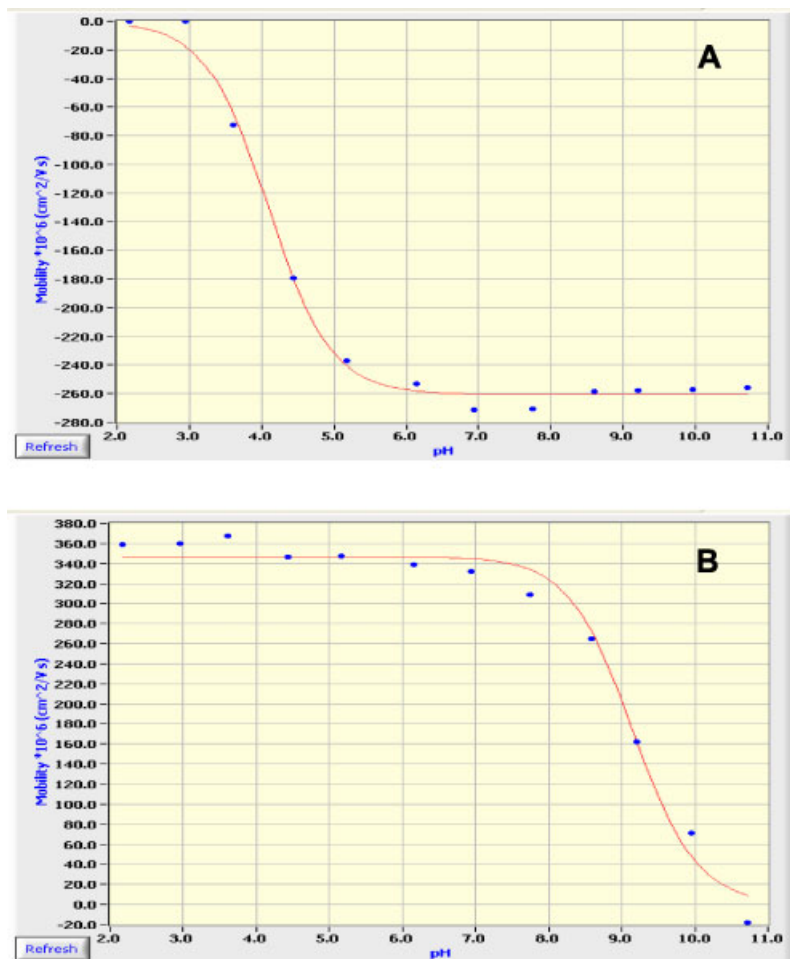


Figure 3. Plot of the effective mobility of (A) benzoic acid and (B) 4-aminopyridine versus running buffer pH. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

vacuum levels is shown in Figure 4. The analysis time decreases as the VAMCE vacuum level increases but the resolution between the DMSO and benzoic acid peaks also decreases with increased VAMCE vacuum level. Eventually increasing bulk flow will degrade the resolution to the point where the calculation of the pK_a value will be adversely affected. For example, reasonable resolution is still obtained at a separation vacuum level of -0.4 psi but is almost completely lost at -1.2 psi. The effect of the separation vacuum level on the calculated pK_a value, the reproducibility of the pK_a measurements, theoretical plates, and the CZE run time for benzoic acid and 4-aminopyridine are shown in Table 1. Good agreement between the calculated pK_a values and the literature pK_a values were obtained at separation vacuum levels of -0.8 psi or lower. Additionally, the reproducibility, as measured by

the % RSD, at the various VAMCE vacuum levels was essentially equivalent as long as the vacuum was maintained at or below -0.8 psi. A broader range of compounds were also examined at three separate separation vacuum levels (Table 2). In general, the best results were obtained using a vacuum of -0.4 psi, although the calculated pK_a values were typically within 0.3 pK_a units regardless of the separation vacuum. A separation vacuum level of -0.4 psi was selected as the best compromise between speed, separation performance, and accuracy of the pK_a estimation. All subsequent experiments were done under -0.4 psi unless mentioned otherwise.

Sample Injection Volumes

A hydrodynamic injection was employed for sample injection by applying a vacuum to the

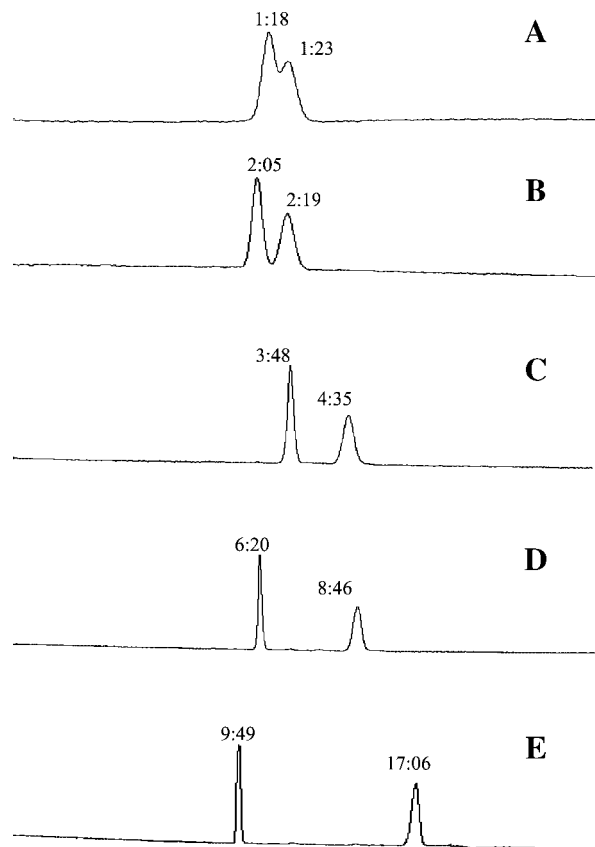


Figure 4. The separation of benzoic acid and DMSO under different vacuum separation levels using pH 5.17 buffer. Vacuum pressures: (A) -1.2 psi; (B) -0.8 psi; (C) -0.4 psi; (D) -0.2 psi; and (E) -0.1 psi.

capillaries for a given period of time. The effect of injected sample volume on the estimation of the pK_a values of the model compounds, benzoic acid, and 4-aminopyridine, by VAMCE was evaluated using an injection vacuum level of -0.2 psi and injection times of 2, 5, 10, and 20 s (Table 3). For benzoic acid, injection times from 2 to 10 s gave

similar pK_a values and similar reproducibility in the pK_a estimation. However, for the basic compound, there was a trend for an increase in the pK_a value with increased injection time. A value of 5 s was selected for the injection time for all subsequent compounds.

Capillary Conditioning

For all the previous experiments the capillaries were given a full conditioning before each run consisting of rinsing the capillaries for 5 min consecutively with 0.1 M NaOH, dd-H₂O and 10 mM borate buffer. The full conditioning step required a total instrument time of 38 min to complete before the CE run could be initiated. Using a separation vacuum level of -0.4 psi the CE run was completed in 5 min. The full conditioning step therefore required roughly eight times longer than the actual CE run. Therefore, following an initial full conditioning, a 5-min rinse with 10 mM borate buffer between consecutive runs was evaluated. The effect of full conditioning and the single 10 mM borate rinse was tested by doing two sets of runs. One set was conducted under the full conditioning before the first run and 10 mM borate buffer-only conditioning for 5 min between runs. The other set was done with full conditioning before each run. For both sets the running buffer plate was changed between each run. The pK_a values obtained for each approach are shown in Table 4. The results demonstrate 10 mM borate conditioning between runs was sufficient to obtain accurate and precise pK_a values. The use of only the 10 mM borate reduced the conditioning time to about 15 min. Therefore, it was possible to estimate the pK_a on eight compounds in roughly 20 min using only the 10 mM borate buffer conditioning step. Data

Table 1. Effect of Vacuum Level on pK_a Value Estimation by VAMCE^a

Assisted Vacuum	Benzoic Acid			4-Aminopyridine			Run Time (min)
	MCE pK_a	%RSD	<i>N</i>	MCE pK_a	%RSD	<i>N</i>	
-0.1 psi	4.12	0.78	4564	9.24	0.19	3880	20.0
-0.2 psi	4.04	0.29	2064	9.21	0.19	2322	10.0
-0.4 psi	4.07	0.62	1764	9.17	0.25	1024	5.0
-0.8 psi	4.29	0.36	697	9.15	0.44	934	2.5
-1.2 psi	4.91	1.12	536	8.74	0.34	940	1.5

^aVAMCE assay conditions: injection = -0.2 psi for 5 s; separation voltage = 4.5 kV and fresh running buffer tray with full conditioning before each run. For each pK_a estimations $n = 3$. Literature pK_a of benzoic acid and 4-aminopyridine are 4.20 and 9.17, respectively. Literature pK_a data obtained from ACD database (<http://www.acdlabs.com>).

Table 2. Effect of Vacuum Level on pK_a Value Estimation by VAMCE^a

Compound	pK _a			Lit. pK _a
	-0.8psi	-0.4psi	-0.2psi	
Nicotinamide	2.83	3.45	3.42	3.56
2-Aminopyrimidine	3.69	3.82	3.79	3.46
Indomethacin	4.79	4.09	4.06	4.30
Naproxen	4.27	4.22	4.28	4.20
<i>p</i> -Anisidine	5.34	5.48	5.36	5.58
2-Methylbenzimidazole	6.30	6.41	6.32	6.29
Uracil	9.28	9.19	9.20	9.45
1-Naphthol	9.97	9.35	9.37	9.30

^aAssay conditions: injection = -0.2 psi for 5 s; separation voltage = 4.5 kV and fresh running buffer tray with full conditioning before each run. Literature pK_a values (see Table 6).

analysis required approximately 10 min per plate, and was performed off-line on a separate computer system.

Buffer Exchange

The running buffers used during the separation provide both a conductive medium and a known pH for the estimation of the pK_a value from the electropherograms. Periodically the running buffers need to be replenished because electrolysis occurs during use, and this can result in a change in the pH of the running buffer solution with repeated usage. The extent of pH change that can occur depends on the total current, the total run time, the buffer strength, and the volume of the buffer reservoirs.⁵⁵ However, changing the buffer between every run uses approximately 8 to 10 mL of each buffer and is also time consuming. To examine for potential buffer depletion effects

Table 3. Effect of Injection Time on pK_a Values Estimation by VAMCE^a

Injection Time (second)	Benzoic Acid		4-Aminopyridine	
	pK _a	%RSD	pK _a	%RSD
2	4.10	0.37	9.17	0.17
5	4.07	0.14	9.24	0.06
10	4.09	0.51	9.32	0.12
20	4.01	0.43	9.51	0.80

^aVAMCE assay conditions: injection = -0.2 psi for indicated times; separation voltage = 4.5 kV, separation vacuum level = -0.4 psi and new running buffer tray with full conditioning were used before each run. For each pK_a value estimation *n* = 3.

Table 4. Effect of Capillary Conditioning Protocol on pK_a Value Estimation by VAMCE^a

	Full Conditioning	Borate Conditioning
	Average pK _a (%RSD)	Average pK _a (%RSD)
Benzoic Acid		
Lane C	4.06 (0.28)	4.07 (0.25)
Lane E	4.04 (0.25)	4.04 (0.29)
Lane G	4.04 (0.29)	4.05 (0.29)
4-Aminopyridine		
Lane D	9.20 (0.38)	9.19 (0.13)
Lane F	9.22 (0.17)	9.22 (0.06)
Lane H	9.21 (0.19)	9.19 (0.13)

^aVAMCE assay conditions: injection = -0.2 psi for 5 s; separation voltage = 4.5 kV, separation vacuum level = -0.4 psi and new running buffer tray with full conditioning were used before each run. Each pK_a value above is the average of three separate runs.

benzoic acid and 4-aminopyridine were repeatedly (*n* = 5) analyzed without changing the running buffer plate. In these experiments a VAMCE vacuum level of -0.2 psi instead of the optimal -0.4 psi was used to exaggerate any depletion effects because the lower vacuum would result in longer run times. The two model compounds were repeated in triplicate on each plate (Table 5). The results show both good reproducibility and good between-run agreement for at least five consecutive runs. Therefore, for routine analysis five runs were employed before changing the running buffer plate.

MCE pK_a Estimation for Test Compounds

A total of 96 commercially available and structurally diverse compounds spanning pK_a values

Table 5. Effect of Running Buffer Change on Reproducibility of pK_a Estimation by VAMCE^a

	Benzoic Acid	4-Aminopyridine
1st run	4.04	9.23
2nd run	4.07	9.25
3rd run	4.07	9.27
4th run	4.07	9.24
5th run	4.07	9.28
Average	4.06	9.25
%RSD	0.33	0.22

^aVAMCE conditions: injection = -0.2 psi for 5 s; separation = 4.5 kV, separation vacuum level = -0.2 psi and full conditioning before the first run and then only 10 mM borate conditioning between runs.

Table 6. pK_a Values Estimated by VAMCE^a

Compound	Lit. pK _a	MCE pK _a	Within-day %RSD (<i>n</i> = 3)	Difference
Metronidazole	2.55 ^a	2.38	1.06	0.17
Diffunisal	3.00 ^b	2.94	0.79	0.06
Salicylic acid	3.01 ^a	2.70	0.57	0.31
Methyl nicotinate	3.13 ^a	3.20	0.65	0.07
2-Fluoroaniline	3.20 ^c	3.23	2.50	0.03
Probenecid	3.40 ^a	3.24	0.82	0.16
2-Aminopyrimidine	3.46 ^d	3.81	0.66	0.35
Ethacrynic acid	3.50 ^a	2.49	1.67	1.01
Tolmetin sodium	3.50 ^b	3.38	0.59	0.12
Nicotinamide	3.56 ^a	3.43	0.34	0.13
Hippuric acid	3.65 ^e	3.43	0.61	0.22
2,6-Dinitrophenol	3.71 ^e	3.43	0.34	0.28
Flurbiprofen	3.80 ^f	3.82	0.84	0.02
p-Aminohippuric acid	3.83 ^b	3.73	0.56	0.10
Furosemide	3.90 ^b	3.56	0.43	0.34
Flufenamic acid	3.90 ^b	4.27	0.54	0.37
<i>N,N</i> -Dimethylnicotinamide	3.92 ^a	3.32	0.46	0.60
4-Chloroaniline	3.99 ^c	4.03	0.52	0.04
Diclofenac sodium	4.01 ^a	3.95	1.17	0.06
Ketoprofen	4.02 ^g	4.06	0.25	0.04
Sulindac	4.03 ^a	3.84	0.60	0.19
Phenindione	4.10 ^b	3.85	0.91	0.25
Quinine				
pK _{a1}	4.10 ^b	4.19	1.01	0.09
pK _{a2}	8.50 ^b	8.51	0.14	0.01
Naproxen	4.20 ^e	4.13	0.98	0.07
2-Benzylamine	4.29 ^a	3.89	0.30	0.40
Indomethacin	4.30 ^a	4.07	1.30	0.23
Phenylbutazone	4.40 ^b	4.23	0.63	0.17
trans-Cinnamic acid	4.44 ^e	4.11	0.00	0.33
Aniline	4.60 ^a	4.80	0.55	0.20
Piroxicam	4.60 ^b	4.94	0.58	0.34
Nicotinic acid	4.76 ^a	4.76	0.32	0.00
<i>N</i> -Methylaniline	4.85 ^c	5.02	0.98	0.17
1,10-Phenanthroline	4.86 ^c	4.55	4.33	0.31
Quinoline	4.97 ^a	5.05	0.23	0.08
Pyridoxine				
pK _{a1}	5.00 ^a	4.88	0.83	0.12
pK _{a2}	8.81 ^a	8.48	0.07	0.33
Aminopyrine	5.00 ^b	5.08	0.11	0.08
Warfarin	5.03 ^c	4.75	0.36	0.28
2-Benzylpyridine	5.13 ^c	5.22	0.58	0.09
8-Chlorotheophylline	5.20 ^a	5.08	0.86	0.12
Isoquinoline	5.40 ^c	5.59	1.00	0.19
p-Anisidine	5.58 ^a	5.52	0.73	0.06
Acridine	5.60 ^a	5.66	1.87	0.06
Sulfamethoxazole	5.81 ^b	6.07	0.58	0.26
Chlorambucil	5.75 ^b	5.01	0.53	0.74
Nalidixic acid	5.94 ^a	5.60	0.41	0.34
Sulfachloropyridazine	6.10 ^b	5.07	0.50	1.03
Dimethadione	6.11 ^a	5.88	1.35	0.23
Triamterene	6.20 ^b	6.24	0.19	0.04
Sulfadimethoxine	6.20 ^h	5.58	1.30	0.62
2-Methylbenzimidazole	6.29 ^a	6.34	0.57	0.05
Flumequine	6.38 ^c	6.09	0.43	0.29
Papaverine	6.39 ^a	6.27	0.00	0.12
2,5-Lutidine	6.43 ^c	6.53	0.49	0.10
4-Chloro-2-nitrophenol	6.45 ^h	6.00	0.93	0.45
3,4-Lutidine	6.47 ^c	6.64	0.46	0.17

(Continued)

Table 6. (Continued)

Compound	Lit. pK _a	MCE pK _a	Within-day %RSD (<i>n</i> = 3)	Difference
Sulfadiazine	6.56 ^a	6.99	0.50	0.43
Trazodone hydrochloride	6.70 ^b	6.76	0.64	0.06
Cimetidine	6.70 ^a	6.75	0.45	0.05
2,4-Lutidine	6.74 ^c	6.75	0.31	0.01
Sulfameter	7.00 ^b	7.21	0.81	0.21
Nitrofurantoin	7.20 ^b	6.96	0.08	0.24
Sulfathiazole	7.23 ^b	7.14	0.16	0.09
Pyrimethamine	7.34 ^b	7.01	0.30	0.33
Sulfamethazine	7.38 ^b	7.53	0.23	0.15
2,4,6-Collidine	7.43 ^c	7.64	0.40	0.21
4-Hydroxybenzaldehyde	7.61 ⁱ	7.94	0.22	0.33
Scopolamine hydrobromide	7.75 ^b	7.69	0.08	0.06
Prilocaine hydrochloride	7.89 ^b	7.92	1.24	0.03
Lidocaine	7.90 ^b	7.92	1.61	0.02
4-Hydroxybenzotrile	7.95 ^j	8.45	0.12	0.50
Azathioprine	7.99 ⁱ	7.63	0.65	0.36
Ranitidine	8.18 ^a	8.29	0.61	0.11
Tranlycpromine	8.20 ^b	8.47	0.07	0.27
Codeine	8.21 ^b	7.81	0.32	0.40
Bendroflumethiazide	8.53 ^a	8.77	0.13	0.24
4-Hydroxybenzamide	8.60 ^a	8.74	0.48	0.14
1-Phenylpiperazine	8.71 ^c	8.37	0.21	0.34
Procaine hydrochloride	8.80 ^b	9.01	0.61	0.21
Dibucaine	8.85 ^b	8.78	0.51	0.07
3-Chlorophenol	9.02 ^e	8.88	0.34	0.14
Sulpiride	9.12 ^b	8.95	0.40	0.17
Acebutolol hydrochloride	9.20 ^b	9.37	0.34	0.17
4-Bromophenol	9.24 ^e	9.12	0.44	0.12
Procainamide hydrochloride	9.26 ^b	9.19	0.79	0.07
1-Naphthol	9.30 ^g	9.34	0.19	0.04
4-Chlorophenol	9.37 ^e	9.25	0.27	0.12
Nadolol	9.39 ^b	9.28	0.37	0.11
Allopurinol	9.40 ^b	9.21	0.33	0.19
Uracil	9.45 ^a	9.18	0.29	0.27
Ethosuximide	9.50 ^b	9.45	0.16	0.05
Atenolol	9.58 ^c	9.42	0.32	0.16
Pindolol	9.70 ^b	9.74	0.59	0.04
Acetaminophen	9.70 ^b	9.46	0.12	0.24
Phenolphthalein	9.71 ^b	9.25	0.22	0.46
(±)-Metoprolol	9.75 ^a	9.36	0.16	0.39
2-Methoxybenzylamine	9.92 ^c	10.30	0.62	0.38
			Average	0.22

VAMCE assay conditions: injection = -0.2 psi for 5 s; separation voltage = 4.5 kV, separation vacuum level = -0.4 psi. Full conditioning used before the first run and then only 10 mM borate conditioning between runs and a fresh running buffer tray was used after every five runs. Each pK_a value above is the average of three separate runs.

^aData from Advanced Chemistry Development, Inc. database (<http://www.acdlab.com>).

^bHansch C, et al., Comprehensive medicinal chemistry, Vol. 6. New York: Pergamon Press; 1990.

^cJia Z, Ramstad T, Zhong M. 2001. Medium-throughput pK_a screening of pharmaceuticals by pressure-assisted capillary electrophoresis. *Electrophoresis* 22:1112-1118.

^dMajor DT, Laxer A, Pischer B. 2002. Protonation studies of modified adenine and adenine nucleotides by theoretical calculations and 15N NMR. *J Org Chem* 67:790-802.

^eDean JA, editor. Lange's handbook of chemistry. New York: McGraw-Hill; 1973 (Table 5.8).

^fKato R. 1984. Non-steroidal anti-inflammatory drugs. In: Yakubutsu-dotai Kenkyukai, editor. Comparative ADME study of drugs, vol. 1. Tokyo, Japan: Maruzen Co., p. 11.

^gPoole SK, Patel S, Dehring K, Workman H, Poole CF. 2004. Determination of acid dissociation constants by capillary electrophoresis. *J Chromatogr A* 1037:445-454.

^hValue from ref. 2.

ⁱValue from ref. 15.

^jLiptak MD, Gross, KC, Seybold PG, Feldgus S, Shields GC. 2002. Absolute pK_a determinations for substituted phenols. *JACS* 124:6421-6427.

from 2.58 to 9.96 were tested by VAMCE using the optimized assay conditions: injection vacuum level, -0.2 psi; injection time, 5 s; VAMCE vacuum level, -0.4 psi; separation voltage, 4.5 kV; and an initial full conditioning followed by only 10 mM borate conditioning between runs. The calculated pK_a values obtained for the compounds by the VAMCE approach are listed and compared to literature values in Table 6. There was relatively good agreement between the literature pK_a values and those estimated by the VAMCE procedure for many compounds with an average absolute deviation of 0.22 pK_a units for the set of 96 compounds. The average absolute deviations for aliphatic carboxylic acid, aromatic carboxylic acids, aliphatic amines, aromatic amines, phenols, aromatic pyridines (mono and polycyclic), and sulfonamides were 0.26, 0.21, 0.17, 0.15, 0.27, 0.20, and 0.29, while the ranges for the absolute differences were 0.02–1.0, 0.00–0.37, 0.02–0.34, 0.03–0.40, 0.04–0.45, 0.01–0.60, and 0.09–0.62, respectively. Additionally, the reproducibility within day, as measured by the %RSD for triplicate determinations, was below 1% for most compounds and was no higher than 4.3% for any compound. The precision of the results demonstrates that a single analysis of a compound is sufficient to obtain estimations of the pK_a values for compounds by the VAMCE approach. Using the current VAMCE methodology it was possible to estimate the pK_a value of

16 compounds the first hour due to the full conditioning step and then approximately 16 to 24 compounds each of the subsequent hours using only the 10 mM borate buffer conditioning step. Data analysis required approximately 10 min per plate and was done using an off-line computer. In an 8-h day, a throughput of 128 to 168 samples can be achieved.

Between-Day Reproducibility

A series of 15 compounds, spanning pK_a values from 2.74 to 9.42, were examined over a 3-day period to determine reproducibility of the VAMCE procedure over time (Table 7). The between-day precision was found to range from 0.17 to 2.59%, with most compounds having a between-day precision of less than 1%. Additionally, the values determined in the between-day precision study agreed, in general, closely with the values previously determined in the single day study (see Table 6).

CONCLUSIONS

The VAMCE methodology was shown to provide a rapid approach for estimating the pK_a values of small organic molecules. The pK_a values found by VAMCE with UV detection were found to be on average within 0.22 pK_a units of those reported in

Table 7. Between-Day Precision for pK_a Estimation by VAMCE^a

Compounds	MCE pK_a				
	Day 1	Day 2	Day 3	Average	%RSD
Salicylic acid	2.75 (0.36)	2.74 (0.56)	2.74 (0.56)	2.74	0.26
Hippuric acid	3.36 (0.62)	3.36 (1.81)	3.35 (1.21)	3.36	0.17
<i>p</i> -Aminohippuric acid	3.78 (0.67)	3.77 (0.41)	3.76 (0.27)	3.77	0.27
Ketoprofen	3.92 (1.78)	3.91 (2.48)	3.94 (3.18)	3.92	0.38
Flufenamic acid	3.92 (1.02)	4.13 (3.05)	4.01 (3.30)	4.02	2.59
Benzoic acid	4.06 (0.14)	4.07 (0.38)	4.04 (0.43)	4.07	0.38
Naproxen	4.12 (0.24)	4.05 (2.16)	4.07 (2.27)	4.09	0.88
Indomethacin	4.25 (0.49)	4.40 (0.92)	4.38 (0.82)	4.33	1.88
4-Nitrophenol	6.13 (1.89)	6.08 (1.52)	6.09 (1.34)	6.10	0.46
Nitrofurantoin	6.86 (0.67)	6.84 (0.80)	7.03 (0.46)	6.85	1.52
Bendroflumethiazide	8.77 (0.17)	8.88 (0.20)	8.84 (0.77)	8.83	0.62
Allopurinol	9.17 (0.19)	9.15 (0.23)	9.15 (0.19)	9.16	0.13
Uracil	9.16 (0.06)	9.19 (0.17)	9.19 (0.11)	9.18	0.19
1-Naphthol	9.30 (0.47)	9.31 (0.22)	9.34 (0.27)	9.31	0.22
Ethosuximide	9.38 (1.09)	9.45 (0.12)	9.43 (0.28)	9.42	0.38

^aSeparation conditions are the same as Table 6. Values in parentheses are within-day %RSD of these $n = 3$ runs.

the literature for a series of 96 compounds. The VAMCE approach was demonstrated to have good within- and between-day accuracy and precision. The methodology allows the estimation of pK_a values for between 128 to 168 compounds in an 8-h period.

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