Internal Standard Capillary Electrophoresis as a High-Throughput Method for pK_a Determination in Drug Discovery and Development

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S *Supporting Information*

ABSTRACT: A novel high-throughput method for determining acidity constants (pK_a) by capillary electrophoresis (CE) is developed. The method, based on the use of an internal standard (IS-CE), is implemented as a routine method for accurate experimental pK_a determination of drugs undergoing physicochemical measurements in drug discovery laboratories. Just two electropherograms at 2 different pH values are needed to calculate an acidity constant. Several ISs can be used in the same buffer and run to enhance precision. With 3 ISs, for

$$pK_{a(TC)} = f(pK_{a(IS_i)}, \mu_i)$$

example, the pK_a of the test compound (TC) can be obtained in triplicate in less than 3 min of electrophoresis. It has been demonstrated that the IS-CE method eliminates some systematic errors, maintaining, or even increasing the precision of the results compared with other methods. Furthermore, pH buffer instability during electrophoretic runs is not a problem in the IS-CE method. It is also proved that after 16 h of electroseparation using the same buffer vial, pH may change by around one unit; but the pK_a calculated by the IS-CE method remains constant. Thus, IS-CE is a powerful high-throughput method for pK_a determination in drug discovery and development.

KEYWORDS: *acidity constant, IS-CE, high-throughput method, pK_a determination, internal standard, capillary electrophoresis*
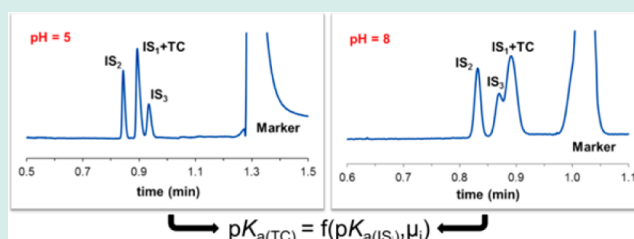
■ INTRODUCTION

Drug discovery has undergone considerable changes with the advent of novel technologies and strategies that have given rise to new opportunities for gathering and integrating information and that can increase the success and efficiency of drug discovery. Pharmaceutical companies synthesize a large number of potential drugs and chemical precursors in relatively short periods.[1,2] Those that are those most suitable are then selected for further testing and development. As a consequence, high-throughput screening for rapid evaluation of compounds is needed as soon as they are available from synthesis.[3−5]

Physicochemical parameters are frequently used as predictors of ADME properties (absorption, distribution, metabolism and excretion) of drugs. One important physicochemical property that affects the pharmaceutical potential of a compound is its acid−base dissociation, defined by its acidity constant (or pK_a on a logarithmic scale). Many potential drugs are weak acids or bases, and their performance in advanced studies depends strongly on their pK_a, since it determines the degree of ionization of the compound under given conditions of pH. Therefore, in drug discovery there is a major need for fast pK_a determination of a large number of compounds.[5,6]

Capillary zone electrophoresis (CZE) has been widely used as a technique for aqueous pK_a determination.[7−15] It is a highly automated technique which requires only small amounts of the sample and reagents. In addition, CZE does not require substances with a high degree of purity because it allows for the separation of impurities and decomposition products from the

main compound. Thus, CZE is an attractive alternative compared to the most common techniques for pK_a determination, such as potentiometric or spectrophotometric titrations, which have some limitations based on the sample quantity, solubility, or purity, among other factors.[13−15]

The classic capillary electrophoresis (CE) method for acidity constant determination involves measuring the mobility of the substance of interest at several pH values which are set by the preparation of suitable buffers at constant ionic strength in different pH ranges.[16,17] However, the classic CZE method is quite slow and not very useful for high-throughput screening. It also has other limitations: interactions between the compound being studied and the different buffers must be avoided; and it requires strict control of temperature to minimize the Joule effect. If such undesired effects are not avoided, deviations in the mobility vs pH curve can result, which affect the final pK_a determination. A faster method to determine acidity constants by CE based on the use of internal standards (ISs) was recently developed by our research group.[18] The IS-CE method is based on the use of an IS with a similar pK_a value to that of the test compound (TC). The IS and the TC are supposed to behave in the same way under the same measurement conditions. Therefore, if they are injected together, the differences in the mobility values of the compounds can be directly related to

differences in their acidity. Provided that the pK_a of the IS is well-known, the pK_a of the drug can be calculated easily. So the IS-CE method benefits from all the advantages of CE as a method for pK_a determination, but in the IS-CE method far fewer measuring points are required. Furthermore, an external accurate measurement of the pH of the buffer solution is not needed because the IS allows the pH to be determined in situ inside the capillary.[18,19]

IS-CE has been applied to determine the acidity constants of simple compounds, such as monoprotic and polyprotic ions with separated pK_a values, as well as more complex ones, ranging from compounds with several close pK_a values to others with acidity constants at the limits of the pH range that is useful in CE.[20−22] The procedure requires a minimum of only 2 CE runs for each pK_a value, which is determined through the ratio between the mobility of the TC and that of the IS. It has been demonstrated that the IS-CE method minimizes the effect of interactions between the TC and the buffer components,[20] and the use of an adequate IS corrects possible systematic experimental errors, such as temperature or pH variations during the determination.[23] In previous work, a set of suitable ISs (acidic and basic monoprotic compounds) with different chemical structure and pK_a values that cover the entire pH range that is useful for CE was established.[20,21] Table 1 lists this proposed set, which is used and tested in the current work.

Table 1. Internal Standard Set[a]

IS	pK_a	IS	pK_a
2-chlorobenzoic acid	2.84	4-nitrophenol	7.09
2,6-dibromo-4-nitrophenol	3.31	vanillin	7.36
4-nitrobenzoic acid	3.37	2,4,6-trimethylpyridine	7.51
2,6-dinitrophenol	3.69	phenobarbital	7.53
3-bromobenzoic acid	3.79	4-hydroxybenzaldehyde	7.61
2,4-dinitrophenol	4.12	lidocaine	7.93
benzoic acid	4.22	clonidine	8.10
ibuprofen	4.49	bupivacaine	8.19
aniline	4.63	3,5-dichlorophenol	8.18
nicotinic acid	4.85	methylparaben	8.35
quinoline	4.93	2-chlorophenol	8.50
4-tert-butylaniline	4.93	1-phenylpiperazine	8.75
warfarin	5.17	N,N-dimethyl-N-benzylamine	8.95
N,N-dimethyl-N-phenylamine	5.17	3-chlorophenol	9.04
pyridine	5.28	diphenydramine	9.08
2,5-dinitrophenol	5.30	procainamide	9.26
sulfacetamide	5.42	4-bromophenol	9.28
acridine	5.55	imipramine	9.37
2,4,6-tribromophenol	6.04	propranolol	9.47
4-tert-butylpyridine	6.03	1-aminoethylbenzene	9.52
papaverine	6.41	paracetamol	9.58
2,4-lutidine	6.81	ephedrine	9.72
trazodone	6.84	phenol	9.89
pilocarpine	7.08	nortriptyline	10.08

[a]Thermodynamic reference pK_a values at 25°C.[20,21]

Although IS-CE is a fast method, it cannot be considered a genuine high-throughput method as several parameters have not yet been optimized. Capillary conditioning processes prior to analysis take some time, and electrophoretic determination could be improved to become faster, particularly at low pH values where the electroosmotic flow is very slow. Con-

sequently, here we sequentially test and optimize several instrumental parameters and different experimental conditions to reduce run time and both the pre- and postconditioning steps as much as possible without losing precision. Furthermore, other important aspects of the implementation of the method for accurate experimental pK_a determination in day-to-day analysis in physicochemical measurement laboratories are checked and improved where possible; for instance, the use of consecutive injections or the stability of the ISs and separation buffers.

■ THEORY

The apparent mobility of a compound (μ_{ap}) is the sum of the electrophoretic (μ_{ep}) and electroosmotic flow (μ_{EOF}) mobilities and can be expressed by means of eq 1

$$\mu_{ap} = \mu_{ep} + \mu_{EOF} \tag{1}$$

Any of these mobilities can easily be calculated from the capillary length to the detector (L_D, usually in cm), the electric field applied (E, in V·cm^{-1}), the observed migration time of the compound (t_m), and an electroosmotic flow marker (t_0). Thus

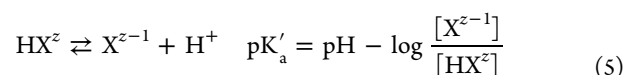$$\mu_{ap} = \frac{L_D}{E \cdot t_m} \tag{2}$$

$$\mu_{ep} = \frac{L_D}{E}\left(\frac{1}{t_m} - \frac{1}{t_0}\right) \tag{3}$$

where E is calculated from the applied potential (V) and the total capillary length (L_T) thus

$$E = \frac{V}{L_T} \tag{4}$$

For an ionizable acid−base compound, the electrophoretic mobility depends on the degree (or degrees) of ionization of the compound. Thus, the general acid−base equilibria for a monoprotic species, HX^z, can be expressed as

$$HX^z \rightleftarrows X^{z-1} + H^+ \quad pK_a' = pH - \log\frac{[X^{z-1}]}{[HX^z]} \tag{5}$$

where z is the charge of the protonated species and pK_a' the logarithmic form of the acidity constant (at a given ionic strength).

The μ_{ep}, also called the effective mobility (μ_{eff}), of a monoprotic compound can be expressed as a function of the pK_a' of the species and the pH of the background electrolyte through the following general equation[8]

$$\mu_{eff} = \frac{\mu_{HX^z} + (\mu_{X^{z-1}})10^{pH-pK_a'}}{1 + 10^{pH-pK_a'}} \tag{6}$$

where μ_{HX^z} and $\mu_{X^{z-1}}$ are the limiting electrophoretic mobilities of the subscripted species.

In the classic CE method, the pH of the buffer solution is measured by potentiometry and related to the mobility of the compound in the same buffered solution measured by CE. In the IS-CE method, both parameters are obtained at the same time in the same CE experiment. The use of an IS allows direct calculation of the pH of the buffered solution inside the capillary. This value is then used to calculate pK_a' of the compound being studied.

For example, for a neutral acid used as the IS, where z is 0, the pH value can be given, by rearranging eq 6, by
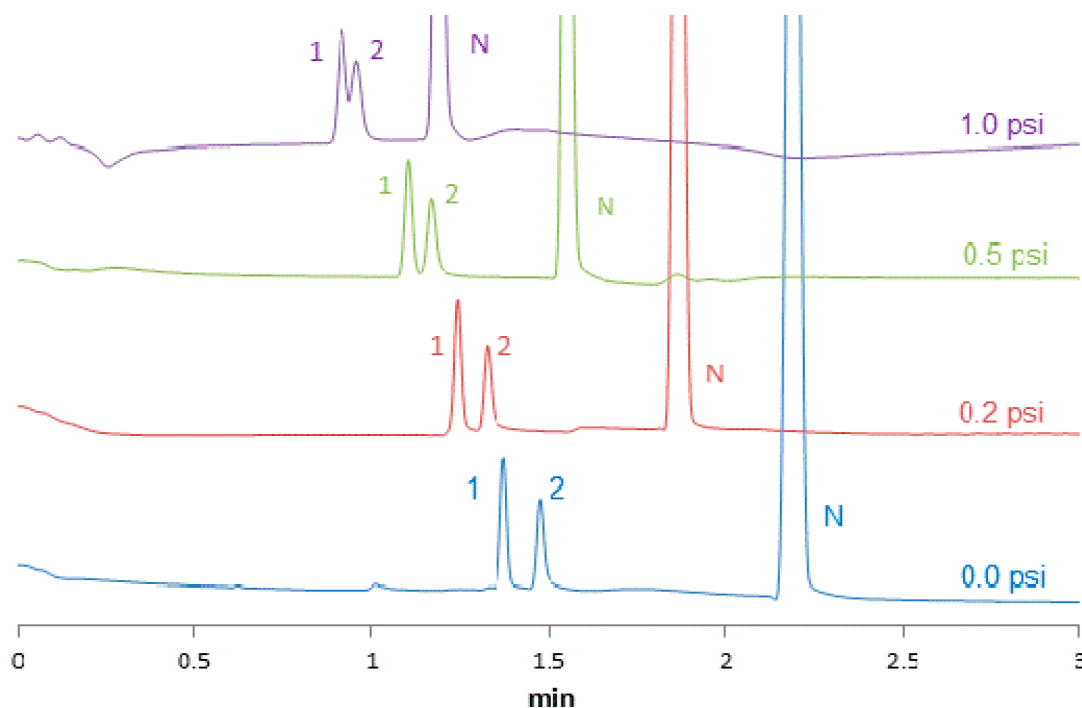
Figure 1. Effect of pressure during the separation on the migration time and resolution. Buffer: HCOOH/HCOO⁻, I = 50 mM, pH = 3.5; compounds, clonidine (1) and imipramine (2) at 100 ppm; neutral marker DMSO (N).

$$pH = pK'_{a(IS)} - \log \frac{\mu_{IS^-} - \mu_{eff(IS)}}{\mu_{eff(IS)}} \tag{7}$$

where $\mu_{eff(IS)}$ is the effective mobility and μ_{IS^-} the limiting mobility (mobility of the fully charged negative species) of the IS. The term corresponding to the uncharged species, μ_{HX}, has zero mobility and is removed from eq 6. Since $pK'_{a(IS)}$ of the IS is well-known, only two runs at two different pH values are needed to calculate the pH: the first where the pH is in the range $pK_a \pm 1$ to calculate the effective mobility; and a second in which it is completely ionized to calculate the limiting mobility.

Once the pH inside the capillary is known, the acidity constant of the TC can be calculated by rearranging eq 6 again:

$$pK'_{a(TC)} = pH - \log \frac{\mu_{HX^z} - \mu_{eff(TC)}}{\mu_{eff(TC)} - \mu_{X^{z-1}}} \tag{8}$$

where μ_{HX^z} and $\mu_{HX^{z-1}}$ refer to the limiting mobilities of the fully charged z and $z - 1$ species of the TC.

In these equations, $pK'_{a(TC)}$ is related to the thermodynamic pK_a by the activity coefficients, calculated by means of the Debye–Hückel equation.[22]

■ **EXPERIMENTAL SECTION**

Apparatus. CE experiments were performed with a P/ACE MDQ Beckman instrument (Palo Alto, CA, USA), equipped with a diode-array spectrophotometric detector. The capillary was made of fused silica, 50 μm I.D., 375 μm O.D., 35.2 cm length (25 cm to the detector) and was obtained from Composite Metal Services Ltd. (Shipley, West Yorkshire, UK). The temperature of the capillary was set to 25.0 °C ± 0.1 °C. The TCs and ISs were injected sequentially (one-by-one before separation) at a hydrodynamic pressure of 0.5 psi for 3 s (1 psi = 6897.76 Pa), and the applied voltage during separation was 20 kV. An additional 0.5 psi of hydrodynamic flow was applied

during separation. The UV detector was set at 214, 254, and 280 nm.

Chemicals. Dimethyl sulfoxide >99.9% (DMSO), 0.5 M sodium hydroxide tritisol, 0.5 M hydrochloric acid tritisol, and sodium dihydrogen phosphate monohydrate >99% were obtained from Merck (Darmstadt, Germany). Anhydrous sodium acetate >99.6% was purchased from J.T. Baker (Deventer, Netherlands). 2-(Cyclohexylamino)ethanesulfonic acid >99% (CHES) and 3-(cyclohexylamino)-1-propanesulfonic acid >98% (CAPS) were purchased from Sigma (St. Louis, MO, USA). 2,2-Bis(hydroxymethyl)-2,2′,2″-nitrilotriethanol >99.9% (BisTris) and sodium formate were bought from Fluka (Buchs, Switzerland). Tris(hydroxylmethyl)amino-methane >99.9% (Tris) was purchased from Aldrich (Milwaukee, WI, USA). Water was purified using a Milli-Q plus system from Millipore (Bedford, MA, USA), to a resistivity of 18.2 MΩcm.

The ISs and TCs used were reagent grade or of chromatographic quality and obtained from Sigma, Fluka, Aldrich, or Carlo Erba (Milan, Italy).

Sample and buffer preparations are detailed in the Supporting Information, section 1.

■ **RESULTS AND DISCUSSION**

To consider IS-CE a high-throughput method for accurate experimental pK_a determination in day-to-day analysis, instrumental parameters and experimental conditions need to be optimized to reduce the run time, conditioning steps and sample preparation as much as possible. IS and buffer solution stability must also be studied to determine how many times they can be used before requiring replacement. The precision and accuracy of the method should also be established.

Instrumental Conditions. In accordance with ideal requirements for the IS-CE method, a CE P/ACE MDQ Beckman instrument was selected as the most adequate for the method. The samples are placed in a different tray from the

buffers and they can be removed easily from their position for storage. Moreover, the samples can be stored directly at 4 °C inside the instrument, and the instrument allows for the application of pressure during separation as well as sequential injecting.

Modifications were made to the experimental conditions and methodology reported in previous work[18−21,23] to minimize the analysis time. The capillary was cut to the minimum length the instrument configuration allows: 25.0 cm to the detector and 35.2 cm total length. To avoid the Joule effect, a voltage of 20 kV was selected as the maximum to maintain linearity between the voltage applied and the current generated (according to Ohm's law) for all the buffers used. Under these capillary and voltage conditions, migration times were reduced compared to those in previous work, down to average analysis times of 3 min for anionic compounds, which are the last compounds eluted.

To avoid the necessity to prepare solutions of specific mixtures of the TC and the appropriate ISs prior to the analysis, sequential injection was used. It must be taken into account that the sample plug length (TC plus IS plugs) should be less than 1% or 2% of the total length of the capillary to avoid overloading. Consequently, the lowest pressure that the instrument allowed (0.5 psi) was applied for the 3 s of injection time.

To speed up the migration of the compounds, pressure was applied during separation: the higher the pressure, the shorter the migration times. However, high pressure may result in overlapping peaks. In order to optimize the pressure, the migration time and separation of several compounds with different structures and limiting mobilities were compared at different pressures (0.0, 0.2, 0.5, and 1.0 psi). The example shown in Figure 1 illustrates as when pressure increases, run time decreases, as does the resolution between peaks. 0.5 psi was chosen as the best compromise between high resolution and a short run time.

Pre- and postconditioning methods (at the beginning of the session; between injections with the same buffer; when the buffer is changed; and at the end of the working session) were optimized to reduce the rinsing time and, as a consequence, total analysis time. Table 2 shows the conditioning sequences after the optimization processes. The total rinsing time was drastically reduced just to obtain reproducible results.

**Table 2. Capillary Conditioning Optimization**

rinse type	CE conditioning (at 20 psi)
beginning of the session	2.0 min NaOH 1.0 M
	0.5 min H$_2$O
	2.0 min buffer
replicates	no rinse
change pH	0.2 min new buffer
end of the session	2.0 min NaOH 0.1 M
	2.0 min H$_2$O

A more detailed description of these processes is available in the Supporting Information (section 2 Instrumental conditions).

Electroosmotic Flow Marker. Although many organic solvents can be used as EOF markers, the common use of DMSO in the syntheses of new drugs together with the fact that it is the most commonly used solvent for preparing compound libraries in the pharmaceutical industry,[24−26] make it appropriate for our purpose. Many potential samples may

already have this EOF marker incorporated, prior to the analysis.[27,28]

To test the performance of the marker, several compounds were totally dissolved in DMSO at a concentration of 1000 μg mL^{-1}. Solutions were then prepared for injection at a concentration of 100 μg mL^{-1} by dilution with water. When the samples were injected, significant peak tailing appeared due to the high concentration of DMSO (Figure 2a). For cationic
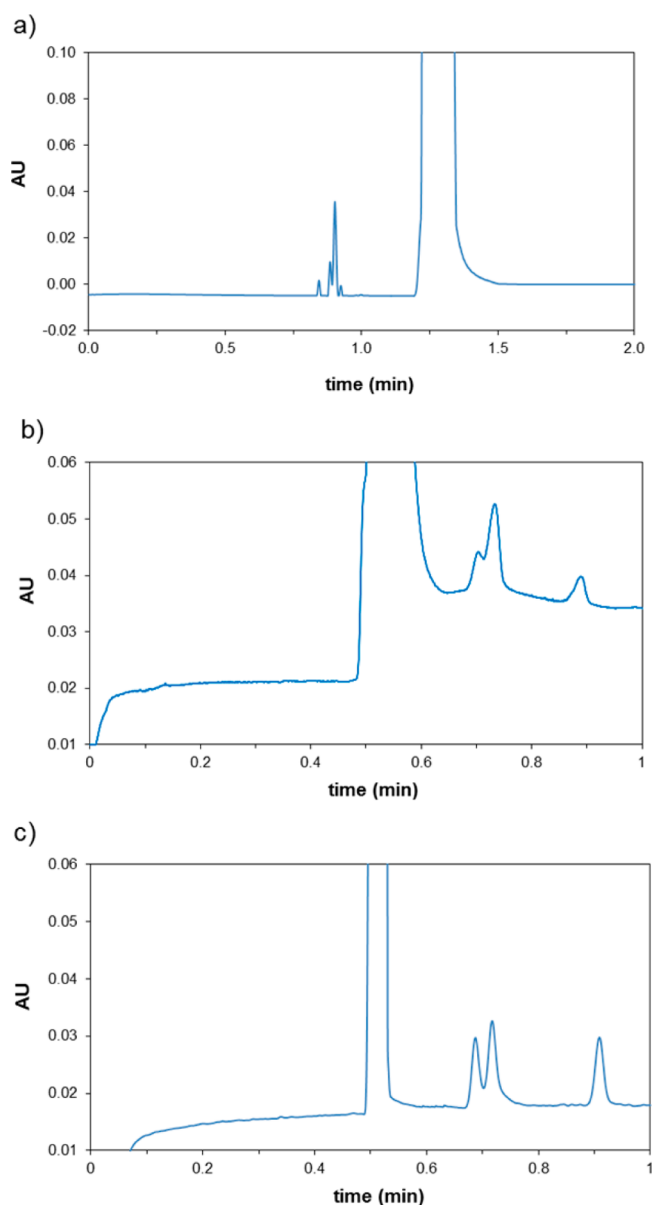
Figure 2. Electropherograms of (a) cationic compounds (metoprolol, diphenidramine, propranolol, ephedrine) at pH = 5.0 and 10% DMSO; (b) anionic compounds (ibuprofen, nicotinic acid, warfarin) at pH = 8.5 and 10% DMSO; (c) anionic compounds (ibuprofen, nicotinic acid, warfarin) at pH = 8.5 and 0.4% DMSO.

compounds no problems were observed because they elute before the EOF marker; however, the anionic compounds were hardly detectable since they were diffused in the tail of the DMSO peak (Figure 2b). Consequently, the quantity of DMSO in the samples was reduced 20-fold. So that 4% DMSO was added to the stock solutions and the final volume was made up with water. After 1/10 dilution, the content of DMSO in the

injection vial was about 0.4%. In these conditions, the large peak tail disappeared, as shown in Figure 2c, thereby increasing the peak efficiency.

Stability of the Buffers. The buffers must satisfy several requirements for meaningful experimental results to be obtained. For use in CE, a buffer must have a good buffering capacity in the pH range of use and a low detection signal.[16] The buffers should also be inert so that they do not interact with the analyte or the walls of the capillary. Moreover, the whole CE system must be filled with the same buffer to keep parameters, such as conductivity, electric field, temperature and pH, as constant as possible.[10,16]

The precise determination of a pK_a by CE requires the measurement of the effective mobility in several electrolytes with a low and constant ionic strength. This ionic strength must be low enough to minimize activity corrections, temperature gradients and viscosity differences; but there must be enough buffer capacity to maintain a fixed pH, even after introduction of the sample.[16,23] Thus, an ionic strength of 0.05 M is a reasonable compromise between the need to minimize Joule heat production inside the capillary and the buffering capacity required. Several running solutions covering the pH range that is useful in CE system were prepared at this ionic strength. Supporting Information Table S1 shows the buffers used, their pK_a value and the stock solution used in their preparation. These buffers have an effective range of approximately two pH units, centered on their pK_a value.

The pH of the buffer can be affected by several factors, such as volatilization (e.g., ammonia buffers), electrolysis when a voltage is applied (almost all buffers), or even CO_2 dissolution (NaOH and highly basic buffers), which cause changes in the pH and migration velocity of the compounds over time.[17,29] Even though with the IS-CE method the pH of the buffer solution inside the capillary can be easily calculated for every run, it is important to know for how many runs a buffer can be used before its properties stray from an acceptable range. Consequently, the recommended buffers were monitored over time to ensure that they kept their initial properties. One way to monitor stability is through the pH value. This is easy to monitor because the pH inside the capillary can be calculated at any time just from knowing the pK_a value and mobilities (limiting and effective) of any compound injected (in fact, of an IS) in a capillary filled with the buffer under study.

The pH values of all the buffers shown in Supporting Information Table S1 were monitored over time (every 5 min for a total of 16 h of electroseparation) using three different ISs of appropriate pK_a from Table 1. Figure 3 shows the average pH obtained with the three ISs against the electrophoretic run time. As can be seen, in all the cases the pH decreases over the run time. This can easily be explained by the electrolysis processes that occur in the separation vials: reactions involving the generation (inlet buffer) and consumption (outlet buffer) of hydrogen ions occur at the electrodes.[30] As explained above, in the IS-CE method alterations in the conditions of analysis can be minimized by the use of an IS that undergoes the same alteration as the TC. This means that these pH variations should not affect the pK_a determination of any compound provided that the TC and IS remain adequately ionized.

Therefore, at the same time as monitoring the pH inside the capillary, the pK_a of two compounds, 2,6-dibromo-4-nitrophenol and acridine, was determined with the same frequency. For 2,6-dibromo-4-nitrophenol, 2-chlorobenzoic acid was used as the IS, and the experiments were performed with an acetic/
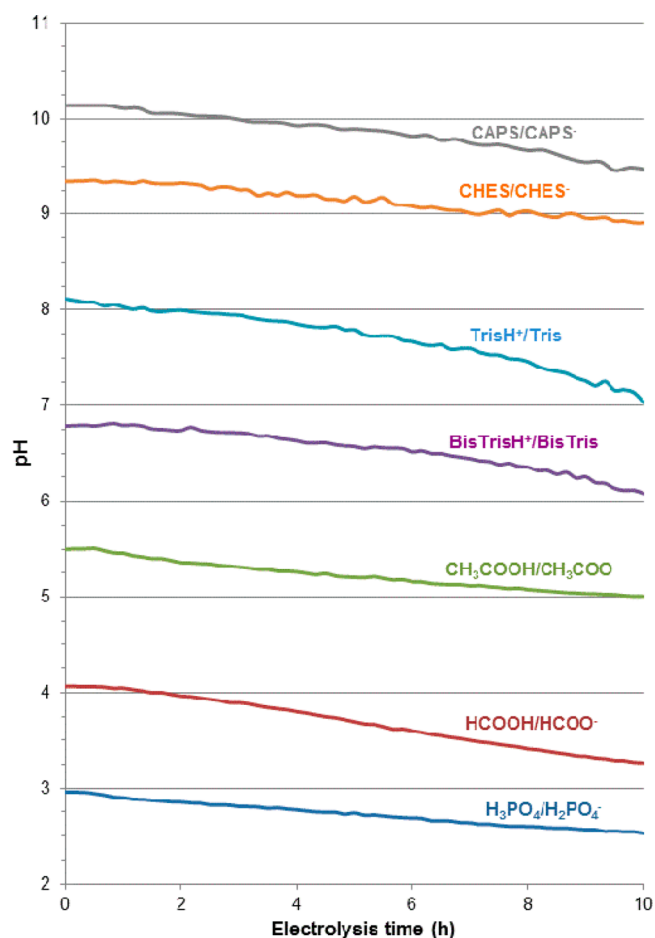

Figure 3. pH of the background electrolytes as a function of electrophoretic run time.

acetate buffer at pH 5.5 and 50 mM ionic strength. In the case of acridine, pyridine was used as the IS and $H_3PO_4/H_2PO_4^-$ as the separation buffer at pH 3.0 (I = 50 mM). Figure 4 shows the pK_a of the compounds (solid line) and the pH of the buffers (dashed line) over the electroseparation time. It can clearly be seen that while the pH decreases during voltage application, the calculated pK_a remains constant all through the 16 h of the study (around 500 injections). This demonstrates that the IS-CE method does not require the buffer vials to be replaced frequently; whereas in other work,[11,17,29] where the classic CE method is used for pK_a determination, pH variation is minimized by replacement of the buffer solutions approximately every 1−2 h of electrodriven separation.

Nevertheless, after several hours of separation, the pH of the buffer may become drastically reduced. For example, Figure 3 shows that the pH of TRIS or CAPS decreases nearly one unit after 10 h of separation. The buffering capacity is then clearly diminished and, what is more important, the IS and TC selected may be in different ionization states from the initial one. In order to prevent this, we recommend buffer replacement after 10 h of use; considering a mean run time of 2 min, this corresponds to 300 injections.

Stability of the Internal Standards. Table 1 shows the pK_a values of the 48 monoprotic acidic[20] and basic[21] ISs established in previous works. The list comprises a reference set of compounds with well-determined acidity constants that facilitates the process of selecting an appropriate IS for a given pK_a determination. Thus, when the pK_a of a TC is to be
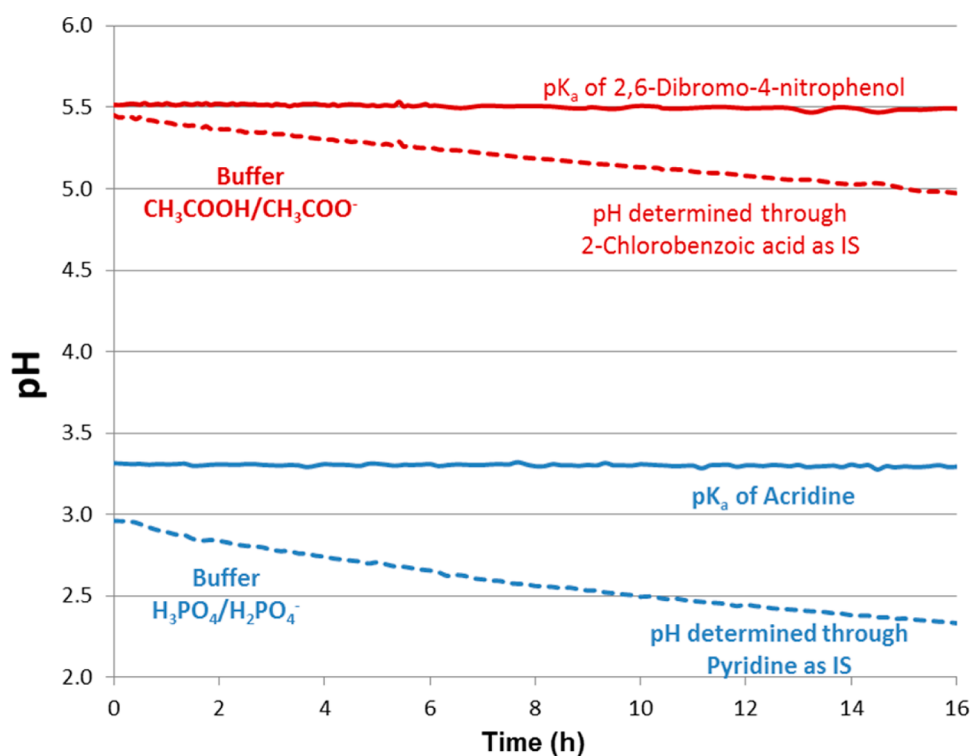
Figure 4. pH of the buffer solution vs electrolysis time. Solid lines correspond to the pK_a of test compounds. Dashed lines are the pH of the buffer calculated using the ISs. Buffers used: pH = 3.0, $H_3PO_4/H_2PO_4^-$; pH = 5.5, CH_3COOH/CH_3COO^-.

determined, one or several appropriate ISs from the sample tray are sequentially injected with the TC. However, the replacement time of the solutions must be known because of possible degradation.

To evaluate the stability of the ISs, each compound was injected once per week at a pH at which it was totally ionized. The peak area and the mobility of the compounds were monitored over this time. Nicotinic acid, N,N-dimethyl-N-phenylamine, pyridine, and 4-tert-butylpyridine showed a decrease of 10% in their area in a few days; so they must be replaced daily. In the case of vanillin, aniline, quinolone, 4-tert-butylaniline, 2,4-lutidine, trazodone, pilocarpine, 2,4,6-trimethylpyridine, bupivacaine, imipramine, and propranolol, weekly replacement is required. Warfarin, 2,4,6-tribromophenol, acridine, papaverine, and procainamide require only monthly replacement. For the rest of ISs, the peak area was still 90% or more after a four-month period.

Precision and Accuracy. The precision of the pK_a values obtained (standard deviation, sd) can be evaluated in several ways. Effective mobilities can be determined using several buffers with pH values close to the pK_a of the TC. For example, the pK_a of mepivacaine was determined using bupicavaine as the IS ($pK_{a(IS)}$ = 8.19) at three pH values near to their pK_a values. Considering the μ_{eff} of the TC and IS at pH values of 7.5, 8.0, and 8.5, the following results were obtained: 7.90, 7.95, and 7.93, respectively. So, final pK_a value obtained for mepivacaine is 7.93 ± 0.03. In this case, four electropherograms are required to determine the acidity constant in triplicate. Alternatively, and much faster, the precision can be improved if more than one IS is used for any pK_a determination. Several ISs can be sequentially injected together with the TC in the same run. For example, the pK_a of mepivacaine was determined using three different ISs (lidocaine, clonidine and bupivacaine) in the same pH buffer and run, obtaining an average pK_a for the three

ISs of 7.92 ± 0.02. In this way, just two runs of 1.1 and 1.6 min are needed to obtain the pK_a value as determined in triplicate, since the four compounds (mepivacaine and the three ISs) are injected together. Figure 5 shows both electropherograms.

To date, we have used the IS-CE method to determine 129 pK_a values for 93 different drugs ($pK_{a(IS-CE)}$). These compounds range from simple structures (with just one acidity constant) to more complex ones with up to four constants. To estimate the accuracy of the experimental values, we correlated them with compiled values obtained from the literature ($pK_{a(lit)}$) at 25 °C and zero ionic strength[20−22,31,32] or measured in our laboratory by other methods. The overall data is presented in Table 2S in the Supporting Information. Figure 6 shows $pK_{a(IS-CE)}$ as a function of $pK_{a(lit)}$ and the fitted line. The equation obtained is

$$pK_{a(IS-CE)} = (0.995 \pm 0.006)pK_{a(lit)} + (0.05 \pm 0.05)$$

$$SD = 0.17 \quad F = 24783 \tag{9}$$

The slope and intercept of the correlation are not significantly different from 1 and 0, respectively, for a 95% confidence level. These fitting results demonstrate the accuracy of the IS-CE method compared with reference literature values.

CONCLUSION

The proposed IS-CE method is a fast and attractive alternative to longer time-consuming methods for the determination of acidity constants, such as potentiometric and spectophotometric titrations, or the classic CZE method. Several instrumental parameters and different experimental conditions were studied and optimized to reduce the time of pK_a determination. With a short capillary of just 25 cm to the detector, reduced capillary conditioning times, 0.4% DMSO marker, hydrodynamic sequential injection at 0.5 psi for 3s, and the application of 20 kV and 0.5 psi during separation, the time
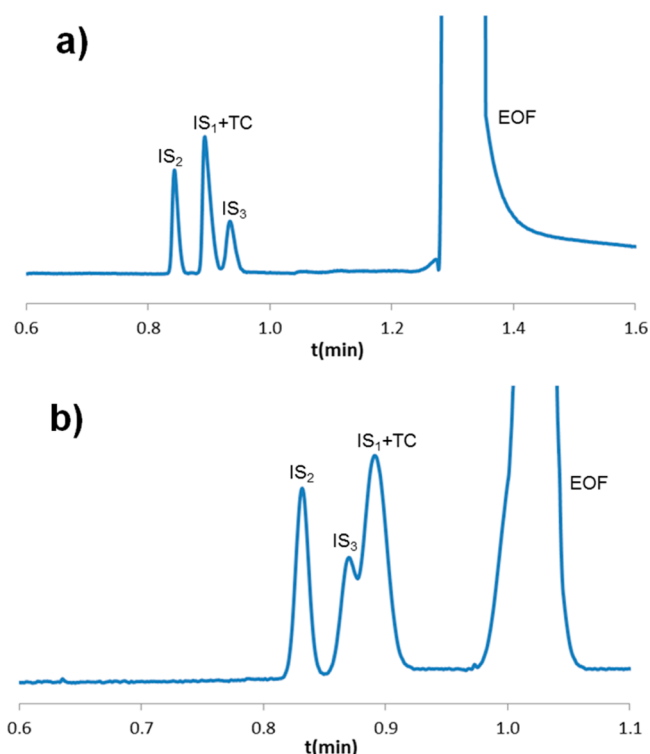
Figure 5. Electropherograms of mepivacaine (TC) and 3 internal standards (IS$_1$, lidocaine; IS$_2$, clonidine; IS$_3$, bupivacaine): (a) pH = 5, limiting mobilities and (b) pH = 8, effective mobilities.
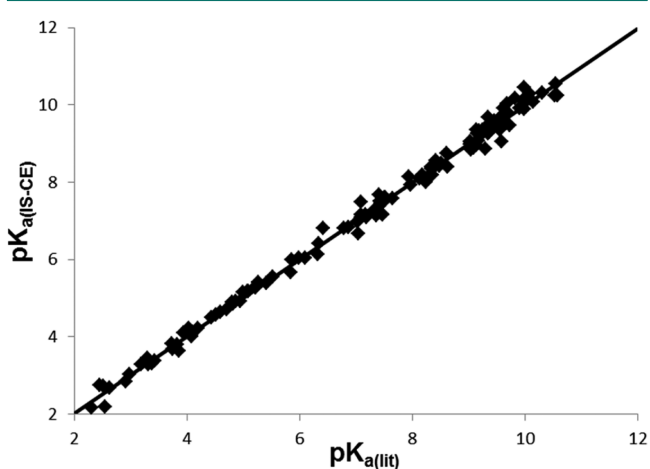
Figure 6. Measured pK_a (p$K_{a(IS-CE)}$) vs literature pK_a (p$K_{a(IS-CE)}$). Fitted line is shown as the solid line.

required for the electropherograms can be reduced in such a way that any pK_a can be obtained in less than 5 min using the proposed buffers and ISs. 129 pK_a values from 93 drugs, ranging from simple structures to more complex ones with up to four constants, were determined by this IS-CE method and satisfactorily correlated with reference values from the literature.

In conclusion, IS-CE is a powerful high-throughput method suitable for routine pK_a determination in drug discovery and development laboratories.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Detailed experimental samples and buffers preparation, instrumental conditions, and precision and accuracy. This material is available free of charge via the Internet at http://pubs.acs.org.

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The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

■ REFERENCES

(1) Comer, W. T. Perspectives in Drug Discovery. *Ref. Module Chem., Mol. Sci. Chem. Eng.* **2013**, DOI: 10.1016/B978-0-12-409547-2.02699-8.

(2) Ganellin, R.; Roberts, S.; Jefferis, R.; Stocks, M. The Small Molecule Drug Discovery Process—From Target Selection to Candidate Selection. *Introd. Biol. Small Mol. Drug Res. Dev.* **2013**, 81−126.

(3) Vachal, P.; Tata, J.; Athanasopoulos, J. High-Throughput Purification Platform in Support of Drug Discovery. *ACS Comb. Sci.* **2012**, *14*, 51−59.

(4) Li, S.; Julien, L.; Tidswell, P.; Goetzinger, W. Enhanced Performance Test Mix for High-Throughput LC/MS Analysis of Pharmaceutical Compounds. *J. Comb. Chem.* **2006**, *8*, 820−828.

(5) Wan, H.; Ulander, J. High-Throughput pKa Screening and Prediction Amenable for ADME Profiling. *Expert Opin. Drug Metab. Toxicol.* **2006**, *2*, 139−155.

(6) Reijenga, J.; van Arno, H.; van Antonie, L.; Teunissen, B. Development of Methods for the Determination of pKa Values. *Anal. Chem. Insights* **2013**, *8*, 53−71.

(7) Cleveland, J. A., Jr.; Benko, M. H.; Gluck, S. J.; Walbroehl, Y. M. Automated pKa Determination at Low Solute Concentrations by Capillary Electrophoresis. *J. Chromatogr. A* **1993**, *652*, 301−308.

(8) Gluck, S. J.; Steele, K. P.; Benkö, M. H. Determination of Acidity Constants of Monoprotic and Diprotic Acids by Capillary Electrophoresis. *J. Chromatogr. A* **1996**, *745*, 117−125.

(9) Jiménez-Lozano, E.; Marqués, I.; Barrón, D.; Beltrán, J. L.; Barbosa, J. Determination of pKa Values of Quinolones from Mobility and Spectroscopic Data Obtained by Capillary Electrophoresis and a Diode Array Detector. *Anal. Chim. Acta* **2002**, *464*, 37−45.

(10) Poole, S. K.; Patel, S.; Dehring, K.; Workman, H.; Poole, C. F. Determination of Acid Dissociation Constants by Capillary Electrophoresis. *J. Chromatogr. A* **2004**, *1037*, 445−454.

(11) Herrero-Martinez, J. M.; Sanmartin, M.; Roses, M.; Bosch, E.; Rafols, C. Determination of Dissociation Constants of Flavonoids by Capillary Electrophoresis. *Electrophoresis* **2005**, *26*, 1886−1895.

(12) Šlampová, A.; Křivánková, L.; Gebauer, P.; Boček, P. Standard Systems for Measurement of pKs and Ionic Mobilities: 1.Univalen Weak Acids. *J. Chromatogr. A* **2008**, *1213*, 25−30.

(13) Babić, S.; Horvat, A. J. M.; Mutavdžić Pavlović, D.; Kaštelan-Macan, M. Determination of pKa Values of Active Pharmaceutical Ingredients. *Trends Anal. Chem.* **2007**, *26*, 1043−1061.

(14) Andrasi, M.; Buglyo, P.; Zekany, L.; Gaspar, A. A Comparative Study of Capillary Zone Electrophoresis and pH-Potentiometry for Determination of Dissociation Constants. *J. Pharm. Biomed. Anal.* **2007**, *44*, 1040−1047.

(15) Barbosa, J.; Barrón, D.; Jiménez-Lozano, E.; Sanz-Nebot, V. Comparison between Capillary Electrophoresis, Liquid Chromatography, Potentiometric and Spectrophotometric Techniques for Evaluation of pKa Values of Zwitterionic Drugs in Acetonitrile−Water Mixtures. *Anal. Chim. Acta* **2001**, *437*, 309−321.

(16) Beckers, J. L.; Bocek, P. The Preparation of Background Electrolytes in Capillary Zone Electrophoresis: Golden Rules and Pitfalls. *Electrophoresis* **2003**, *24*, 518−535.

(17) Fuguet, E.; Reta, M.; Gibert, C.; Rosés, M.; Bosch, E.; Ràfols, C. Critical Evaluation of Buffering Solutions for pKa Determination by Capillary Electrophoresis. *Electrophoresis* **2008**, *29*, 2841−2851.

(18) Fuguet, E.; Ràfols, C.; Bosch, E.; Rosés, M. A Fast Method for pKa Determination by Capillary Electrophoresis. *Chem. Biodiversity* **2009**, *6*, 1822−1827.

(19) Fuguet, E.; Ràfols, C.; Bosch, E.; Rosés, M. Fast High-Throughput Method for the Determination of Acidity Constants by Capillary Electrophoresis: I. Monoprotic Weak Acids and Bases. *J. Chromatogr. A* **2009**, *1216*, 3646−3651.

(20) Cabot, J. M.; Fuguet, E.; Ràfols, C.; Rosés, M. Fast High-Throughput Method for the Determination of Acidity Constants by Capillary Electrophoresis. II. Acidic Internal Standards. *J. Chromatogr. A* **2010**, *1217*, 8340−8345.

(21) Fuguet, E.; Ràfols, C.; Rosés, M. A Fast High Throughput Method for the Determination of Acidity Constants by Capillary Electrophoresis. 3. Basic Internal Standards. *J. Chromatogr. A* **2011**, *1218*, 3928−3934.

(22) Cabot, J. M.; Fuguet, E.; Ràfols, C.; Rosés, M. Determination of Acidity Constants by the Capillary Electrophoresis Internal Standard Method. IV. Polyprotic Compounds. *J. Chromatogr. A* **2013**, *1279*, 108−116.

(23) Gravador, R. S.; Fuguet, E.; Ràfols, C.; Rosés, M. Temperature Variation Effects on the Determination of Acidity Constants through the Internal Standard-Capillary Electrophoresis Method. *Electrophoresis* **2013**, *34*, 1203−1211.

(24) Avdeef, A. Physicochemical Profiling (Solubility, Permeability and Charge State). *Curr. Trends Med. Chem.* **2001**, *1*, 277−351.

(25) Lipinski, C. A. Solubility in Water and DMSO: Issues and Potential Solutions. *Biotechnol.: Pharm. Aspects* **2004**, *1*, 93−125.

(26) Lipinski, C. Drug Solubility in Water and Dimethylsulfoxide. *Methods Princ. Med. Chem.* **2008**, *37*, 257−282.

(27) Wan, H.; Holmén, A.; Någård, M.; Lindberg, W. Rapid Screening of pKa Values of Pharmaceuticals by Pressure-Assisted Capillary Electrophoresis Combined with Short-End Injection. *J. Chromatogr. A* **2002**, *979*, 369−377.

(28) Lipka, E.; Folly-Klan, M.; Charton, J.; Vaccher, M.-P.; Bonte, J.-P.; Vaccher, C. Determination of pKa Values of Benzimidazole Derivatives from Mobility Obtained by Capillary Electrophoresis. *J. Pharm. Biomed. Anal.* **2010**, *53*, 1267−1271.

(29) Foulon, C.; Duhal, N.; Lacroix-Callens, B.; Vaccher, C.; Bonte, J. P.; Goossens, J. F. Determination of pK_a Values of Benzoxa-, Benzothia-, and Benzoselena-Zolinone Derivatives by Capillary Electrophoresis. Comparison with Potentiometric Titration and Spectrometric Data. *Eur. J. Pharm. Sci.* **2007**, *31*, 165−171.

(30) Zhu, T.; Sun, Y.; Zhang, C.; Ling, D.; Sun, Z. Variation of the pH of the Background Electrolyte as a Result of Electrolysis in Capillary Electrophoresis. *J. High Resolut. Chromatogr.* **1994**, *17*, 563−564.

(31) Box, K.; Bevan, C.; Comer, J.; Hill, A.; Allen, R.; Reynolds, D. High-Throughput Measurement of P K a Values in a Mixed-Buffer Linear pH Gradient System. *Anal. Chem.* **2003**, *75*, 883−892.

(32) Box, K. J.; Comer, J. E. A. Using Measured pKa, LogP and Solubility to Investigate Supersaturation and Predict BCS Class. *Curr. Drug Metab.* **2008**, *9*, 869−878.