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A fast high throughput method for the determination of acidity constants by capillary electrophoresis. 3. Basic internal standards

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ABSTRACT

A set of 25 monoprotic bases is proposed as internal standards for pK_a determination by capillary electrophoresis. The pK_a of the bases is determined and compared with available literature data. The capillary electrophoresis internal standard method offers numerous advantages over other typical methods for pK_a determination, especially of analysis time and buffer preparation. However, it requires disposing of appropriate standards with reference pK_a value. The set of bases established in this work together with the set of acids previously established provide a reference set of compounds with well-determined acidity constants that facilitate the process of selecting appropriate internal standards for fast pK_a determination by capillary electrophoresis in high throughput screening of pharmaceutical drugs. In addition, the performance of the method when acidic internal standards are used for the determination of acidity constants of basic internal standards has also been tested. Although higher errors may be expected in this case, good agreement is observed between determined and literature values. These results indicate that in most cases structural similarity between the analyte and the internal standard might not be an essential requirement in the internal standard method.

1. Introduction

Drug discovery has undergone considerable changes with the addition of new technologies and strategies, such as combinatorial chemistry, high throughput screening, and robotics. These technologies have made possible synthesis of millions of potential drugs for thousands of pharmaceutical applications. Physicochemical and physiological property profiles of candidate compounds must be established during early discovery phases to select the most appropriate ones and reduce the number of failures in later stages of the development process. As a consequence, there is an increasing demand of methods for high throughput screening, allowing the analysis of a high number of compounds in a very short period of time [1–5].

One property that affects the pharmaceutical potential of a compound is, among others, the acidity constant (or pK_a in its common logarithmic form) [3,6]. The aqueous dissociation constant is an important parameter, as the ionization of a compound is associated with other key physicochemical properties such as lipophilicity and solubility. It is also well known that the ionization state of a drug affects absorption, distribution, metabolism and excretion (ADME) [3,4].

Recently, we have developed a new method for fast determination of acidity constants by capillary electrophoresis (CE) [7]. This method is based on the use of internal standards (IS), compounds with well-known pK_a values similar to the ones of the analyte, which offers advantages over other methods for pK_a determination. On one hand, it has the advantages of the technique: in CE very low amounts of solvents and samples are needed, and there is no need of high purity because it is a separation technique [8–14]. On the other hand, it also has other advantages conferred by the own methodology: there is no need of pH measurement of the buffers, few measurements are needed for a good precision, and systematic errors during the experiments or specific interactions of the analytes with the buffers can be minimized by the use of appropriate internal standards [7]. One of the main drawbacks of the capillary electrophoresis classical method for pK_a determination is the need to make separate experimental runs at different buffered pH values to determine the relative concentration of ionized species at each pH. This rate-limiting step, which does not apply to potentiometric titration methods, neither applies to the internal standard method.

In order to facilitate the process of selecting an adequate internal standard for a given determination, we proposed a set of 24 acidic internal standards covering all the useful pH range in CE. This set of compounds belonged to different chemical families and their pK_a values were well established [15]. In a similar way, in this work

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we establish a set of basic compounds that can be used as internal standards. For this purpose we propose a set of 25 monoprotic bases with different functional groups and structures and establish their pK_a values. In addition, we also validate the applicability of the method in terms of structural similarity between the analyte and the internal standard. To this end, several determinations where both analyte and IS have similar pK_a values but they have very different nature (i.e., the analyte is a base and the IS is an acid) have been performed, and results have been evaluated.

2. Theory

The effective electrophoretic mobility, μ_{eff}, of a monoprotic neutral base (B) and a monoprotic neutral acid (HA) can be expressed as a function of their acidity constants and the pH of the background electrolyte (BGE) by Eqs. (1) and (2), respectively [16]:

$$\mu_{eff} = \frac{\mu_{BH^+}}{1 + 10^{pH - pK'_a}} \quad (1)$$

$$\mu_{eff} = \frac{\mu_{A^-}}{1 + 10^{pK'_a - pH}} \quad (2)$$

where μ_{BH^+} is the mobility of the protonated base, μ_{A^-} the mobility of the deprotonated acid, and pK'_a is related to the thermodynamic pK_a through Eq. (3) for a base and Eq. (4) for an acid:

$$pK_a = pK'_a + \log\ \gamma_{BH^+} \quad (3)$$

$$pK_a = pK'_a - \log\ \gamma_{A^-} \quad (4)$$

provided that pK'_a involves the activity of the hydrogen ion and the concentration of acidic and basic species, respectively. In these equations γ is the activity coefficient of the subscript species and corrects the effect of the ionic strength on solute ionization.

Activity coefficients are usually estimated by Debye–Hückel equation, which depend on the ionic strength (I) of the solution:

$$-\log\ \gamma_{BH^+} = -\log \gamma_{A^-} = \frac{Az^2\sqrt{I}}{1 + Ba\sqrt{I}} \quad (5)$$

where A and B depend on the solvent dielectric constant and temperature (their values are 0.509 and 0.33, respectively, in water at 25 °C), z is the charge of the ion, and a is the hydrated radius of the ion. The value of a depends on the hydrated ion, although a value of 4.5 Å (value for hydrogen ion) is commonly taken for most ions. This equation is valid for ionic strength values lower than 0.2 M. As usual, activity coefficients of neutral species ($z = 0$) are assumed to be unity.

For pK_a determination through the internal standard method, rearrangement of Eqs. (1) and (2) provides the following equations:

$$pK'_a = pH - \log\frac{\mu_{BH^+} - \mu_{eff}}{\mu_{eff}} = pH - \log\ Q \quad (6)$$

$$pK'_a = pH + \log\frac{\mu_{A^-} - \mu_{eff}}{\mu_{eff}} = pH + \log\ Q \quad (7)$$

For a given compound, Q is a quotient that involves its limiting mobility (i.e., of the fully charged form, μ_{BH^+} or μ_{A^-}) and its effective mobility (i.e., the mobility measured at a pH where the ionized and the neutral form of the compound coexist, μ_{eff}). Eq. (6) is applied to basic compounds, whereas Eq. (7) is applied to acidic ones. These mobility values are directly calculated from the migration times of the analyte (t_m) and the electroosmotic flow marker (t_0) through Eq. (8):

$$\mu = \frac{L_T L_D}{V}\left(\frac{1}{t_m} - \frac{1}{t_0}\right) \quad (8)$$

where L_T and L_D are the total and effective capillary length, respectively, and V is the applied voltage.

2.1. Determination of the acidity constant of basic compounds with basic internal standards

When both, the analyte (AN) and the internal standard (IS) are monoprotic bases, two different forms of Eq. (6) can be defined, one for each compound (AN or IS). If these two equations are subtracted, we obtain Eq. (9), that allows the direct calculation of the pK_a of the analyte.

$$pK'_{a,AN} = pK'_{a,IS} - \log\ Q_{AN} + \log Q_{IS} \quad (9)$$

This equation is not pH dependent, so there is no need to measure the pH of the electrophoretic buffers, and the limiting and effective mobilities of the analyte and the internal standard are the only parameters to be determined.

Finally, the thermodynamic pK_a value is easily obtained substituting Eq. (3) for both the analyte and the internal standard into Eq. (9):

$$pK_{a,AN} = pK_{a,IS} - \log\ Q_{AN} + \log Q_{IS} \quad (10)$$

Eq. (10) allows the calculation of the pK_a value of a monoprotic base using another monoprotic base as internal standard. It is not pH dependent, and thus pH does not need to be measured. Moreover, it does not need activity coefficient corrections because the activity coefficients of the analyte and the internal standard cancel out.

2.2. Determination of the acidity constant of basic compounds with acidic internal standards

In a similar way, the pK_a of a basic compound can be determined through an acidic internal standard. In this case Eq. (6) is defined for the analyte and Eq. (7) for the internal standard. When these two equations are subtracted we obtain Eq. (11):

$$pK'_{a,AN} = pK'_{a,IS} - \log\ Q_{AN} - \log Q_{IS} \quad (11)$$

Again, this equation is not pH dependent. To calculate the pK_a of the analyte, the exact pK_a of the IS and the limiting and effective mobilities of the analyte and internal standard are the only parameters needed. If Eq. (3) (for the analyte) and Eq. (4) (for the internal standard) are substituted into Eq. (11), the ionic strength correction is performed, so the aqueous pK_a of the analyte is obtained. Eq. (12) allows the calculation of the pK_a of a basic analyte through an acidic internal standard, being BH^+ the protonated analyte and A^- the deprotonated internal standard:

$$pK_{a,AN} = pK_{a,IS} - \log\ Q_{AN} - \log Q_{IS} + \log \gamma_{BH^+} + \log \gamma_{A^-} \quad (12)$$

or from Eq. (5)

$$pK_{a,AN} = pK_{a,IS} - \log\ Q_{AN} - \log Q_{IS} - \frac{1.018\sqrt{I}}{1 + 1.5\sqrt{I}} \quad (13)$$

It can be observed that Eq. (11) is not pH dependent, but it depends on the ionic activity coefficient of analyte and internal standard.

3. Experimental

3.1. Apparatus

Experiments were performed with an Agilent Technologies (Santa Clara, CA, USA) capillary electrophoresis system, equipped with a diode-array spectrophotometric detector. A fused-silica capillary of 50 μm I.D., 375 μm O.D. and 48.5 cm of total length (40 cm to the detector), obtained from Composite Metal Services Ltd (Ilkley, England), was used to carry out the experimental mobility determinations. The temperature of the capillary was kept at 25.0 °C (±0.1 °C). Samples were injected hydrodynamically, and the applied voltage was 20 kV. UV detection was carried out at 214 nm.

3.2. Reagents

Benzyl alcohol (p.a), sodium hydroxide 0.5 M, hydrochloric acid 0.5 M, and potassium chloride (>99.5%) were from Merck (Darmstadt, Germany). Sodium acetate anhydrous (>99.6%) was purchased from J.T. Baker (Deventer, Holland). CHES (2-(cyclohexylamino)ethanesulfonic acid) (>99%), and CAPS (3-(cyclohexylamino)-1-propanesulfonic acid) (>98%) were from Sigma (St Louis, MO, USA). BisTris (2,2-Bis(hydroxymethyl)-2,2′,2″-nitrilotriethanol), and sodium formate were from Fluka (Buchs, Switzerland). Tris (Tris(hydroxymethyl)aminomethane) (>99.9%) was purchased from Aldrich (Milwaukee, WI, USA). Water was purified by a Milli-Q plus system from Millipore (Bedford, MA, USA), with a resistivity of 18.2 MΩ cm.

The test solutes employed were: aniline, quinoline, 4-*tert*-butylaniline, *N*,*N*-dimethyl-*N*-phenylamine, pyridine, acridine, 4-*tert*-butylpyridine, papaverine, 2,4-lutidine, trazodone, pilocarpine, 2,4,6-trimethylpyridine, lidocaine, clonidine, bupivacaine, quinine, 1-phenylpiperazine, *N*,*N*-dimethyl-*N*-benzylamine, diphenhydramine, imipramine, procainamide, propranolol, 1-aminoethylbenzene, ephedrine, nortriptyline, 4-bromophenol, 3-chlorophenol, 3,5-dichlorophenol, paracetamol, 2-chlorophenol, methylparaben, 4-hydroxybenzaldehyde, vanillin, warfarin, sulfacetamide, benzoic acid, and ibuprofen. All the compounds were reagent grade or better and were obtained from Sigma, Fluka, or Carlo Erba (Milano, Italy).

3.3. Procedure

Capillary conditioning methodology was reported previously [17]. Briefly, before the first use it was conditioned with 1 M NaOH, water and the running buffer; when the buffer was changed it was rinsed with water and the new buffer; and between runs it was rinsed for 3 min with running buffer. At the end of the working session the capillary was flushed with water.

Several running buffers covering the pH range between 3 and 12 and 0.05 M ionic strength were prepared. The employed buffers, its pK_a value, the covered pH range, the stock solutions used as well as their preparation is explained elsewhere [17].

Stock solutions of the analytes were prepared at a concentration of 1 mg L^{-1} in water or a mixture of water/methanol when they were not soluble in water itself. After that, they were diluted with water to a concentration of 100 μg mL^{-1}. Benzyl alcohol was added (100 μg mL^{-1}) and used as EOF marker for the calculation of the mobilities.

All running buffers and samples were filtered through a 0.45 μm pore size nylon filter (Whatman, Maidstone, Kent, UK) and stored at 4 °C until used.

4. Results and discussion

4.1. Reference set of basic internal standards

In order to set up a list of basic internal standards to be used in routine analysis we selected 25 monoprotic compounds of different chemical nature. The set of compounds together with their structures and literature pK_a values are shown in Table 1. According to the pK_a values, these compounds cover the most useful pH range in CE, so they can be used to determine any pK_a between 4 and 11.

The next step was to establish the reference pK_a of these compounds through the internal standard method. Most of the literature pK_a values were taken from a compilation [18] based on the most extensive pK_a values databases [19–22]. Only when a pK_a was not found in these sources, the values were taken from experimental determinations from literature [23–27], or were experimentally determined in our laboratory [17,28]. Although the literature values have been taken from reliable bibliographic sources, sometimes different pK_a values can be found for the same compound, and it is difficult to ascertain which value is the correct one. For this reason we applied the iterative process we established previously for acids [15], starting with the corresponding literature pK_a value for each internal standard ($pK_{a,IS}$)to calculate the pK_a of each compound through Eq. (10), and using our own experimental electrophoretic data ($\mu_{BH^+,AN}$, $\mu_{BH^+,IS}$, $\mu_{eff,AN}$, and $\mu_{eff,IS}$) to calculate the *Q* ratios. In this process compounds have been used indistinctly as analytes or as internal standards in the following way: to determine the pK_a of a given compound of Table 1, the neighboring compounds of the table (which have a similar pK_a value) have been used as internal standards. This procedure has been done for each compound in the list, using the corresponding neighbors as internal standards. The next step is to determine the limiting mobilities, injecting both compounds together (the analyte and the internal standard) at a pH where they are totally ionized, and also determine the effective mobilities, injecting them together at a pH where they are only partially ionized. Eq. (10) provides then a new pK_a for the analyte, through the electrophoretic data and the literature pK_a of the internal standard.

This new pK_a is used then as pK_a of the internal standard to start a new iteration step, using the same electrophoretic data. This process is repeated until pK_a values obtained through two consecutive iterations differ in less than 0.02 pK_a units.

As an example Table 2 shows the iterative process for aniline, quinoline and 4-*tert*-butylaniline, although this process has been performed for all the compounds in Table 1.

In case of aniline, three other compounds have been used as internal standard (quinoline, 4-*tert*-butylaniline, and *N*,*N*-dimethyl-*N*-phenylamine), or in case of quinoline also three other compounds (aniline, 4-*tert*-butylaniline, and pyridine) have been used as internal standard. To start the iteration process for aniline, the literature values of the three internal standards (4.87, 4.95, and 5.07 respectively) are used in Eq. (10) as $pK_{a,IS}$, together with the mobility data to determine a new pK_a for aniline. In this way, three different $pK_{a,AN}$ values are obtained for aniline, which are shown in Table 2 (4.57, 4.64 and 4.53). These three values are averaged, and the average (4.58) is used as new pK_a for aniline in the second round of the iteration process. In the same way, the average values calculated for the rest of compounds (4.91 for quinoline, 4.87 for 4-*tert*-butylaniline, 5.17 for *N*,*N*-dimethyl-*N*-phenylamine, etc.) are used as new $pK_{a,IS}$ for the second iteration (see the $pK_{a,IS}$ values in the second iteration column). Now, using these values in Eq. (10), 3 new pK_as are obtained for aniline (4.61, 4.57, and 4.63), and the average of these values (4.60) will be used in the third iteration. At the end of the iteration process a new list of pK_a values, which may be slightly different from literature values, is obtained. The iterative process was stopped after a maximum of six iterations. In this point the differences between the average values of iteration 5 and iteration 6 were lower than 0.02 pK_a units for all compounds.

Table 1 shows the final pK_a values, which are the reference values for the proposed set of internal standards. The number of internal standards used (*N*), the standard error obtained, and the limiting mobility of each reference compound are also indicated. The difference between the literature and the final pK_a values obtained after the iterative process is lower than 0.1 pK_a units for most of the compounds. Only bupivacaine, 1-phenylpiperazine, and *N*,*N*-dimethyl-*N*-benzylamine show differences between 0.1 and 0.2 pK_a units. The advantage of using this procedure is that during the iterative process, the initial pK_a values, which could be uncertain, converge to the correct final value, which is consistent with the measured electrophoretic mobilities for the whole set of internal standards.

Table 1
Set of basic internal standards with their structures, literature $pK_{a,lit}$ values, reference pK_a values at 25 °C and zero ionic strength, limiting mobilities μ_{BH^+} and the respective standard deviations. *N* is the number of internal standards used in the iteration process.

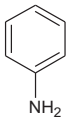
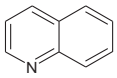
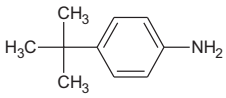
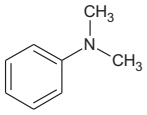
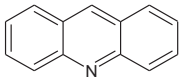
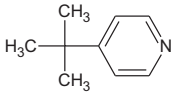
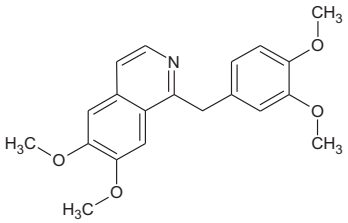
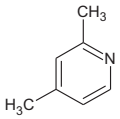
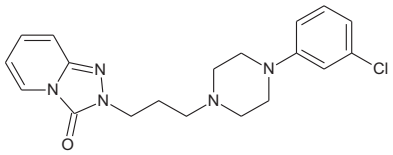
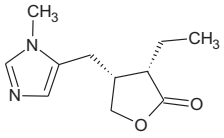
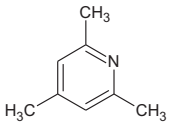
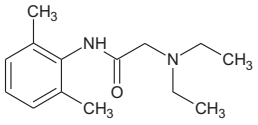
Compound	Structure	$pK_{a,lit}$	Reference	*N*	pK_a	μ_{BH^+} •10^4(cm^2 min^{-1} V^{-1})
Aniline		4.60	[18]	3	4.63 ± 0.02	102.5 ± 0.7
Quinoline		4.87	[18]	3	4.93 ± 0.01	102.4 ± 1.0
4-*tert*-Butylaniline		4.95	[18]	4	4.93 ± 0.01	75.6 ± 1.0
N,*N*-Dimethyl-*N*-phenylamine		5.07	[18]	4	5.17 ± 0.02	96.1 ± 0.8
Pyridine		5.22	[18]	6	5.28 ± 0.01	138.8 ± 1.7
Acridine		5.52	[23]	4	5.55 ± 0.06	85.2 ± 1.3
4-*tert*-Butylpyridine		5.99	[18]	4	6.03 ± 0.03	91.2 ± 0.8
Papaverine		6.34	[18]	4	6.41 ± 0.07	52.3 ± 0.6
2,4-Lutidine		6.79	[23]	4	6.81 ± 0.05	104.3 ± 0.3
Trazodone		6.87	[17]	4	6.84 ± 0.05	49.9 ± 0.5
Pilocarpine		7.08	[24]	5	7.08 ± 0.02	76.2 ± 1.1
2,4,6-Trimethylpyridine		7.43	[18]	5	7.51 ± 0.03	93.1 ± 1.6
Lidocaine		7.96	[25]	5	7.93 ± 0.01	62.2 ± 0.5

Table 1 (*Continued*)

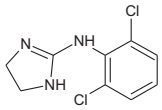
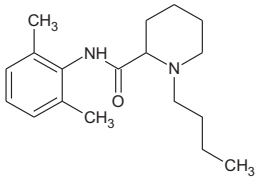
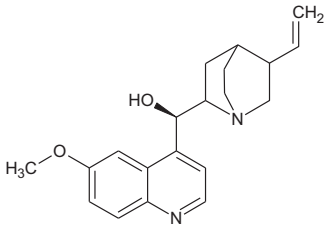
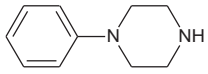
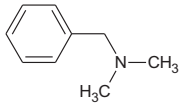
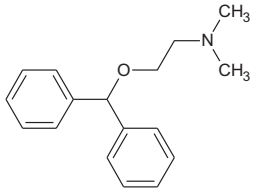
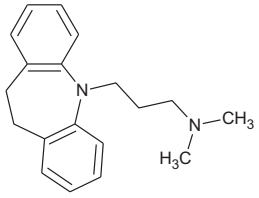
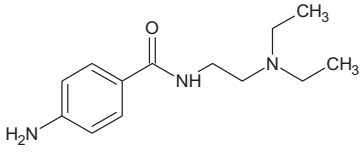
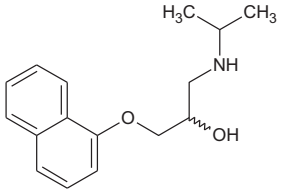
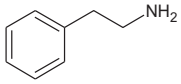
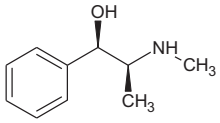
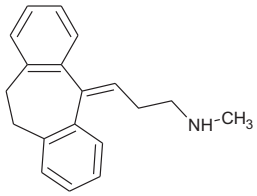
Compound	Structure	$pK_{a,lit}$	Reference	*N*	pK_a	μ_{BH^+} •10^4(cm^2 min^{-1} V^{-1})
Clonidine		8.12	[26]	5	8.10 ± 0.04	75.4 ± 0.7
Bupivacaine		8.35	[27]	5	8.19 ± 0.03	53.0 ± 2.1
Quinine		8.48	[17]	3	8.45 ± 0.05	52.4 ± 0.4
1-Phenylpiperazine		8.60	[23]	4	8.75 ± 0.02	81.5 ± 3.8
N,*N*-Dimethyl-*N*-benzylamine		9.14	[18]	7	8.95 ± 0.04	93.9 ± 3.3
Diphenhydramine		9.17	[17]	4	9.08 ± 0.02	63.11 ± 2.5
Imipramine		9.30	[28]	3	9.37 ± 0.02	59.9 ± 2.1
Procainamide		9.35	[17]	4	9.26 ± 0.03	61.5 ± 2.7
Propranolol		9.48	[17]	4	9.47 ± 0.00	56.8 ± 0.3
1-Aminoethylbenzene		9.49	[17]	3	9.52 ± 0.01	86.6 ± 0.2

Table 1 (*Continued*)

Compound	Structure	$pK_{a,lit}$	Reference	*N*	pK_a	μ_{BH^+} •10^4(cm^2 min^{-1} V^{-1})
Ephedrine		9.66	[26]	4	9.72 ± 0.02	72.4 ± 0.7
Nortriptyline		10.14	[17]	3	10.08 ± 0.01	59.4 ± 0.7

4.2. Determination of the pK_a of basic compounds through acidic internal standards

Another objective of this work was to test whether the similarity in terms of compounds structure and functionality between the analyte and the internal standard affects the obtained pK_a. For this purpose we have determined the pK_a of some of the bases of the above presented set, using acidic compounds as internal standard. One additional goal of this procedure is to relate the pK_a values of the set of basic internal standards established in this work with the pK_a values of the set of acidic internal standards previously established [15]. That is to say, to combine the two pK_a scales (one for neutral acids and the other for neutral bases) in one unique acid–base pK_a scale.

These kinds of determinations imply small modifications of the general procedure. When the analyte and the internal standard have the same charge (two monoprotic bases of similar pK_a, for example), only two CE analysis are needed: a first one at a pH where both bases are totally ionized which provides the limiting mobility (μ_{BH^+}), and a second one at a pH where both compounds are partially ionized to obtain the effective mobility (μ_{eff}). However, when the analyte and the internal standard are not of the same nature more measurements are required. In this case we have a monoprotic base and a monoprotic acid of similar pK_a. Three electrophoretic injections are needed then: a first one at low pH values to get the limiting mobility of the protonated basic analyte (μ_{BH^+}), a second one at a pH close to the pK_a of the compounds to obtain μ_{eff} of both compounds, and a third one at high pH values to get the limiting mobility of the ionized acidic internal standard (μ_{A^-}). The mobility of the analyte at this pH will be zero, and the mobility of the internal standard at low pH will also be zero, since in the respective conditions they will be in their neutral form. In addition, ionic activity coefficient correction is required (see Eq. (12)). Thus, this procedure is more complex and we recommend using analytes and internal standards of the same type (both neutral acids or neutral bases) whenever possible. They might be also similar in structure and properties to minimize differences in ionic activity coefficient and also buffer interactions. However, we have tested the feasibility of using analytes and standards of different types because it is not always possible to find an internal standard similar to the analyte.

Table 3 shows the results obtained. $pK_{a,AN,exp}$ is the pK_a directly determined in these experiments and pK_a is the reference pK_a established in Table 1. ΔpK_a measures the difference between $pK_{a,AN,exp}$ and pK_a. In some cases internal standards of different nature have been selected to determine the pK_a of the same compound, as for example for bupivacaine, whose pK_a has been determined using two different phenols and one paraben. In all cases very small differences are observed between both pK_a values, being always lower than 0.1 units. The results obtained are very good, although this fact is in part surprising for different reasons. First of all, in these experiments the possible errors in the measurement of the limiting mobilities are not compensated by the use of an internal standard, because the two limiting mobilities have been measured at different pH conditions. Only the measurement of the effective mobilities has been done in the same injection for both compounds. Secondly, the nature of the analyte and the internal standard is different, and specific interactions of the analyte such as interactions with the buffer constituents or with the capillary wall, for example, may not be compensated by the IS. These are the main reasons why higher differences between the experimental pK_a and the reference one were expected. Nevertheless, good agreement is observed, so it is concluded that the structural similarity between

Table 2
Iterative procedure for the establishment of the reference pK_a values from the literature initial pK_a values.

Analyte	Internal standard	Iteration 1		Iteration 2		Iteration 3		Iteration 4		Iteration 5		Iteration 6	
		$pK_{a,IS}$	$pK_{a,AN}$	$pK_{a,IS}$	$pK_{a,AN}$	$pK_{a,IS}$	$pK_{a,AN}$	$pK_{a,IS}$	$pK_{a,AN}$	$pK_{a,IS}$	$pK_{a,AN}$	$pK_{a,IS}$	$pK_{a,AN}$
Aniline	Quinoline	4.87	4.57	4.91	4.61	4.88	4.59	4.91	4.61	4.91	4.61	4.92	4.62
	4-*tert*-Butylaniline	4.95	4.64	4.87	4.57	4.91	4.60	4.90	4.60	4.92	4.61	4.92	4.62
	N,*N*-Dimethyl-*N*-phenylamine	5.07	4.53	5.17	4.63	5.14	4.60	5.17	4.64	5.17	4.63	5.19	4.65
	Average		4.58		4.60		4.60		4.61		4.62		4.63
Quinoline	Aniline	4.60	4.90	4.58	4.88	4.60	4.90	4.60	4.89	4.61	4.91	4.62	4.91
	4-*tert*-Butylaniline	4.95	4.95	4.87	4.87	4.91	4.91	4.90	4.90	4.92	4.92	4.92	4.92
	Pyridine	5.24	4.89	5.25	4.90	5.27	4.92	5.27	4.92	5.28	4.94	5.29	4.94
	Average		4.91		4.88		4.91		4.91		4.92		4.93
4-*tert*-Butylaniline	Aniline	4.60	4.91	4.58	4.89	4.60	4.91	4.60	4.90	4.61	4.92	4.62	4.92
	Quinoline	4.87	4.87	4.91	4.91	4.88	4.88	4.91	4.91	4.91	4.91	4.92	4.92
	N,*N*-Dimethyl-*N*-phenylamine	5.07	4.83	5.17	4.93	5.14	4.90	5.17	4.93	5.17	4.93	5.19	4.95
	Pyridine	5.24	4.89	5.25	4.90	5.27	4.92	5.27	4.92	5.28	4.93	5.29	4.94
	Average		4.87		4.91		4.90		4.92		4.92		4.93

Table 3
pK_a of basic compounds determined through an acidic internal standards. Mobilities ($\mu \times 10^4$) are expressed in $cm^2\ V^{-1}\ min^{-1}$.

Analyte	Internal standard	$pK_{a,IS}$	$\mu_{BH^+,AN}$	$\mu_{eff,AN}$	$\mu_{eff,IS}$	$\mu_{A^-,IS}$	$pK_{a,AN,exp}$	ΔpK_a
Bupivacaine	2-Chlorophenol	8.50	55.5	20.8	−41.5	−81.9	8.12	0.07
Bupivacaine	Methylparaben	8.35	55.4	21.2	−40.7	−69.7	8.12	0.07
Bupivacaine	3,5-Dichlorophenol	8.18	55.6	21.3	−51.2	−76.8	8.10	0.09
2,4,6-Trimethylpyridine	4-Hydroxybenzaldehyde	7.61	95.1	49.6	−41.7	−86.0	7.45	0.06
2,4-Lutidine	Vanillin	7.36	110.9	39.0	−33.0	−81.5	6.76	0.05
Acridine	Warfarin	5.17	76.9	54.9	−37.2	−59.5	5.62	−0.07
N,*N*-Dimethyl-*N*-phenylamine	Sulfacetamide	5.42	98.6	69.6	−19.7	−75.7	5.17	0.00
Quinoline	Benzoic acid	4.22	108.5	90.6	−58.1	−97.8	4.92	0.01
Aniline	Ibuprofen	4.49	110.3	74.6	−29.5	−65.1	4.56	0.07

the analyte and the internal standard is not an essential requirement in the internal standard method. However, this is only true when the analyte does not suffer specific interactions. In that case the two compounds would act in a different way under the analysis conditions, so deviations in mobility would not be compensated, obtaining then clear deviations in the calculated pK_a.

5. Conclusions

A reference set of 25 basic internal standards of different nature that cover all the useful pH range in CZE has been established. Together with the reference set of acidic internal standards provided in an earlier work, analysts have at their disposal a wide set of compounds of well-known pK_a to select in a simple and fast way the best internal standard for a given determination. These two sets can be combined into one unique set of reference compounds (some acidic and some basic standards) for routine determination of acidity constants through the internal standard method.

It has been proved that in absence of specific interactions of the analyte the only requirement to select an internal standard is the similarity between the pK_a values, being the nature or the structure of the compounds not relevant for the determination, provided that ionic strength is carefully controlled and the ionic activity coefficient correction appropriately done. However, we recommend the use of analytes and internal standards of similar nature and functionality, or even with similar structure when possible, in order to avoid activity coefficient corrections and deviations caused by specific interactions, which are not always easily detectable.

The internal standard method is a fast alternative to other methods for pK_a determination. Among other advantages, this method requires few electrophoretic runs for the determination of an acidity constant, and the exact measure of the pH of the buffer solutions is not needed. Once solutions of electrophoretic buffers at different pH values (not measured), and solutions of internal standards are prepared, routine analysis can be performed just selecting the appropriate buffers and internal standard for a given analyte.

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