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Fast high-throughput method for the determination of acidity constants by capillary electrophoresis. II. Acidic internal standards

Joan Marc Cabot, Elisabet Fuguet, Clara Ràfols, Martí Rosés*

Departament de Química Analítica, Institut de Biomedicina de la Universitat de Barcelona (IBUB), Universitat de Barcelona, Martí i Franquès 1-11, E-08028 Barcelona, Spain

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ABSTRACT

A fast method for the determination of acidity constants by CZE has been recently developed. This method is based on the use of an internal standard of pK_a similar to that of the analyte. In this paper we establish the reference pK_a values of a set of 24 monoprotic neutral acids of varied structure that we propose as internal standards. These compounds cover the most usual working pH range in CZE and facilitate the selection of adequate internal standards for a given determination. The reference pK_a values of the acids have been established by the own internal standard method, i.e. from the mobility differences between different acids of similar pK_a in the same pH buffers. The determined pK_a values have been contrasted to the literature pK_a values and confirmed by determination of the pK_a values of some acids of the set by the classical CE method. Some systematic deviations of mobilities have been observed in NaOH buffer in reference to the other used buffers, overcoming the use of NaOH in the classical CE method. However, the deviations affect in a similar degree to the test compounds and internal standards allowing thus, the use of NaOH buffer in the internal standard method. This fact demonstrates the better performance of the internal standard method over the classical method to correct mobility deviations, which together with its fastness makes it an interesting method for the routine determination of accurate pK_a values of new pharmaceutical drugs and drug precursors.

1. Introduction

The knowledge of the acidity constant K_a (or in logarithmic scale, pK_a) of chemical compounds with acid–base properties is essential in many fields. In fact, the neutral and ionic forms of the chemical substances can have very different physicochemical and biological properties, the acidity constant being sometimes decisive for a given application. This is the case in the pharmaceutical industry, in which the pK_a values have a particular importance [1–3]. Many drugs are weak acids or bases and new applications will depend on their particular physicochemical properties such as acidity, hydrophobicity, and solubility [3,4]. Therefore, pharmaceutical companies must characterize a great number of potential drugs and chemical precursors in a relatively short time to select those which are more suitable for their further test and development. Need of rapid patents of the promising drugs is evident for such companies, which normally compete for similar compounds and applications, thus justifying the increasing demand of fast and reliable physicochemical characterization techniques of potential drugs [1,2].

There are several methodologies for the determination of acidity constants. The ones most used are based on potentiometric [5–7], spectrophotometric [5,8] and electrophoretic measurements [9–15]. Capillary electrophoresis (CE) has been widely used to determine acidity constants. It is a highly automatized technique that uses small amounts of samples and reagents. In addition, impurities are not a problem because they can be easily separated from the analyte during the electrophoretic process [13–17].

Recently, our research group has developed a new method to determine acidity constants by capillary zone electrophoresis (CZE) [18,19], faster and more efficient than the classical one. This method is based on the use of a reference compound or internal standard (IS), with nature and pK_a similar to those of the analyte. The reference compound and the analyte behave in a similar manner in the conditions of the analysis and therefore, the difference between the values of their relative mobilities is directly related to their different acidity. In comparison with other CE methods, the internal standard method requires less electrophoretic measurements and there is no need to measure the pH value of the buffer solution. In fact, for routine pK_a estimation the analyte and the internal standard can be injected at only two different pH values: a first one where the analyte and the IS are partially ionized (at pH = p$K_a \pm 1$) in order to calculate the effective mobility (μ_{eff}), and a second one in which both are completely ionized to calculate the limiting mobility (μ_{A^-} or μ_{BH^+}) for monoprotic compounds. From these mobility

* Corresponding author. Tel.: +34 93 403 92 75; fax: +34 93 402 12 33.
E-mail addresses: marti.roses@ub.edu, marti@apolo.qui.ub.es (M. Rosés).

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measurements the pK_a can be directly obtained if the pK_a of the IS is well known. The CE internal standard method is, therefore, an attractive alternative to other methods, which are more expensive because they need higher amounts of pure sample and solvents, and they are more time consuming [5–15]. The main purpose of this paper is to establish the pK_a of a series of acids with different functional groups and structures, covering all the useful pH range in CE, which can potentially be employed as internal standards for the fast and efficient knowledge of the pK_a of new drugs and precursors.

2. Theory

The effective electrophoretic mobility, μ_{eff}, of a monoprotic neutral acid HA can be related to the acidity constant K_a and pH through the following equation [16]:

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

where μ_{A^-} is the mobility of the completely deprotonated species, and pK'_a is related to the thermodynamic pK_a and the activity coefficient of the anionic species γ_{A^-}, which corrects the effect of the ionic strength (I), as follows:

$$pK_a = pK'_a - \log \gamma_{A^-} \quad (2)$$

The pK_a of the test compound can be easily obtained from Eq. (1):

$$pK'_a = pH + \log \frac{\mu_{A^-} - \mu_{eff}}{\mu_{eff}} = pH + \log Q \quad (3)$$

For an acid HA, Q is the coefficient of the relative amounts of the neutral and ionic forms, related to μ_{A^-} and μ_{eff}. Such mobilities ($cm^2\,V^{-1}\,min^{-1}$) can be directly calculated from the retention time of the analyte t_m and the electroosmotic flow marker t_0 (min) by means of the expression:

$$\mu = \frac{L_T L_D}{V}\left(\frac{1}{t_m} - \frac{1}{t_0}\right) \quad (4)$$

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

In the internal standard method, Eq. (3) is applied to both, the analyte (AN) and the internal standard (IS). The difference between the two equations obtained leads to:

$$pK'_{a,AN} - pK'_{a,IS} = \log Q_{AN} - \log Q_{IS} \quad (5)$$

This equation is independent of pH. Therefore, when injecting both compounds using a given buffer solution in which they are all completely deprotonated (to obtain μ_{A^-}), and a second buffer solution with a different pH where they are partially ionized (to obtain μ_{eff}), p$K'_{a,AN}$ can be calculated despite the pH is not exactly known (since it is not involved in the equation):

$$pK'_{a,AN} = pK'_{a,IS} + \log Q_{AN} - \log Q_{IS} \quad (6)$$

To determine p$K'_{a,AN}$, the value of p$K'_{a,IS}$ is obviously needed, and it should be as accurate as possible.

Notice, that if the ionic forms of the analyte and internal standard have the same charge (such as in all acids studied in this work) then Debye–Hückel equation predicts the same activity coefficient correction for Eq. (2). In this instance, pK'_a can be replaced by pK_a in Eq. (6) for both analyte and internal standard. In other words, the pK_a (or pK'_a) value of the analyte is obtained in the same working conditions (ionic strength) than the pK_a value of the internal standard used to calculate it.

3. Experimental

3.1. Apparatus

Capillary electrophoresis experiments have been performed using a P/ACE MDQ Beckman instrument (Palo Alto, CA, USA), equipped with a diode-array spectrophotometric detector. The fused-silica capillaries were 50.2 cm length (40 cm to the detector) × 50 μm ID and were obtained from Composite Metal Services Ltd. (Shipley, West Yorkshire, UK). The temperature of the capillary was 25.0 ± 0.1 °C. The samples were injected at a hydrodynamic pressure of 0.5 psi for 3 s, and the applied voltage was 20 kV.

Before the injections, the capillary was conditioned according to the following steps [17,18]: 1.0 M NaOH (20 min), H_2O (10 min), and finally the buffer to be used in the experiment (20 min). Between consecutive injections, the capillary was washed with the same buffer (3 min), and after 4–5 injections, the vial containing the buffer was replaced. When a different buffer was used, the capillary was washed with water (10 min) and afterwards, with the new buffer solution (20 min). At the end of each session, the capillary was washed with 0.1 M NaOH (10 min) and water (15 min).

3.2. Reagents

Benzyl alcohol (analytical-reagent grade), 0.5 M sodium hydroxide, 0.5 M hydrochloric acid, and potassium chloride (>99.5%) were from Merck (Darmstadt, Germany). Anhydrous sodium acetate (>99.6%) was purchased from J.T. Baker (Deventer, The Netherlands). 2-(Cyclohexylamino)ethanesulfonic acid (CHES, >99%), and 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS, >98%) were from Sigma (St. Louis, MO, USA). 2,2-Bis(hydroxymethyl)-2,2′,2″-nitrilotriethanol (BisTris), and sodium formate were from Fluka (Buchs, Switzerland). Tris(hydroxylmethyl)amino-methane (Tris, >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: 2-chlorobenzoic acid, 2,6-dibromo-4-nitrophenol, 4-nitrobenzoic acid, 2,6-dinitrophenol, 3-bromobenzoic acid, 2,4-dinitrophenol, benzoic acid, ibuprofen, nicotinic acid, warfarin, 2,5-dinitrophenol, sulfacetamide, 2,4,6-tribromophenol, 4-nitrophenol, vanillin, 4-hydroxybenzaldehyde, phenobarbital, 3,5-dichlorophenol, methylparaben, 2-chlorophenol, 3-chlorophenol, 4-bromophenol, paracetamol, and phenol. All the compounds were reagent grade or of chromatographic quality and were obtained from Sigma, Fluka, or Carlo Erba (Milan, Italy).

3.3. Preparation of samples and buffers

The buffers employed covered practically all the useful CE pH range (from 3 to 12). They are shown in Table 1, together with the corresponding pH range and their stock solutions. The buffer solutions have been prepared as described in previous work [17]. Briefly, to prepare the buffers at the desired pH and constant ionic strength, 0.5 M HCl, 0.5 M NaOH or 0.5 M KCl was added to 50 mL of the stock solution (0.1 M). Finally, the solution obtained was diluted by adding water up to a volume 100 mL, thus allowing keeping a constant ionic strength of 50 mM.

The internal standard solutions were prepared at a concentration of 1000 μg mL^{-1}, in water or in a methanol/water mixture (when they were not soluble in water). Afterwards they were diluted to 100 μg mL^{-1} with water. Benzyl alcohol (100 μg mL^{-1}) was then added as the EOF marker to determine the mobilities.

All the samples and buffers were filtered through a nylon mesh 0.45 μm porous size (Whatman, Maidstone, UK) and stored at 4 °C until used.

Table 1
Buffer solutions used to determine acidity constants with the internal standard and classical methods by capillary electrophoresis.

Buffer	pK_a	pH range	Stock solution (I = 50 mM)
HCOOH/$HCOO^-$	3.75	2.60–4.80	0.1 M HCOONa + 0.5 M HCl
CH_3COOH/CH_3COO^-	4.76	3.70–5.80	0.1 M CH_3COONa + 0.5 M HCl
$BisTrisH^+$/BisTris	6.48	5.50–7.50	0.1 M BisTrisHCl + 0.5 M NaOH
$H_2PO_4^-/HPO_4^{2-}$ [a]	7.21	5.80–8.20	0.1 M NaH_2PO_4 + 0.1 M Na_2HPO_4
$TrisH^+$/Tris	8.08	7.00–9.00	0.1 M TrisHCl + 0.5 M NaOH
$H_3BO_3/H_2BO_3^-$ [a]	9.24	8.20–10.40	0.1 M $Na_2B_4O_7$ + 0.5 M HCl
CHES/$CHES^-$	9.50	8.40–10.10	0.1 M CHESNa + 0.5 M HCl
CAPS/$CAPS^-$	10.40	9.40–11.60	0.1 M CAPSNa + 0.5 M HCl
NaOH[b]		11.8–13.00	0.1 M NaOH + 0.5 M KCl

[a] Used only in the classical method.
[b] Not recommended for the classical method (see text).

4. Results and discussion

Table 2 lists the acids selected as internal standards together with their literature pK_a values (p$K_{a,lit}$) at 25 °C and zero ionic strength [20–24]. They are of diverse chemical nature and cover the pH range most useful for CE. We selected pK_a values from general sources which are compilations of pK_a values [20,21]. Only when the pK_a of the compound was not found in this reference literature, it was taken from specific research articles [22–24]. Since different pK_a values can be found in the literature for a same compound, it was difficult to choose one reference value for each internal standard. To set up a list with reliable pK_a values, we applied an iterative process with our own experimental electrophoretic data. The advantage of using this procedure is that during the refining process, the initial pK_a values, which could be no good enough, converge to the correct final value (consistent with the measured electrophoretic mobilities for the whole set of internal standards).

The procedure to establish the pK_a reference values was performed as follows. The pK_a value for each acid (p$K_{a,AN}$) was determined from the electrophoretic measurements using a series of appropriate acids from the same set in Table 2 with p$K_{a,IS}$ = p$K_{a,AN}$ ± 1, used as internal standards. 4–7 internal standards were generally used for each compound. In a first approach the literature pK_a values of the internal standard were used as p$K_{a,IS}$ in Eq. (6) for the calculation of p$K_{a,AN}$. Then the p$K_{a,AN}$ values obtained with the different internal standards for the same analyte were averaged. This procedure provided a new list of pK_a values for all the studied compounds, slightly different from the initial literature ones. An iterative process was then applied to obtain a new pK_a value for each compound, from the already measured electrophoretic mobilities and the new obtained pK_a values for the internal standards (instead of the literature ones). A new set of refined average pK_a values was thus obtained. The procedure was repeated until achieving pK_a values differing only ≤0.01 units between consecutive refined values.

Table 3 shows the evolution of the pK_a values of some compounds of Table 2 through the consecutive refining steps. For example, the pK_a of 2-chlorobenzoic acid was calculated from the p$K_{a,lit}$ values of 2,6-dibromo-4-nitrophenol, 4-nitrobenzoic acid, 2,6-dinitrophenol, and 3-bromobenzoic acid, used as internal standards (N = 4), and the corresponding electrophoretic results. The calculated pK_a values of 2-chlorobenzoic acid from these four internal standards were 2.91, 2.91, 2.86 and 2.77, respectively, with an average value of 2.86 (see the first value in the column of p$K_{a,R1}$ shown in Table 3). At the same time, the pK_a of 2,6-dibromo-4-nitrophenol was calculated from the p$K_{a,lit}$ values of 2-chlorobenzoic acid, 4-nitrobenzoic acid, 2,6-dinitrophenol, 3-bromobenzoic acid, 2,4-dinitrophenol and benzoic acid (N = 6) obtaining p$K_{a,R1}$ = 3.32. Similarly, the p$K_{a,R1}$ of 4-nitrobenzoic acid was calculated from the p$K_{a,lit}$ values of five different internal stan-

Table 2
Literature (p$K_{a,lit}$) and experimental reference (p$K_{a,ref}$) pK_a values at 25 °C and zero ionic strength of the acidic internal standards, together with the mobilities of the completely deprotonated species μ_{A^-}. N is the number of internal standards used in the determination.

Compound	N	p$K_{a,lit}$	Ref.	p$K_{a,ref}$	$\mu_{A^-} \times 10^4$ (cm^2 min^{-1} V^{-1})
2-Chlorobenzoic acid	4	2.92	[20]	2.84 ± 0.02	−137.1 ± 0.5
2,6-Dibromo-4-nitrophenol	6	3.38	[20]	3.31 ± 0.03	−122.8 ± 1.1
4-Nitrobenzoic acid	5	3.43	[20]	3.37 ± 0.01	−138.1 ± 0.5
2,6-Dinitrophenol	7	3.74	[20]	3.69 ± 0.01	−149.6 ± 0.9
3-Bromobenzoic acid	8	3.83	[20]	3.79 ± 0.02	−134.1 ± 1.0
2,4-Dinitrophenol	7	4.10	[20]	4.12 ± 0.02	−140.8 ± 1.4
Benzoic acid	7	4.19	[20]	4.22 ± 0.03	−146.2 ± 1.9
Ibuprofen	6	4.43	[23]	4.49 ± 0.02	−95.7 ± 0.6
Nicotinic acid	7	4.82	[21]	4.85 ± 0.03	−143.8 ± 1.6
Warfarin	6	5.08	[23]	5.17 ± 0.04	−84.8 ± 2.3
2,5-Dinitrophenol	4	5.22	[20]	5.30 ± 0.05	−144.2 ± 0.8
Sulfacetamide	4	5.27, 5.32	[23,24]	5.42 ± 0.05	−114.7 ± 0.4
2,4,6-Tribromophenol	5	6.10	[20]	6.04 ± 0.08	−116.0 ± 1.3
4-Nitrophenol	5	7.18	[20]	7.09 ± 0.05	−144.1 ± 0.6
Vanillin	6	7.40	[21]	7.36 ± 0.06	−122.8 ± 1.6
4-Hydroxybenzaldehyde	6	7.51	[20]	7.61 ± 0.04	−137.2 ± 1.7
Phenobarbital	6	7.46	[22]	7.53 ± 0.04	−113.0 ± 1.1
3,5-Dichlorophenol	7	8.18	[20]	8.18 ± 0.04	−130.1 ± 1.8
Methylparaben	7	8.37	[20]	8.35 ± 0.03	−122.1 ± 1.7
2-Chlorophenol	7	8.51	[20]	8.50 ± 0.04	−145.0 ± 1.1
3-Chlorophenol	6	9.02	[20]	9.04 ± 0.01	−144.6 ± 1.3
4-Bromophenol	5	9.36	[19]	9.28 ± 0.01	−136.4 ± 1.2
Paracetamol	4	9.45, 9.75	[21,23]	9.58 ± 0.02	−110.7 ± 0.8
Phenol	3	9.99	[20]	9.89 ± 0.01	−150.7 ± 0.3

Table 3
Procedure for establishment of the reference pK_a values (p$K_{a,ref}$) from the literature initial pK_a values (p$K_{a,lit}$) after several pK_a refinements (p$K_{a,Ri}$).

Compound	N	p$K_{a,lit}$	p$K_{a,R1}$	p$K_{a,R2}$	p$K_{a,R3}$	p$K_{a,R4}$	p$K_{a,ref}$
2-Chlorobenzoic acid	4	2.92	2.86	2.85	2.85	2.84	2.84 ± 0.02
2,6-Dibromo-4-nitrophenol	6	3.38	3.32	3.32	3.31	3.31	3.31 ± 0.03
4-Nitrobenzoic acid	5	3.43	3.40	3.38	3.38	3.37	3.37 ± 0.01
2,6-Dinitrophenol	7	3.74	3.69	3.69	3.69	3.69	3.69 ± 0.01
3-Bromobenzoic acid	8	3.83	3.80	3.79	3.79	3.79	3.79 ± 0.02
2,4-Dinitrophenol	7	4.10	4.12	4.12	4.12	4.12	4.12 ± 0.02
Benzoic acid	7	4.19	4.20	4.20	4.21	4.21	4.22 ± 0.03
Ibuprofen	6	4.43	4.47	4.48	4.48	4.49	4.49 ± 0.02
Nicotinic acid	7	4.82	4.80	4.84	4.84	4.85	4.85 ± 0.03
Warfarin	6	5.08	5.13	5.15	5.15	5.16	5.17 ± 0.04
⋮	⋮	⋮	⋮	⋮	⋮	⋮	⋮

dards, etc. Once the column of p$K_{a,R1}$ values was completed, the procedure was repeated for each compound with the same electrophoretic data and internal standards, but using the new p$K_{a,R1}$ values instead of the literature pK_a values. A new series of p$K_{a,R2}$ values is thus obtained. The refining process was stopped after calculating the p$K_{a,R5}$ series, which was almost equal to p$K_{a,R4}$ (difference $\leq$0.01). The last column shows the p$K_{a,R5}$ values, which have been considered the reference ones (p$K_{a,ref}$). The standard error obtained for the use of N internal standards is also indicated.

For each compound μ_{eff} and μ_{A^-} were calculated in buffers with an appropriate pH from the experimental migration times by means of Eq. (4), and the corresponding p$K_{a,AN}$ was obtained from Eq. (6). The reference μ_{A^-} value was determined as the average of all the limiting mobilities obtained using the N internal standards. Table 2 shows all the limiting mobilities and the experimental pK_a values (p$K_{a,exp}$) with the corresponding standard deviation.

As shown in Table 2, the experimental results obtained in this paper agree well with the literature ones; in general they differ in less than 0.1 units. Table 2 provides, therefore, a series of monoprotic acidic internal standards with a well established pK_a value, useful to determine pK_a of test compounds through the internal standard method.

To further validate the method, the pK_a values of some of the studied compounds were also determined by the classical CE method. In the classical method, the effective mobility of the analyte is measured at pH values close to its pK_a, at some pH values much higher than pK_a and usually, although not strictly needed for a neutral acid, at some pH values much lower than pK_a. The plot of the mobility vs. pH gives a sigmoidal curve with an inflection point at pH = pK_a. In our case, the mobility was determined at pH increments of about 0.2 pH units in the middle of the curve, and at pH increments of about 0.5 pH units at the beginning and at the end of the curve. Fitting the corresponding effective mobility to pH through Eq. (1), the pK_a and μ_{A^-} parameters are obtained.

In particular, the pK_a values of paracetamol (with two quite different literature pK_a values) and phenol (with a 0.1 pK_a difference between literature and experimental values) were checked. When determining the pK_a values by the classical method, an abnormal behaviour was found at the end of the curve (see Fig. 1). The mobilities determined using NaOH as buffer showed a shift to more negative values than expected. It is difficult to discard NaOH because it is apparently innocuous and specific interactions with analytes have not been reported. For this reason CHES/CHES$^-$ and CAPS/CAPS$^-$ buffers (see Table 1) were supposed to be the cause of the deviation in the plots of Fig. 1. They are buffers with a more complex structure and could exhibit a selective capacity of interaction greater than that of the NaOH buffer. However, it is generally accepted that the zwitterionic buffers such as CHES and CAPS do not present interactions with acids of this type [17]. In addition, both have a similar chemical nature and it is not easy to ascertain from Fig. 1 that they promote deviations.

In order to test the effect of NaOH as buffer on the mobility–pH curves, we selected three other acids from Table 2, of different nature and lower acidity constants. This would allow drawing a baseline in the high pH region, where the buffers under study may present problems. These compounds are 3-bromobenzoic acid, 2,5-dinitrophenol, and 4-nitrophenol, with pK_a values of 3.79, 5.30, and 7.09, respectively.

Fig. 2 depicts the mobility–pH curves for such three compounds. It is clearly observed that when $H_2PO_4^-/HPO_4^{2-}$ and $H_3BO_3/H_2BO_3^-$ are used as buffers, their experimental points follow the same trend as the ones of CHES/CHES$^-$ and CAPS/CAPS$^-$ buffers. No deviation is observed in this case. In addition, a negative deviation in the mobility is clearly observed when NaOH buffer is employed, very similar to the negative deviation observed for paracetamol and phenol. These results allow conclusion that NaOH buffers are not adequate to determine pK_a values with the classical method. We do not know the exact reason of this behaviour, although, we presume it may be caused by attack of

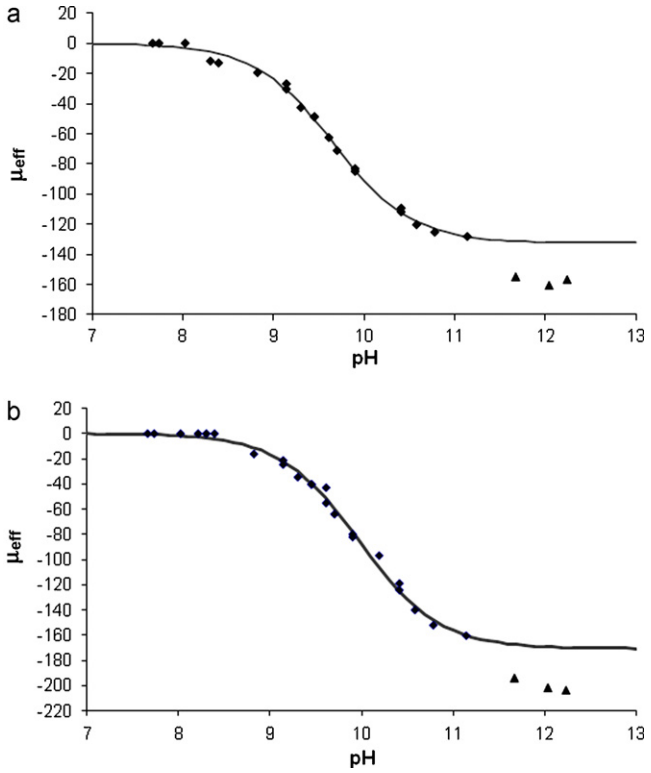

Fig. 1. Mobility (cm^2 min^{-1} V^{-1} × 10^4) vs. pH plots for (a) paracetamol and (b) phenol. The employed buffers are: (♦) TrisH$^+$/Tris, CHES/CHES$^-$, and CAPS/CAPS$^-$, and (▲) NaOH. Solid lines correspond to the fitting to Eq. (1).

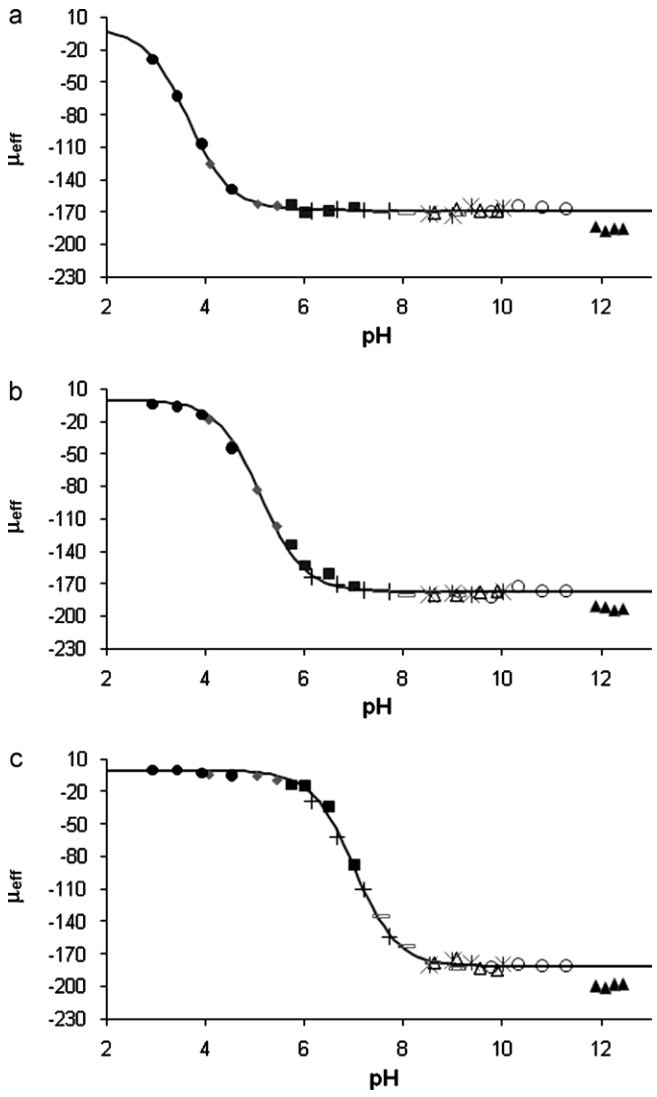

Fig. 2. Mobility ($cm^2\ min^{-1}\ V^{-1} \times 10^4$) vs. pH plots for (a) 3-bromobenzoic acid, (b) 2,5-dinitrophenol, and (c) 4-nitrophenol. The employed buffers are: (●) $HCOOH/HCOO^-$, (♦) CH_3COOH/CH_3COO^-, (■) $BisTrisH^+$/BisTris, (+) $H_2PO_4^-/HPO_4^{2-}$, (−) $TrisH^+$/Tris, (*) $CHES/CHES^-$, (△) $H_3BO_3/H_2BO_3^-$, (○) $CAPS/CAPS^-$, (▲) NaOH. Solid lines correspond to the fitting to Eq. (1).

the NaOH buffer to the silica capillary wall that modifies solute mobilities.

The pK_a values of the compounds of Figs. 1 and 2 were then calculated by fitting the experimental data to Eq. (1) but excluding the points obtained with NaOH buffer. The curve obtained from the fit is plotted as a solid line in Figs. 1 and 2, and the obtained pK_a values are listed in Table 4, where they are compared to those obtained using the internal standards method. It can be seen that they are practically equal.

Table 4
Comparison of experimental pK_a values at 25 °C and zero ionic strength, obtained through the internal standard method (Table 2) and through the classical method (statistics of the fitting to Eq. (1) are also shown).

	IS method	Classical method			
Compound	p$K_a \pm s$	p$K_a \pm s$	r^2	s	F
Paracetamol	9.58 ± 0.02	9.57 ± 0.02	0.996	2.85	4509.0
Phenol	9.89 ± 0.01	9.89 ± 0.03	0.994	4.22	3377.5
3-Bromobenzoic acid	3.79 ± 0.02	3.73 ± 0.01	0.995	2.29	6133.9
2,5-Dinitrophenol	5.30 ± 0.05	5.22 ± 0.02	0.996	3.86	8075.7
4-Nitrophenol	7.09 ± 0.05	7.09 ± 0.02	0.997	4.15	11458

In order to see if NaOH buffer had any effect in the measurements performed in the IS method, those analysis performed at high pH values with NaOH as buffer were repeated using CAPS/$CAPS^-$. It was observed that although the limiting mobility (μ_{A^-}) was shifted to more negatives values for NaOH than for CAPS, the same pK_a values were obtained independently of the buffer used. The reason is that although the analyte shows mobilities in NaOH more negative than those observed in CAPS, the same happens with the internal standard, so the effect is balanced and the same final pK_a value is obtained. Therefore, NaOH can be used to determine pK_a values using internal standard method proving the better performance of this method over the classical one to deal with mobility shifts caused by unexpected effects and interactions that affect in a similar degree to the analyte and the internal standard.

5. Conclusions

A reference set of monoprotic neutral acids with different functional groups and structures, and reference pK_a values that cover all the useful pH range in CE, has been established. These compounds can be systematically employed as internal standards for routine and accurate determination of pK_a values in a fast way. They can be especially interesting for the pK_a determination of new drugs and drug precursors in the pharmaceutical industry, which may need to deal with a lot of compounds in a short time.

For routine analysis of neutral acidic compounds an internal standard with a pK_a similar to the one of the analyte can be selected from the set presented in Table 2. Then, both compounds are injected together in a buffer with a pH where they are partially ionized and in a buffer with a pH where they are totally ionized. The electrophoretic mobilities in these conditions are calculated from the migration times, and finally the pK_a of the analyte is calculated through Eq. (6). This procedure provides a pK_a value for the analyte as accurate as the one of the internal standard. Higher precision and accuracy can be obtained selecting several adequate internal standards from the list, and averaging the results obtained.

It has been also observed, that the internal standard method compensate some systematic deviations of mobility, which is a clear advantage over the classical CE method. In fact, it has been proved that mobilities determined using NaOH as buffer show a shift to more negative values than expected from other buffers. The NaOH buffer is, therefore, not suitable for accurate determinations when the classical method is used, whereas these problems are overcome with the internal standard method.

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