Determination of the aqueous pKₐ of very insoluble drugs by capillary electrophoresis: Internal standards for methanol-water extrapolation

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A fast determination of acidity constants (pKₐ) of very insoluble drugs has become a necessity in drug discovery process because it often produces molecules that are highly lipophilic and sparingly soluble in water. In this work the high throughput internal standard capillary electrophoresis (IS-CE) method has been adapted to the determination of pKₐ of water insoluble compounds by measurement in methanol/aqueous buffer mixtures. For this purpose, the reference pKₐ values for a set of 46 acid-base compounds of varied structure (internal standards) have been established in methanol-water mixtures at several solvent composition levels (with a maximum of 40% methanol). The IS-CE method has been successfully applied to seven test drugs of different chemical nature with intrinsic solubilities lower than 10⁻⁸ M. pKₐ values have been determined at different methanol/aqueous buffer compositions and afterwards Yasuda-Shedlovsky extrapolation method has been applied to obtain the aqueous pKₐ. The obtained results have successfully been compared to literature ones obtained by other methods. It is concluded that the IS-CE method allows the determination of aqueous pKₐ values using low proportions of methanol, becoming then more accurate in the extrapolation procedure than other reference methods.

1. Introduction

New technologies and strategies for drug discovery and development have increased considerably in the last decades, creating new opportunities for gathering and integrating information to increase the success and efficiency of drug discovery. Consequently, pharmaceutical companies synthesize a great number of potential drugs and chemical precursors in a relative short time. ADMET (Absorption, Distribution, Metabolism, Excretion and Toxicity) and DMPK (Drug Metabolism and Pharmacokinetics) studies frequently use physicochemical parameters for the understanding and simulation of drug properties and processes, to select those compounds most suitable for further test and development. Therefore, there is a need of high throughput screening analytical methods for rapid evaluation of potential drug candidates as soon as they are synthesized [1,2].

The acidity dissociation constant (or pKₐ in logarithmic scale) is an important physicochemical parameter that plays an important role in the ADMET and DMPK studies since it determines the ionization degree of a potential drug at the target pH. In fact, the neutral and ionic forms of a compound can have very different physicochemical and biological properties, being the drug pKₐ sometimes decisive for a given application [2–4]. Modern techniques of drug discovery often produce molecules that are highly lipophilic and sparingly soluble in water. Poor aqueous solubility becomes a drawback in the physicochemical characterization because many assays require the drugs to be in aqueous solution during measurement. Therefore, the determination of the acidity constant of this kind of drugs can be a serious problem [2,4–6]. Different analytical techniques are commonly used to determine pKₐ of very insoluble compounds, such as potentiometry and spectrophotometry. A very common practice in these cases is to use aqueous-organic solvent mixtures to dissolve the drug, and then determine the pKₐ at different ratios of the solvent mixtures. The pKₐ in water is then estimated by extrapolation to 0% of organic solvent [7–10].

Capillary electrophoresis is a particularly convenient separation technique for pKₐ determinations because low amounts of sample are needed, information about concentration is not required, and it handles both impure and complex samples [11]. The classic CE method for pKₐ measurement is based on the relationship between the electrophoretic mobility of an ionic compound and the pH of the background electrolyte solution [12–20]. The inflection

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point of the mobility vs. pH curve corresponds to the pK_a value of the compound. To obtain reliable pK_a values, the mobility measurements must be done in several buffers of adequate and constant ionic strength, and well-known pH. Some years ago, a high-throughput CE method to determine acidity constants based on the use of internal standards (ISs) was developed [21]. Among other advantages, the internal standard capillary electrophoresis (IS-CE) method does not need an accurate measure of the pH of the electrophoretic buffers, and few measurements are needed for a single pK_a determination (just two, at two different pH values). In addition, experimental errors due to putative interactions between the test compounds and buffers and other systematic errors -like temperature or buffer pH variations due to electrolysis processes during run analysis- are minimized by the use of an adequate internal standard [21]. The IS-CE method works well for many different kinds of drugs [22], the pK_a of a compound can be determined within 5 min, and a novel automated instrument has been developed for this purpose [23].

Regarding solubility, the IS-CE method has been evaluated for the determination of pK_a values of sparingly soluble compounds. It has been demonstrated that the pK_a values of compounds with solubility values of about 10⁻⁸ mol L⁻¹ can be determined directly in aqueous buffers [24]. Nevertheless, mixtures of aqueous buffers and organic solvents should be used as electrophoretic background electrolytes to apply the method to less soluble compounds.

It is well-known that the pK_a value of a substance dissolved in a solvent mixture changes according to the nature and content of organic solvent in the mixture. For this reason, to apply the IS-CE method using solvent mixture-based buffers it is mandatory to determine the pK_a values of the compounds established as ISs [25,26] in the same medium. Many water-miscible organic solvents have been employed for this purpose, such as alcohols or acetonitrile. Methanol has similar properties to water, and it can solubilize a large number of compounds insoluble in water itself. So far, it is thought to be the least error-prone of the common co-solvents since its general effect on pK_a values has been studied so extensively [7–9,27–29]. The IS-CE method is not an absolute determination method. The pK_a of the test compound is measured as a difference to the one of the IS. The same process is used to establish the pK_a of the internal standards in a given medium or condition: the selected set of compounds are used equally as ISs or as test compounds. Thus, reliable pK_a values of some acids and bases in the solvent mixtures are needed to anchor the IS-CE pK_a scale, i.e., pK_a values of some of the ISs in the methanol-water mixtures used must be known (from reliable literature for instance) or determined by an absolute method (e.g., potentiometry).

In this work, the pK_a of monoprotic neutral acetic or basic ISs were determined in different methanol-water mixtures at several levels of methanol by capillary electrophoresis and anchored to potentiometrically determined pK_a values. Afterwards, the performance of the method to determine acidity constants of very low soluble drugs (solubility below 10⁻⁶ mol L⁻¹) were evaluated by comparison of the results to the literature ones obtained by other methods.

2. Material and methods

2.1. Apparatus

Capillary electrophoresis experiments were done with a P/ACE MDQ Beckman instrument (Palo Alto, CA, USA), equipped with a diode-array spectrophotometric detector. Capillary was made of fused silica and was obtained from Composite Metal Services Ltd (Shipley, West Yorkshire, UK). The dimensions are 50 μm I.D., 375 μm O.D., and 35.2 cm length (25 cm to the detector). The temperature of the capillary was set to 25.0 ± 0.1 °C. Test compounds and internal standards were injected sequentially at a hydrodynamic pressure of 0.5 psi for 3 s (1 psi = 6894.76 Pa), and the applied voltage during separation was 20 kV. In order to speed up analysis, an additional hydrodynamic pressure of 1.0 psi was applied during separation. Optimized capillary conditionings were described elsewhere [21]. Briefly, at the beginning of the session capillary was conditioned with 1.0 mol L⁻¹ NaOH (2.0 min), H₂O (0.5 min) and buffer (2 min); 0.2 min with new buffer when pH was changed; and at the end of the session 0.1 mol L⁻¹ NaOH (2.0 min) and H₂O (2.0 min). Capillary was not rinsed between consecutive runs with the same background electrolyte.

Potentiometric pK_a determinations were performed in an 888 Titrando potentiometer from Metrohm (Herisau, Switzerland), equipped with a combined pH electrode and a burette also from Metrohm, a tempering beaker, and a temperature-controlled water bath (J. P. Selecta, Abrera, Spain).

2.2. Chemicals and solvents

Dimethyl sulfoxide >99.9% (DMSO), methanol HPLC grade, 0.5 mol L⁻¹ sodium hydroxide, 0.5 mol L⁻¹ hydrochloric acid, and sodium dihydrogenphosphate monohydrate >99% were from Merck (Darmstadt, Germany). Anhydrous sodium acetate >99.6%, 2-(cylohexylamino)ethanesulfonic acid >99% (CHES), and 3-(cylohexylamino)-1-propanesulfonic acid >98% (CAPS) were from Sigma (St. Louis, MO, USA); 2,2-bis(hydroxymethyl)-2.2′-nitrilotriethanol >99.9% (BiTris) and sodium formate were from Fluka (Buchs, Switzerland). Tris(hydroxymethyl)aminomethane >99.9% (Tris) was purchased from Aldrich (Milwaukee, WI, USA). Potassium hydrogen phthalate standard for volumetric analysis, ACS, ISO 99.95–100.05% was from Panrec (Castellar del Vallés, Spain). Water was purified by a Milli-Q plus system from Millipore (Bedford, MA, USA), with a resistivity of 18.2 MΩcm.

All studied drugs and internal standards were reagent grade or purer, and were purchased from Sigma, Aldrich, Fluka, Merck, or Carlo Erba.

2.3. Potentiometric titrations

A total of 25 mL of an approximately 0.005 mol L⁻¹ solution of the compound in the appropriate methanol-water mixture were placed in the thermostated beaker for the titration. Once the solution had reached 25 °C, the titration was performed by 0.1 mol L⁻¹ sodium hydroxide or 0.1 mol L⁻¹ hydrochloric acid, depending on the nature of the acid-base compound from pH 2 to pH 12, or vice versa. The titrand was solved in the same methanol-water media as the titrant. An inert gas (N₂) was continuously passed through the titrant solution to eliminate CO₂. All solutions (titrands and titrants) were prepared with pure methanol and boiled water. 0.1 mol L⁻¹ sodium hydroxide solution was previously standardized with potassium hydrogen phthalate. 0.1 mol L⁻¹ hydrochloric acid solution was standardized using Tris as primary standard. The potentiometric system was calibrated with the aqueous standard reference solutions at pH 2, 4, 7, and 9 at 25 °C. Glass electrode was conditioned for each methanol-water composition storing it at least for 24 h in the solvent mixture. In the potentiometric measurements, a minimum of 20 s and a maximum of 60 s were established as equilibration time between consecutive additions. Solvent evaporation was avoided closing the reservoir in which determination was done and shortening as much as possible the total analysis time (around 5–10 min each determination).

2.4. Preparation of samples and buffers for CE determinations

Stock solutions of sodium dihydrogenphosphate, sodium formate, sodium acetate, BisTrisH⁺, TrisH⁺, CHES⁻, CAPS⁻ or sodium
hydroxide were prepared in aqueous media at 0.25 mol L⁻¹. In order to obtain methanol-water BGE solutions at the desired pH and ionic strength, a known amount of 0.5 mol L⁻¹ HCl, 0.5 mol L⁻¹ NaOH, or 0.5 mol L⁻¹ KCl was added to 5 mL of the corresponding stock solution. Next, pure methanol was added to have the desired composition (v/v), and afterwards the content of the volumetric flask was diluted with a methanol-water mixture of the same v/v proportion up to 25 mL. Ionic strength was kept constant at 0.05 mol L⁻¹.

Buffer solutions covering practically all the useful pH range (from 2 to 12.5 separated within intervals of 0.5 pH units) were prepared at 10, 20, 30 and 40% (v/v) methanol mixtures. Stock solutions of test compounds (TC) and ISs were prepared at a concentration of 1 mg mL⁻¹ in water or in a methanol/water mixture (when they were not soluble in water itself). 4% of DMSO was added as electrospray flow (EOF) marker. Afterwards, a 1/10 dilution in water of the stock solution was prepared for injection (100 mg L⁻¹; 0.4% DMSO). All buffers and compound solutions were filtered through a nylon mesh 0.45 µm porous size (Whatman, Maidstone, UK) and stored at 4 °C until their use.

### 2.5. Mobility calculation

Mobility values (m² V⁻¹ s⁻¹) were directly calculated from the migration times of the test compound or internal standard (tₘ) and the electrospray flow marker (DMSO, t₀) through Eq. (1):

\[
\mu = \frac{l₁t₀}{V} \left( \frac{1}{tₘ} - \frac{1}{t₀} \right)
\]

where l₁ and l₀ are the total and effective capillary length respectively (m), V is the applied voltage (V) and migration times are expressed in seconds.

### 2.6. pKₐ determination

#### 2.6.1. Determination of the pKₐ of reference test compounds by potentiometry

Since pH values were measured in the methanol-water media in reference to standards in water (\(\gamma_p\), pH), they were converted to pH values referred to the same methanol-water mixture (\(\gamma_p\), pH) by means of the δ term correction [30] which includes the medium effect and the differences in the liquid junction potentials in the two media, according to Eq. (2).

\[
\gamma_p = \gamma_p^- \Delta pH - \delta
\]

pKₐ was calculated through the titration data, taking into account the mass and charge balances of the species in equilibrium. Autoprotolysis of the methanol-water solvent (pKₐ) was considered in the calculation [34]. Activity corrections at each titration point were done through the mean activity coefficient of each ion (\(γ_p^-\)), which was calculated through the Debye-Hückel equation, according to Eq. (3).

\[
\log γ_p^- = -\frac{az^2\sqrt{I}}{1 + a₀B\sqrt{I}}
\]

where z is charge number of the ion, and I is the ionic strength of the solution. Values of the A and a₀B Debye–Hückel parameters for the different methanol-water mixtures are given in Table 1 together with pKₐ, δ values and some other relevant macroscopic parameters.

### 2.6.2. Determination of the pKₐ of test compounds by the IS-CE method

The optimized procedure used for acidity constants determination by the IS-CE method has already been reported [21]. Briefly, the method is based on the use of an IS with pKₐ similar to that of test compound (ΔpKₐ < 1), choosing as first approximation for the pKₐ of the test compound the prediction by an appropriate software (ACD/Labs in our case) [42]. Then, mobilities of the IS and the test compound are determined in at least two different buffers for a monoprotic acid-base compound: a buffer in which the analyte and the IS are completely ionized (actual mobility); and a second buffer (or more buffers) in which both compounds are partially ionized (pH in the range pKₐ ± 1, effective mobility). From these mobility measurements the pKₐ of the test compound can be directly obtained if the pKₐ of the IS is well known from equations (4) (monoprotic neutral bases) and (5) (monoprotic neutral acids).

\[
pK_{a,IS} = pK_{a,TC} - \log \left( \frac{\mu_{BIS} + \mu_{eff}}{\mu_{eff}} \right)_{TC} + \log \left( \frac{\mu_{BIS} + \mu_{eff}}{\mu_{eff}} \right)_{IS}
\]

(4)

\[
pK_{a,TC} = pK_{a,IS} + \log \left( \frac{\mu_K + \mu_{eff}}{\mu_{eff}} \right)_{TC} - \log \left( \frac{\mu_K + \mu_{eff}}{\mu_{eff}} \right)_{IS}
\]

(5)

In Eq. (4), TC and IS are monoprotic bases, \(\mu_{BIS}\) is the actual mobility of the corresponding base and \(\mu_{eff}\) is the effective mobility. In a similar way, Eq. (5) accounts for the pKₐ determination of acidic monoprotic compounds (TC) through acidic internal standards (IS). In this last equation \(\mu_K\) is the actual mobility of the acidic TC or IS.

These two Eqs. (4) and (5) are valid in case that IS and TC have the same nature, i.e. they have the same charge, which is the case of this work. When equations are applied, the pKₐ of the TC is obtained at the same ionic strength as the pKₐ of the IS. When the nature of the two compounds is not the same, activity coefficient corrections are needed, as explained elsewhere [26]. Thus, direct use of Eqs. (4) and (5) to establish the ISs pKₐ values provides two independent subsets of ISs, one for monoprotic neutral acids (Eq. (5)) and another one for monoprotic neutral bases (Eq. (4)).

For the determination of the aqueous pKₐ of low soluble compounds, the IS-CE method was applied in methanol-water buffers. The pKₐ of the target TC was determined at different methanol compositions (10, 20, 30, 40% v/v), and the aqueous pKₐ was obtained by extrapolation to 0% methanol through the Yasuda–Shedlovsky method [51, 52] presented in Eq. (6).

\[
pK_a + \log [H_2O] = a \frac{100}{\varepsilon} + b
\]

Table 1

<table>
<thead>
<tr>
<th>% MeOH (v/v)</th>
<th>x_acet</th>
<th>log [H₂O]</th>
<th>p (kg dm⁻³)</th>
<th>e</th>
<th>a₀B</th>
<th>δ</th>
<th>pKₐ</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>0.000</td>
<td>1.74</td>
<td>0.995</td>
<td>78.30</td>
<td>0.53</td>
<td>1.50</td>
<td>0.00</td>
</tr>
<tr>
<td>10.0</td>
<td>0.047</td>
<td>1.70</td>
<td>0.983</td>
<td>75.05</td>
<td>0.56</td>
<td>1.53</td>
<td>0.01</td>
</tr>
<tr>
<td>20.0</td>
<td>0.100</td>
<td>1.65</td>
<td>0.969</td>
<td>71.37</td>
<td>0.59</td>
<td>1.57</td>
<td>0.03</td>
</tr>
<tr>
<td>30.0</td>
<td>0.160</td>
<td>1.59</td>
<td>0.955</td>
<td>67.49</td>
<td>0.64</td>
<td>1.61</td>
<td>0.05</td>
</tr>
<tr>
<td>40.0</td>
<td>0.229</td>
<td>1.52</td>
<td>0.939</td>
<td>63.39</td>
<td>0.70</td>
<td>1.66</td>
<td>0.09</td>
</tr>
</tbody>
</table>
Table 2
Potentiometric pKₐ values (standard deviations) of internal standard for the neutral acids and conjugated cationic acids of the neutral bases selected for anchoring IS-CE relative pKₐ scales.

<table>
<thead>
<tr>
<th>% Methanol (v/v):</th>
<th>0.0%</th>
<th>10.0%</th>
<th>20.0%</th>
<th>30.0%</th>
<th>40.0%</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-chlorobenzoic acid</td>
<td>2.84 (0.02)</td>
<td>2.96 (0.01)</td>
<td>3.14 (0.02)</td>
<td>3.35 (0.01)</td>
<td>3.58 (0.01)</td>
</tr>
<tr>
<td>Benzoic acid</td>
<td>4.17 (0.01)</td>
<td>4.27 (0.01)</td>
<td>4.39 (0.01)</td>
<td>4.59 (0.02)</td>
<td>4.80 (0.01)</td>
</tr>
<tr>
<td>Vanillin</td>
<td>7.37 (0.02)</td>
<td>7.45 (0.01)</td>
<td>7.60 (0.01)</td>
<td>7.70 (0.01)</td>
<td>7.87 (0.02)</td>
</tr>
<tr>
<td>Quinoline</td>
<td>4.92 (0.02)</td>
<td>4.80 (0.03)</td>
<td>4.66 (0.03)</td>
<td>4.43 (0.03)</td>
<td>4.23 (0.01)</td>
</tr>
<tr>
<td>4-tert-butylpyridine</td>
<td>6.09 (0.02)</td>
<td>5.91 (0.03)</td>
<td>5.68 (0.02)</td>
<td>5.59 (0.02)</td>
<td>5.20 (0.02)</td>
</tr>
<tr>
<td>2,4-lutidine</td>
<td>6.80 (0.01)</td>
<td>6.61 (0.01)</td>
<td>6.42 (0.01)</td>
<td>6.24 (0.00)</td>
<td>5.98 (0.02)</td>
</tr>
</tbody>
</table>

In this equation, log [H₂O] is the logarithm of the water molar concentration in a given solvent mixture, and ε is the dielectric constant of the solvent mixture. From the plot of the pKₐ vs. 100/ε a linear relationship should be obtained, and extrapolation to pure water would provide the aequous pKₐ of the compound [7].

2.6.3. Determination of the pKₐ of the internal standards

Since the pKₐ of the internal standards is not known in methanol-water mixtures it must be previously determined by an independent technique for all set members. Alternatively, the relative pKₐ difference values between all set members can be determined by the IS-CE method, as described in the previous subsection, and the pKₐ scale anchored to the known pKₐ value of one or several of the set members. This last method has been used in this work, anchoring the IS-CE scale to the pKₐ of three selected neutral acids and three selected cationic acids whose pKₐ values were previously determined by potentiometric titrations in the same studied methanol-water mixtures.

3. Results and discussion

3.1. Potentiometric determination of the pKₐ of the anchoring acid-base compounds

Three different neutral acids and three different neutral bases of varied acid-base strength were selected as anchor compounds for the two sub-sets of internal standards (the 23 neutral acids and the 23 neutral bases). The potentiometrically determined pKₐ values of these acid-base compounds in the different methanol-water mixtures are presented in Table 2. The results are the mean of three independent titrations and the interday-titration standard deviation is given too. The intraday-titration standard deviations for the different data points are 0.01–0.04, similar or slightly larger than the interday-titration ones. Thus, we estimate the standard deviation of the determined pKₐ values to be less than 0.05.

The pKₐ variation with the percentage of methanol (v/v) is plotted in Fig. 1. The variation of pKₐ is as expected [43]. The pKₐ of neutral acids / conjugated anionic bases (HA/A⁻ pairs) increases with the increase of methanol percentage, whereas the pKₐ of neutral bases / conjugated cationic acids (B/H⁺ pairs) decreases with the increase of methanol percentage. The variation of pKₐ values is combination of three different effects: i) the decrease of the dielectric permittivity of the media with the increase of methanol percentage (which does not favor ionic dissociation); ii) the variation of the intrinsic basicity of the solvent (or ability of the particular methanol-water mixture to accept dissociated hydrogen ions); iii) and the specific solvation effects of the different acid-base species by methanol and water. Decrease of dielectric permittivity affects acid dissociation (HA) because of the increase in the number of ions in the dissociation process (HA + S ⇌ HS⁻ + A⁻), being S the methanol-water solvent; or in other words, decrease of dielectric permittivity will increase electrostatic interaction between HS⁻ and A⁻ and will shift the equilibria to the left. However, decrease of dielectric permittivity practically does not affect dissociation of cationic acids because there is no variation in the number of ions (BH⁺ + S ⇌ B + HS⁺). Therefore, pKₐ values decrease with the methanol content because of combination of the increase in the basicity of the methanol-water media and possible specific solvation effects. In the case of neutral acids, the effect caused by the dielectric permittivity surpasses the effect of solvent basicity and leads to a moderate increase of the values. Overall variation of the pKₐ values for the reference acid-base compounds in the studied range of solvent compositions (0–40% methanol) is an increase of 0.5–0.8 units for neutral acids and a decrease of 0.7–0.9 units for cationic acids. More detailed and rigorous discussion of these effects can be found elsewhere [43–45].

3.2. Establishment of the pKₐ of the internal standards

Working with methanol-water buffers implies establishing reference pKₐ values for the set of ISs in the range of methanol-water working compositions (0–40% v/v). The procedure followed to establish these pKₐ values at each composition is based on the IS-CE methodology and has been used already in previous works to determine the aqueous pKₐ of the reference set of ISs [25,26]. Briefly, it consists of an iterative process in which the compounds intended to be ISs are used indistinctly as TCs or as ISs in the following way: to determine the pKₐ of a given compound of the set, the neighbouring compounds (with pKₐ differing less than 1 pKₐ unit) are used as ISs. Effective and actual mobilities of TC and IS were measured in methanol-water buffers of adequate pH (although not accurately known), and finally the pKₐ of the TC was calculated through Eq. (4) or 5 using initial approximate pKₐ values for the ISs (e.g., the pKₐ values in pure water). When several ISs were used for a TC, the final pKₐ value was the average of all determinations. This was done for each compound of the set. After this first calculation, the whole process was repeated, and the pKₐ of each of the compounds was calculated again with the same mobility data and equations 4 or 5 but using this time the average pKₐ of the ISs obtained in the previous calculation. From this second round of calculations a new averaged pKₐ was generated for each compound, which in turn, was used again in Eqs. (4) and (5) to calculate a third round of pKₐs. This process was repeated until the pKₐ differences between consecutive rounds was lower than 0.02 units for all compounds. The refinement process was applied separately for the acidic and the basic ISs, and the pKₐ values were determined at the four different methanol-water compositions studied: 10, 20, 30, and 40% of methanol (v/v). The procedure leads to coherent, but relative set of pKₐ values for each solvent composition minimizing the different pKₐ differences between the acidic or basic ISs (Eq. (5)) or for the ISs (Eq. (4)) as ISs. Although exact and precise pKₐ differences can be obtained with this procedure, the exact pKₐ value of each individual compound of the set cannot be accurately known because the initial values used in the iterative process were only approximate. The acidity scale of pKₐ differences needs to be anchored to reference compounds of well-known pKₐ values.

The anchoring procedure is widely used in pKₐ determination in many non-aqueous solvents where the activity of the solvated
hydrogen ion (or pH) is difficult to measure and then pKₐ is measured as a relative pKₐ value. To obtain absolute pKₐ values, the measured relative values must be linked to “anchor compounds”, for which the absolute values are known, by shifting the relative pKₐ of the anchor compound to its absolute pKₐ. Then, the pKₐ difference in the shifting of the anchor compound is applied to the rest of the acid-base compounds of the same set of relative pKₐ values. For instance, the pKₐ relative scale of neutral acids in acetonitrile have been anchored to the pKₐ value of picric acid, and the relative scale of neutral bases (cationic acids) to the pKₐ
value of pyridinium ion [57]. Anchoring procedure is usually done by a unique anchor compound for each scale. However, for minimizing errors we anchored each studied scale to three different acids averaging the pkₐ differences. The potentiometrically determined absolute pkₐ values of 2-chlorobenzoic acid, benzoic acid, and vanillin were used to anchor the relative pkₐ scale for neutral acids obtained electrophoretically. In the same way, the potentiometric absolute pkₐ values for the conjugated acids of quinoline, 2,4-lutidine, and 4-tert-butylpyridine were used for anchoring the relative electrophoretic scale of cationic acids-neutral bases pairs. The potentiometrically determined pkₐ values of 2-chlorobenzoic acid, benzoic acid, and vanillin have been used to anchor the acids scale and quinoline, 2,4-lutidine, and 4-tert-butylpyridine have been used for the bases scale. The anchoring process consists on shifting the electrophoretically obtained pkₐ in each solvent composition (acid or base set) to minimize differences between the electrophoretic and potentiometric pkₐ values of the reference anchoring compounds. The obtained pkₐ values for the different acid-base compounds and studied solvent mixtures are presented in Table 3. The RSD of the mobility for the ionized species was below 5% in all cases. The set of internal standards is the same established for water in previous works [25,26], excluding nicotinic acid (in fact a diprotic acid) and clonidine because the results obtained with them differed from the ones of the other standards (of similar pkₐ value) in some methanol-water mixtures. Comparison of the pkₐ values of the internal standards in Table 3 to the previous published ones for the same standards for pure water shows very small differences (< 0.05 pkₐ units). The differences come out from the new recalculation of pkₐ without nicotinic acid and clonidine, and because of the new anchoring. Previous ISs sets data [25,26] were anchored to literature pkₐ values, whereas the new set has been anchored to the potentiometrically determined reference pkₐ values.

Since the potentiometric reference pkₐ values were calculated at zero ionic strength by ionic activity coefficients correction and the same correction applies to the electrophoretic pkₐ values, the anchored values of Table 3 are at zero ionic strength too. All results are obtained at 25 °C from the differences of at least three different determinations with three neighbouring compounds as ISs. Standard deviations are also given. There is a good agreement between potentiometric and electrophoretic values of the reference anchoring compounds which is presented in Fig. 1. The agreement
These arithmic nation solvent, water drugs stances, charge units 2-chlorobenzoic acid methanol. The acidity of methanol is about ±0.05 pKa units which we estimated to be the maximum error of both potentiometric and capillary electrophoresis determinations.

The trend in the pKa variation with the percentage of methanol in the solvent is similar to the one observed with the potentiometric pKa values. Neutral bases decrease the pKa of their conjugated acids with the increase in the methanol percentage because of the effect of the increase of the basicity of the solvent, and this decrease is from 0.2 to 0.9 pKa units, between 0 and 40% methanol. The effect of the decrease of the dielectric permittivity of the medium on the neutral acids counteracts this decrease of pKa, and, globally, acids increase their pKa with the increase of the methanol percentage, up to a maximum of 0.8 pKa units for 2-chlorobenzoic acid and ibuprofen. Notice the very small increase for ortho- and para-phenols, with a maximum increase of 0.2 pKa units for 2,6-dinitrophenol and only 0.02 for 2,4-dinitrophenol. We attribute the small increase to the delocalization of the negative charge of the phenolate ion into the nitro groups, that decrease the electrostatic interactions, which are favored by the decrease in the dielectric permittivity.

3.3. Determination of acidity constant of very insoluble drugs

The test compounds chosen for the evaluation of the performance of the IS-CE method in solvent mixtures include 7 substances, most of them commercially available drugs (Fig. 2). These drugs (4 bases and 3 acids) were selected as their insolubility in water makes impossible to perform an aqueous IS-CE determination [24]. Table 4 shows the compounds together with the logarithmic form of their intrinsic solubility (logS_{int}, S in mol L^{-1}). These solubility values were collected from several sources [31,46] or predicted through ACD/Percepta software [42] when experimental data was not available in literature. Compounds are listed in decreasing order of solubility, from mephenamic acid with logS of −6.34 to amiodarone with logS of −8.17. The pKa values of the drugs determined at the four percentages of methanol (10, 20, 30 and 40% v/v) are shown in Table 4. Each pKa was determined following the description in Section 2.5.2. As an example, the two electropherograms at 20% MeOH at pH 5.0 (partially ionized) and pH 9.5 (totally ionized) are shown in Fig. 3. Calculating the mobili-
ties for TC (glyburide) and IS (2,5-dinitrophenol) at these 2 pH values, the pKₐ at 20% MeOH of glyburide was determined by means of Eq. (5). The pKₐ value at 0% MeOH was determined by extrapolation. Table 4 also shows the extrapolation to 0% obtained by using the Yasuda-Shedlovsky equation, and the fitting parameters of the regression curve. ε values were calculated as specified in bibliography [34,47]. We chose Yasuda-Shedlovsky equation rather than polynomial fitting because of two reasons: on the one hand, Yasuda-Shedlovsky is a linear equation that requires less input data than polynomial equations, and on the other hand, it is a universal equation for any experimental technique used for aqueous pKₐ extrapolation [7,9,10,48–52]. It is worth mentioning that exceptional precision was obtained in the Yasuda-Shedlovsky extrapolation. This might be attributed to the advantage of using an internal standard of a similar structure in the determination, and the minimum evaporation losses during the mobility measurement in the CE process. Yasuda-Shedlovsky plots for the studied insoluble drugs are presented in Fig. 4.

Shown in Table 5 is a comparison for the selected drugs between the aqueous pKₐ values obtained by the IS-CE method to the ones obtained by other methods. For most of the compounds multiple literature values (pKₐ[H±]) were reported, determined by a variety of methods and experimental conditions. Only the pKₐ of sertaconazole was not found in any reliable source, so comparison is not possible. When mentioned in literature, information regarding the nature and percentages of co-solvent used for the pKₐ determination is shown.

After literature data analysis [28,29,31,53–56], it can be concluded that at these solubility levels all methods (potentiometry, spectrophotometry and the classic CE method) require the use of co-solvent (mostly methanol) and extrapolation methods to obtain the pKₐ in water of the studied drug set. The only exception is the classical CE method coupled to a MS detector (CE-MS), because the high sensitivity of the technique allows the direct determination

### Table 4

<table>
<thead>
<tr>
<th>Compound</th>
<th>pKₐ IS-CE</th>
<th>pKₐ (H±)</th>
<th>Method</th>
<th>Co solvent (%v/v) range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mefenamic acid</td>
<td>4.12 (0.04)</td>
<td>4.22 [31]</td>
<td>GLpKa spectrophotometry</td>
<td>MeOH, n.s.</td>
</tr>
<tr>
<td>Mefenamic acid</td>
<td>4.04 (0.05)</td>
<td>4.10 [31]</td>
<td>GLpKa n.s.</td>
<td>MeOH, n.s.</td>
</tr>
<tr>
<td>Sertaconazole</td>
<td>6.15 (0.04)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Glyburide</td>
<td>5.39 (0.01)</td>
<td>5.22 [53]</td>
<td>CE-MS</td>
<td>None</td>
</tr>
<tr>
<td>Loperamide</td>
<td>8.90 (0.05)</td>
<td>8.90 [31]</td>
<td>GLpKa spectrophotometry</td>
<td>MeOH, n.s.</td>
</tr>
<tr>
<td>Terfenadine</td>
<td>9.30 (0.05)</td>
<td>9.25 [31]</td>
<td>GLpKa potentiometry 35</td>
<td>MeOH, 50–70%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9.74 [54]</td>
<td>Potentiometry 55</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9.31 [29]</td>
<td>Multiplexed CE</td>
<td>MeOH, 40–60%</td>
</tr>
<tr>
<td>Amiodarone</td>
<td>8.61 (0.04)</td>
<td>8.76 (0.09) [28]</td>
<td>GLpKa spectrophotometry</td>
<td>MeOH, 46–60%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8.80 (0.02) [55]</td>
<td>Potentiometry</td>
<td>MDM, 36–53%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8.73 [55]</td>
<td>Potentiometry</td>
<td>MeOH, n.s.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10.12 (0.15) [56]</td>
<td>Potentiometry</td>
<td>MeOH, n.s.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8.73 [31]</td>
<td>GLpKa spectrophotometry</td>
<td>MeOH, 40–50%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8.85 [28]</td>
<td>Classic CE</td>
<td>MeOH, 50%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8.62 [29]</td>
<td>Multiplexed CE</td>
<td>MeOH, 50–60%</td>
</tr>
</tbody>
</table>

n.s.: not specified in the reference.

*MDM: mixture that consists of equal volumes of methanol, 1,4-dioxane and acetonitrile.
in aqueous buffers at very low concentration levels. Although CE-MS is a less common and more expensive and complex technique. Results obtained by the IS-CE method show good agreement with the ones of literature. However, there is an important difference regarding the percentage of methanol needed to perform the determinations. In the IS-CE method the percentages of methanol in the buffers ranged from 10 to 40%, whereas in the other methods (potentiometric titrations, spectrophotometric titrations, and classic CE method) percentages ranging from 40% to 70% of methanol were needed for the same compounds. One of the reasons why the IS-CE allows the use of lower methanol content compared to other methods is because low concentration of sample is needed (5–100 mg L\(^{-1}\)) is enough. At these concentrations, detection using diode-array spectrophotometric detector can be performed correctly. The use of DMSO, appropriate MeOH/H\(_2\)O mixtures, and reported strategies to increase solubility [24] is enough to solubilize the TCs at concentration of 100 mg L\(^{-1}\). Briefly, solubility is increased by previous ionization using the required amount of HCl or NaOH and if precipitation in capillary is detected, the measurement of the effective mobility can be done at a new pH closer to the pH where ionic form predominates, which implies a higher ionization degree of the drug. As the amount of compound injected into the capillary is very small, many times precipitation can be avoided if the compound is previously ionized and solubilized, even when pH and media change inside the capillary.

Despite \(pK_a\) values in this work were determined in four solvent mixtures and using only one IS, this method allows the determination of more data points and using more than one IS [21]. This is very useful for a further result validation for a specific target in drug discovery and development.

Apart from its quickness and the low organic solvent content needed, another big advantage of the IS-CE method in solvent mixtures is that the pH of the buffer solution is calculated inside the capillary in each determination by means of the IS, directly obtaining the pH of the solution. In other methods like potentiometry, spectrophotometry and the classical CE, pH is measured with a glass electrode generally calibrated with aqueous buffers, while sample measurements are done in solvent/water mixtures. Therefore, a correction of the pH scale is needed to transform the obtained \(p\text{H}^+\) into the desired \(p\text{H}\). Using the IS-CE method we avoid approximations or tedious pH-meter calibrations, obtaining then faster and more accurate \(pK_a\) values.

4. Conclusions

The applicability of the internal standard capillary electrophoresis method, previously developed for fast and high-throughput \(pK_a\) determination in water, has been extended to highly insoluble compounds setting the \(pK_a\) values of the internal standards in methanol–water mixtures (0–40% of methanol). The relative acid and base scales have been properly anchored to potentiometrically determined \(pK_a\) values of reference compounds in the studied methanol-water mixtures. Therefore, the proposed set of compounds can be systematically employed as internal standards for routine and accurate measurements of \(pK_a\) by capillary electrophoresis in methanol–water media. In particular, it is very appropriate for determination of the aqueous \(pK_a\) of water insoluble compounds from extrapolation of the methanol-water \(pK_a\) values by the Yasuda-Shedlovsky method.

The usefulness of the method has been tested for some commercial drugs with aqueous solubility under 10\(^{-6}\) mol L\(^{-1}\). Their aqueous \(pK_a\) values have been easily determined through the Yasuda-Shedlovsky extrapolation, using methanol as co-solvent in the 10% to 40% (v/v) range. Due to the different possibilities of the IS-CE method to solubilize the sample, it is possible to work in lower methanol composition ranges than in other techniques, which implies lower extrapolation error. The obtained results point out that the IS-CE method is a fast and reliable alternative to other usual methods for the determination of aqueous acidity constants of very insoluble compounds.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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