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# Extension of the Internal Standard Method for Determination of Thermodynamic Acidity Constants of Compounds Sparingly Soluble in Water by Capillary Zone Electrophoresis

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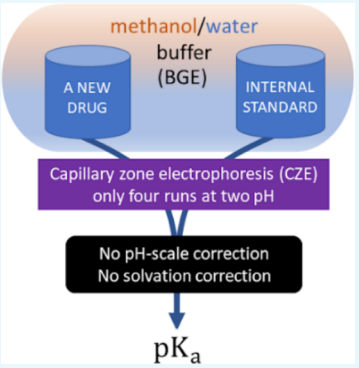
**ABSTRACT:** The paper extends applicability of the internal standard method published in 2009 (Fuguet E. *et al., J. Chromatogr. A* **2009,** *1216*(17), 3646). Although the original capillary zone electrophoresis method was suggested to determine thermodynamic acidity constants of compounds sparingly soluble in aqueous solutions by carrying out only runs at two different pH values (*i.e.*, without the need to perform many experiments over the appropriate pH range including the form of a low-ionized analyte), we proved that the approach also virtually overcomes any interactions of the analyte in mixed solvents, so that the experiments can be carried out in a methanol−water buffer where the solubility is much better. Applicability of the extended method is illustrated on six selected $\beta$-blockers.




## INTRODUCTION

A common method for determining p$K_a$ of monoprotic weak bases by capillary zone electrophoresis (CZE) is based on changes in the analyte mobility with the variation of buffer pH: a series of experiments with electrolytes are conducted over the appropriate pH range ($\approx$p$K_a \pm 2$) at a constant ionic strength.[1−3] The theory of electrophoretic mobility states that[4]

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

where $\mu_{eff}$ is the effective electrophoretic mobility, $\mu_{BH^+}$ is the electrophoretic mobility of the fully protonated base, p$K_a'$ is the negative decadic logarithm of the mixed acidity constant. Equation 1 indicates that by plotting the observed electrophoretic mobility (calculated from migration times in the electropherogram) against pH, a sigmoidal curve is obtained where its inflection point represents p$K_a'$. There are many spreadsheet calculators that can help to calculate p$K_a'$ by fitting the curve.[5]

Calculation of the effective electrophoretic mobility $\mu_{eff}$ in capillary zone electrophoresis is based on measurement of two migration times: apparent migration time (of the analyte) $t_m$ and a migration time of the electroosmotic flow (an EOF marker) $t_{EOF}$

$$\mu_{eff} = \frac{L_D L_T}{U} \cdot \left( \frac{1}{t_m} - \frac{1}{t_{EOF}} \right) \quad (2)$$

where $L_D$ is the length from the capillary inlet to the center of the detection window, $L_T$ is the total capillary length, and $U$ is the applied separation voltage.

Because the experiments are carried out in an electrolyte, typically an aqueous buffer, in order to get the thermodynamic p$K_a$, the obtained value should be corrected to the activity coefficient for ions in dilute (up to 0.075 mol/L) electrolyte solutions at 25 °C according to the Debye−Hückel theory of nonideality of electrolyte solution. For bases, it holds

$$pK_a = pK_a' + \log \gamma \quad (3)$$

where $\gamma$ is the activity coefficient of the buffer species, calculated as $\log \gamma = -0.5085 z^2 \sqrt{I} / (1 + 1.64\sqrt{I})$ where $z$ is a charge number and $I$ is the ionic strength of the solution.

Compounds that are slightly soluble in water may require experiments in mixed solvents. A mixture of water and methanol is usually employed.[6] However, in a mixed solvent (*e.g.*, of the volume fraction $\phi$ of methanol in water), an experimentally accessed acidity constant ${}^{s}_{w}pK_a$ (solvent−water

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acidity constant) is related to a pure organic solvent ${}^{s}_{s}pK_a$ (solvent−solvent acidity constant) by a formula[7]

$$ {}^{s}_{s}pK_a = {}^{s}_{w}pK_a - \delta \quad (4) $$

where

$\delta = (0.09\phi - 0.11\phi^2)/(1 - 3.15\phi + 3.51\phi^2 - 1.35\phi^3)$.

Therefore, a way to obtain the thermodynamic $pK_a = {}^{w}_{w}pK_a$ (water−water acidity constant) from ${}^{s}_{s}pK_a$ is a correction to solvation effects. The literature suggests several extrapolations to estimate ${}^{w}_{w}pK_a$. A common approach is the Yasuda−Shedlovsky equation that relates ${}^{s}_{s}pK_a$ with reciprocal relative electric permittivity of an aqueous binary solvent.[8] In this case, an extrapolation to zero content of organic solvent is performed from series of experiments with different amounts of water. Alternatively, an empirical linear equation was suggested for acids belonging to the same family when specific solvation effects in solvent (S) and in water (W) can be linearly related with the acidity of the acid

$$ {}^{w}_{w}pK_a = \frac{{}^{s}_{s}pK_a - b_s}{a_s} \quad (5) $$

where parameters $a_S$ and $b_S$ can be calculated from the organic solvent amount (for bases or acids from data of Rived *et al.*[9]). Another approach to the conversion of methanolic $pK_a$ values to ${}^{w}_{w}pK_a$ for structurally similar compounds was also presented.[10]

**Internal Standard in Capillary Zone Electrophoresis for $pK_a$ Determination.** In 2009, Fuguet *et al.* introduced a method for the determination of acidity constants by capillary zone electrophoresis (CZE) with an internal standard (IS): only two pairs of electrophoretic runs are required to determine the acidity constant: (i) at pH, where the analyte and internal standard are fully ionized and (ii) at a different pH where both of them are partially ionized. The authors emphasized that the main advantage of the method is that it is not pH-dependent, so there is no need to know the exact pH of the buffer solutions—it is only important that the pH is identical for runs with an analyte and the internal standard. They measured acidity constants of various amines and phenols ($pK_a$ range 7.1−9.6) and compared them to the literature.[11]

In a study, the authors measured $pK_a$ of weak acids[12] and proposed a set of 24 monoprotic weak acids of various structures as internal standards. Later, the same authors established a set of 25 basic internal standards[13] and the method was extended for polyprotic compounds.[14] The authors claimed the IS-CE method suitable also for sparingly soluble compounds,[15] as other reference methods require the use of aqueous−organic solvent buffers and extrapolation (corrections) to obtain a thermodynamic $pK_a$. Temperature variations in CZE were studied by the same team in 2013[16] with a conclusion that the IS-CE method also compensates uncontrolled temperature fluctuations (*e.g.*, due to Joule heat) inside the capillary. The authors obtained reliable acidity constant values at the desired temperatures. Cabot *et al.*[17] enhanced the IS-CE method as a high-throughput method (3 min runs) by calculating pH from electrophoretic mobilities of multiple internal standards and applying pressure. Despite depletion of BGE ("buffer instability"), the authors confirmed that the method eliminates this systematic error. Later, the authors introduced an automated analyzer for $pK_a$ determination.[18]

The goal of this paper is to demonstrate that the IS-CE method in principle can eliminate the influence of the contingent interactions of an analyte with nonrecommended buffers and even compensate the solvation effect of an analyte in mixed solvents, which means that, within experimental precision, the method yields correct values of thermodynamic acidity constants, though the data are measured in methanol−water mixed solvent and no corrections are taken.

## THEORY

Many popular electrolytes used in capillary electrophoresis are Good's buffers (derivatives of ethane−sulfonic acids), mainly for separation purposes, where the goal is to obtain the resolution of compounds with close mobilities, for example, MES, Bis-Tris, ACES, MOPS, HEPES, CHES, and TAPS.[19]

Several authors studied electrophoretic mobilities measured in common buffers and found that some common inorganic buffers may exhibit unpredictable migration behavior (*e.g.*, phosphate[20]). Buffers suitable for $pK_a$ determination by CZE were reported by Poole *et al.*, who recommended mostly inorganic buffers for electrophoretic $pK_a$ determination: sodium phosphate, acetate, and boric, phosphoric acid, acetic, and formic acid (for pH > 10 butylamine).[2] Later, other researchers concluded that "phosphate and borate buffers should be avoided to determine the mobility of amines with aqueous $pK_a$ higher than 8, at least in solutions with high methanol content".[6] Critical evaluation of buffers for capillary electrophoresis was presented in 2008 by Fuguet *et al.* who did not recommend ammonium salts, organoammonium salts, and hydrogen phosphate/phosphate because they may interact with a wide range of compounds. Also, dihydrogen phosphate/hydrogen phosphate, MES, HEPES, and borates showed specific interaction.[21] In 2009, Fuguet *et al.* suggested for $pK_a$ determination the following set of buffers: formate, acetate, Bis-Tris, CHES, and CAPS.[11] Also, for $pK_a$ determination, the use of univalent anionic/cationic buffers with only one counterion (sodium/chloride) was recommended.[22,23] Later, Cabot *et al.* observed systematic electrophoretic mobility deviations of weak bases at pH > 9 in some buffers (TAPS and CHES).[12] Nevertheless, their observations proved that the IS-method showed a better performance compared to the common approach because such a deviation was compensated.

**Principle of the Internal Standard Capillary Electrophoresis Method (IS-CE Method).** This method requires in principle two electrophoretic runs: a first one at a pH, where both analyte and internal standard are totally ionized (as protonated bases, $pH < pK_a - 2$) to calculate their actual ionic mobilities and a second one at another pH where both are partially ionized ($pH \approx pK_a$); the mobility of the partially ionized form should be approximately 50% lower compared to the totally ionized form in order to calculate $pK_a$ correctly. As noted above, the method is not pH-dependent, so an accurate measure of the pH of the buffer solutions is not needed because the solution, where both the compounds are measured, has identical pH and composition.[11]

As the authors stated "One of the main advantages of using an internal standard is that some systematic errors are compensated". The following equations will show the calculation of the IS-CE method and how it eliminates the activity coefficient correction. In an analogous manner, it can



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eliminate the corrections for the solvation effect in mixed solvents.

**Activity Coefficient Correction.** For a base, eq 1 can be rearranged introducing a variable $Q > 0$ ($\mu_{BH^+} > \mu_{eff}$)

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

and in combination with eq 3 we get

$$pK_a = pH - \log Q + \log \gamma \quad (7)$$

Because eq 7 holds for both the analyte (AN) and internal standard (IS), log $\gamma$ is subtracted[13]

$$pK_a(AN) = pK_a(IS) - \log Q(AN) + \log Q(IS) \quad (8)$$

which proves that $pK_a$(AN) is pH- and $\gamma$-independent because the activity coefficients of the buffer are identical for the IS and analyte. Such an elimination of the activity coefficient may fail at basic analytes with acidic internal standards, which was also discussed in Fuguet 2011;[13] however, using a weak acid as an internal standard for $pK_a$, determination of a base is not a common approach.

**Water-Solvent pH Scale Correction.** From eq 4, ${}^{s}_{s}pK_a$ can be easily estimated (calculated) from any experimental value ${}^{s}_{w}pK_a$ knowing the methanol volume fraction $\phi$. Clearly, the correction $\delta$ is identical for both the internal standard (IS) and the analyte (AN), thus after a rearrangement with a help of 8 we get

$$\begin{aligned} &{}^{s}_{s}pK_a(AN) - {}^{s}_{s}pK_a(IS) \\ &= {}^{s}_{w}pK_a(AN) - {}^{s}_{w}pK_a(IS) \\ &= -\log Q(AN) + \log Q(IS) \end{aligned} \quad (9)$$

which proves that the experimental data (a calculated difference of log $Q_s$) will directly give the difference of ${}^{s}_{s}pK_a$(IS) and ${}^{s}_{s}pK_a$(AN) without the presence of $\delta$ because eq 9 turns into eq 8.

**Mixed Solvent (Solvation) Correction.** Calculation of the coefficients $a_S$ and $b_S$ for amines gives[9] $a_s = (1 - 0.476v + 0.209v^2)/(1 - 0.4v + 0.158v^2)$ and $b_s = (-0.458v + 0.477v^2)/(1 - 1.674v + 0.69v^2)$ ($v$ is the volume fraction of methanol in the mixture with water). As shown in Figure 1, we plot the course of eq 5 on methanol content for two bases ${}^{w}_{w}pK_a$(IS) = 9.48 (*e.g.*, propranolol) and a hypothetical base with ${}^{w}_{w}pK_a$(AN) = 9.00.

In Figure 1, one can see (i) the coefficient $a_S$ is practically constant and close to 1 (dotted line) and (ii) the graphs of ${}^{s}_{s}pK_a$ course for both bases (solid and dashed lines, resp.) decrease in parallel lines. A calculated difference of both ${}^{s}_{s}pK_a$ is 0.48−0.46 within the range of 0−70% (v/v) of methanol. Therefore

$$\begin{aligned} &{}^{s}_{s}pK_a\ (AN) - {}^{s}_{s}pK_a\ (IS) \\ &\approx {}^{w}_{w}pK_a\ (AN) - {}^{w}_{w}pK_a\ (IS) \\ &= -\log Q(AN) + \log Q(IS) \end{aligned} \quad (10)$$

Again, this leads to an elimination of $b_S$ and practically also $a_s$. It means that the difference of log $Qs$ can be used for a direct calculation of ${}^{w}_{w}pK_a$ in methanol−water solutions because eq 10 turns into eq 8 (within the experimental error).

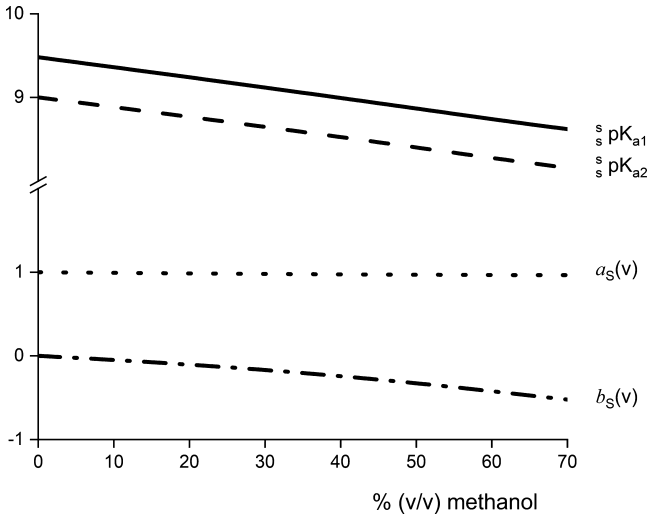


**Figure 1.** Graph of eq 5 (correction to solvation effects for methanol−water solvent) for two hypothetical bases. Two lower traces are plots of parameters $a_S$ (dotted line) and $b_S$ (dash-dot line); the upper two traces show the course of ${}^{s}_{s}pK_a$ for bases with thermodynamic acidity constants of ${}^{w}_{w}pK_a$ 9.00 (dashed line) and 9.48 (solid line), respectively.

## RESULTS AND DISCUSSION

**Buffer Choice.** Selection of a buffer and its concentration for experiments in CE is practically limited due to Joule heating; to keep Ohm's law valid (constant resistance of the solution), high concentrations of multiple-charged species should be avoided. In this work, the course of Ohm's law for the buffers (concentration 0.025 M) was recorded at a continuous increase of voltage and showed deviations from a linear course for U > 15 kV.

Our starting experiments about an effect of voltage on mobility +5, +10, and +20 kV (gradient 151−606 V/cm) proved that at +20 kV, calculated electrophoretic mobilities exhibited higher values (approx. by +10%) in comparison to +10 or +5 kV (also after correction to voltage ramp[24]) for all the analytes and common buffers tested. Because the effect was observed also for electro-osmotic flow mobility, and even after setting the thermostat to 15 °C, it is likely that excessive Joule heating and inefficient heat dissipation caused the viscosity decrease inside the capillary, which affected the species electrophoretic movement. Despite the fact that the IS-CE method should eliminate such a shift similar to the temperature effect,[16] the voltage +10 kV (where the Ohm's plot was strictly linear) was selected for all the following runs for $pK_a$ determination in order to avoid any unpredictable migration behavior. Because the compounds studied were monoprotic bases with $pK_a$ around 9.5, pairs of buffers with pH values between 6.0 and 9.5 were always chosen ($c$ = 25 mM).

**Ammonium Buffer and Triethylamine Buffer.** The acidity constant of atenolol 9.54[25] is close to that of propranolol 9.48,[25] so one would expect their electrophoretic mobilities to be similar, which was confirmed by experiments with all the $\beta$-blockers in the carbonate buffer (pH = 9.5) (≈+13 × $10^{-9}$ $m^2$/V·s, data not shown). However, our additional experiments with other buffers showed that



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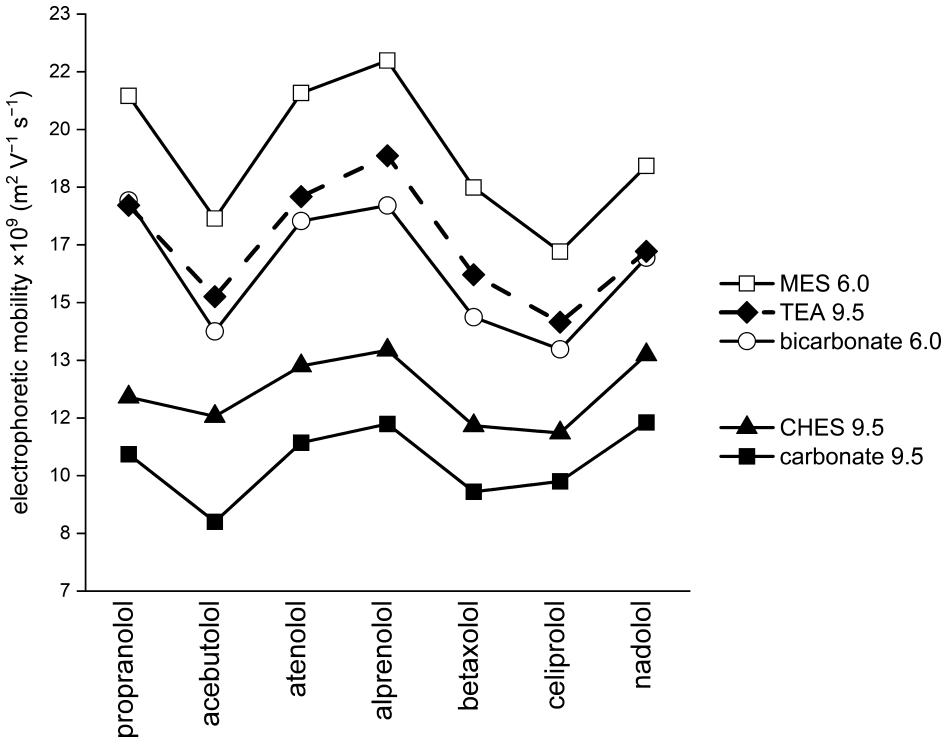


**Figure 2.** Electrophoretic mobilities of all the analytes at +10 kV in various buffers (10−25 mM). The dashed trace (full diamonds) of analytes in TEA is compared to other buffers of pH = 9.5 (full trace, CHES—closed triangles and carbonate—closed squares) and at pH = 6.0 (MES—open squares, bicarbonate—open circles). The lines connect points for clarity only.

ammonium buffer pH = 9.5 exhibited systematically higher electrophoretic mobility at all the voltages for all the $\beta$-blockers, which was mostly pronounced for atenolol ($\approx$+17 × $10^{-9}$ $m^2$/V·s) in comparison to propranolol ($\approx$+15 × $10^{-9}$ $m^2$/V·s). An explanation can be the presence of the amide functional group of atenolol in contrast to propranolol.

Further experiments at +10 kV with different buffers (pH = 9.5) revealed a systematic positive shift in electrophoretic mobility (by 60−100%) of all the $\beta$-blockers in BGE of triethylamine (TEA) buffer (Figure 2, the dashed line). Another interesting systematic increase in mobility ($\approx$3.5 × $10^{-9}$ m2/V·s) was also observed for MES at pH = 6.0 in comparison to bicarbonate at pH = 6.0, suggesting an interaction of the protonated bases with MES. This is in a general agreement with findings of Fuguet *et al.*[21] where the authors concluded, among others, that ammonium and alkylammonium buffers are not recommended for p$K_a$ determination by CZE (see Theory above).

An important consequence of the observations for determining the p$K_a$ of weak bases is that TEA buffer (pH = 9.5) cannot be combined with, for example, bicarbonate buffer (pH = 6.0) by the IS-CE method, as the algorithm would fail, because the electrophoretic mobility around p$K_a$ would be higher than the mobility of the fully protonated base and the variable $Q$ becomes negative (see eq 6).

**p$K_a$ Determination.** Several series of measurements of six $\beta$−blockers ($N$ = 7−14) were carried out with aqueous buffers and buffers in mixed solvents 10−50% (v/v) with propranolol as the internal standard (p$K_a$ = 9.48 of propranolol was taken as an average from a review[25]). The experimental values are graphically shown in Figure 3. The calculations were performed according to eq 8 without any correction to activity coefficient or solvent interactions and were statistically evaluated (Tables 1, 2).

Table 1 compares coefficients of determination ($R^2$) of p$K_a$ vs methanol % (v/v) in BGE for individual $\beta$-blockers. All $R^2$ are close to zero and $p$-values were always ≫0.05, which means that, at level $\alpha$ = 0.05, the slope was NOT significantly different from zero and there was no statistically significant correlation (Table 1).

This finding suggests that there is no systematic change in p$K_a$ values in the mixed solvents (no increase/decrease in p$K_a$) depending on the methanol content in BGE as predicted from Figure 1 (eq 5) for $^{s}_{s}pK_a$ of an individual base.

In Table 2, the p$K_a$ values for each $\beta$-blocker of the two groups (group 1 = aqueous buffers and group 2 = methanol−water buffers) were statistically tested by independent sample tests of equality ($t$-test and Mann−Whitney $U$ test). Both the parametric and nonparametric tests proved no statistically significant differences at level $\alpha$ = 0.05. Therefore, both the data sets belong to the same populations and they could be pooled. Then, average acidity constants calculated from the pooled data ($N$ = 13−22) were compared to values from the literature (Table 3).

## CONCLUSIONS

The results showed that triethylamine buffer cannot be recommended as a background electrolyte for measuring the p$K_a$ of weak bases by capillary electrophoresis because extreme values of electrophoretic mobility in the basic region may exceed values for electrophoretic mobility of the fully protonated form and the IS-CE algorithm fails.

If a suitable internal standard is selected, the IS-CE method can be used even for (i) other buffers that are not recommended for the traditional approach because contingent



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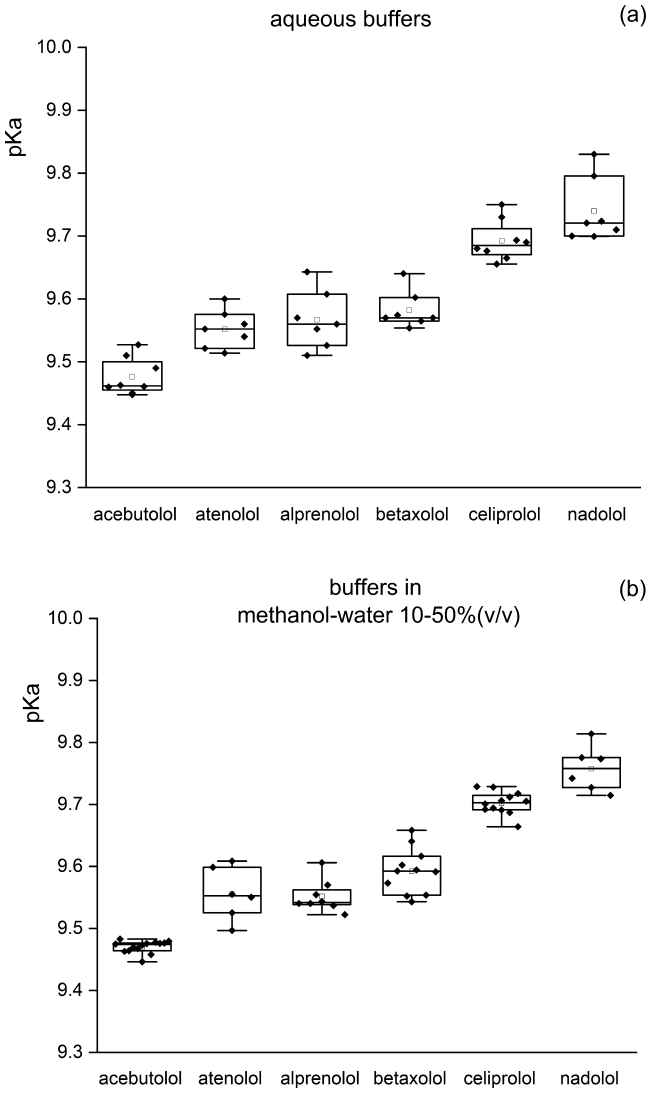


**Figure 3.** Box-and-whiskers plots with all the individual experimental values of p$K_a$ determination for six $\beta$-blockers. Values obtained in aqueous buffers are shown in (a) and those obtained in methanol−water buffers 10−50% (v/v) are in (b). All the measured data are shown at each box ($N$ = 7−14).

**Table 1. Statistical Evaluation of a Linear Fit of a Dependence of p$K_a$ on Methanol Content in BGE [10−50% (v/v)]**

| | $R^2$ | $p$ ($F$-test) |
|---|---|---|
| acebutolol | 0.010 | 0.655 |
| atenolol | 0.121 | 0.243 |
| alprenolol | 0.077 | 0.318 |
| betaxolol | 0.041 | 0.419 |
| celiprolol | 0.073 | 0.249 |
| nadolol | 0.174 | 0.156 |

interactions with BGE can be compensated and (ii) analytes with low solubility in water because the runs can be safely performed in methanol−water mixed solvents. The latter advantage may overcome problems with acidity constant determination of many newly synthetized compounds with limited water solubility.

Based on error propagation, the experimental error of the determined acidity constant (calculated according to eqs 2, 6, and 8) is only by 0.02 higher than the uncertainty of the internal standard p$K_a$.

**Table 2. Statistical Evaluation of Results in Aqueous BGE vs Methanol−Water BGE[a]**

| | $p$ ($t$-test) | $p$ (MW $U$ test) |
|---|---|---|
| acebutolol | 0.51 | 0.71 |
| atenolol | 0.85 | 0.94 |
| alprenolol | 0.43 | 0.45 |
| betaxolol | 0.53 | 0.53 |
| celiprolol | 0.40 | 0.26 |
| nadolol | 0.49 | 0.28 |

[a]Results of $t$-test and Mann−Whitney $U$ test of equality of data from Figure 3. Equality of p$K_a$ for a $\beta$-blocker in aqueous buffer 3a and methanol−water buffer 3b was always a null hypothesis. Because $p$-values were always $\gg$0.05, $H_0$ was always accepted.

**Table 3. Comparison of the Determined p$K_a$ to the Literature[a]**

| | p$K_a$ values (reference) | this work |
|---|---|---|
| propranolol | 9.53, 9.40, 9.57, 9.51, 9.32, 9.72, 9.43, 9.45, 9.23, 9.40, 9.50, 9.7, 9.45, 9.59[26] | 9.48 (IS) |
| acebutolol | 9.40, 9.67, 9.67, 9.4, 9.52[26] | 9.47 ± 0.01 |
| atenolol | 9.60, 9.58, 9.25, 9.56, 9.54, 9.54, 9.55, 9.6, 9.6, 9.60[26] | 9.55 ± 0.02 |
| alprenolol | 9.6, 9.63, 9.62[26] | 9.56 ± 0.02 |
| betaxolol | 9.21 | 9.59 ± 0.02 |
| celiprolol | 9.7 | 9.70 ± 0.01 |
| nadolol | 9.39, 9.67, 9.4 | 9.75 ± 0.03 |

[a]p$K_a$ values in the second column were mostly found in a review,[25] if not stated otherwise. The half-widths of the confidence interval in the last column were calculated according to Student ($\alpha$ = 0.05).

## EXPERIMENTAL SECTION

The CE experiments were carried out using an Agilent CE G-1600 equipped with DAD (190−600 nm) (Agilent Technologies, Waldbronn, Germany) and data software supplied by the manufacturer (Chemstation). An untreated fused silica capillary of 50 $\mu$m internal diameter (Simplus Capillaries MicroSolv, USA) was used with a total length 33 cm, effective length 24.5 cm. For buffer preparation, a pH meter Orion 370 (Thermo Electron Corp., USA) was utilized.

Chemicals were purchased from various manufacturers: nadolol, atenolol, betaxolol hydrochloride, and alprenolol hydrochloride from EDQM (Strasbourg, France), propranolol hydrochloride, acebutolol hydrochloride, CHES, MES, and mesityl oxide (MSO, an EOF marker) from Fluka (Buchs, Switzerland), sodium bicarbonate, celiprolol hydrochloride, sodium dihydrogen phosphate, citric acid, and methanol HPLC grade from Sigma Aldrich (St. Louis, MO, USA), triethyl amine (TEA), and ammonium hydroxide from Lach:ner (Czech Republic). Standards of $\beta$-blockers (propranolol, atenolol, alprenolol, nadolol, acebutolol, celiprolol, and betaxolol) of concentration 0.2 mg/mL were prepared in a buffer of pH = 6 and dissolver in an ultrasonic bath. Injection was performed for 2 s at 20 mbar, the diode-array detector was set to 240 nm (MSO maximum) and 204 nm (for several $\beta$-blockers where the wavelength 240 nm was not sensitive enough).

Various concentrations of methanol in buffers MES (pH = 6.0) and CHES (pH = 9.5) (10−50% v/v) were prepared by dissolution of the buffer salt in water and methanol.



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### Author Contributions

The manuscript was written through contributions of all authors.

### Notes

The authors declare no competing financial interest.

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